

**Studies on the Role of the Distal Sequences Involved in the
Expression of the Human Growth Hormone/Chorionic
Somatomammotropin Gene Family**

BY

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in Partial Fulfillment of the Requirements
for the Degree of**

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**Department of Physiology
Faculty of Medicine
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Winnipeg, Manitoba**

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**Studies on the Role of the Distal Sequences Involved in the Expression of the Human
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**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University
of Manitoba in partial fulfillment of the requirements of the degree
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Dedication

To my sister Indu

and

to the Scientific Community

ABSTRACT

The human growth hormone/chorionic somatomammotropin (GH/CS) gene family offers an interesting model to study mechanisms involved in tissue-specific transcriptional regulation of genes. Despite their high sequence homology, the members of GH/CS gene family are predominantly expressed in two different tissues: the GH-N gene is expressed in pituitary, whereas the CS-L, CS-A, GH-V and CS-B genes are expressed in placenta. A series of highly conserved regions within the GH/CS gene locus were identified by other investigators and were suggested to play a role in the differential and tissue-specific transcriptional regulation of the GH/CS genes. Presented here is the characterization of sequences/elements residing within the GH/CS locus, as well as those found in the flanking regions.

Previous work in our laboratory identified PSF (PSF-A/-B) elements which are involved in the pituitary repressor mechanism for the CS/GH-V genes. Part of the present thesis work is aimed at the identification of the PSF-DNA binding proteins using conventional protein purification methods combined with PSF-specific DNA affinity chromatography. Characterization of partially purified proteins using southwestern blotting suggested that PSF proteins could be in the molecular range of 50-70 kDa. Further analysis of the PSF-DNA sequence revealed that these elements are recognized likely by nuclear factor-1 (NF-1) or NF-1-like proteins. NF-1 belongs to a family of proteins whose molecular sizes range from 50 to 70 kDa. Thus, these characteristics strongly suggest that PSFs could be NF-1 or NF-1-like.

An AP-1 element is identified in the upstream sequences of the GH/CS genes using DNase I protection assays. Attempts were also made to characterize the role of this AP-1 element through the use of transient transcription analysis.

The enhancer/enhancer-like sequences located downstream of CS-L, CS-A and CS-B genes were shown to contain multiple elements for transcriptional enhancer factor-1

(TEF-1). The presence of these sites implicated TEF-1, which is expressed ubiquitously, and TEF-5, which is expressed abundantly in placenta, in modulating the placenta-specific expression of the CS genes. A series of deletion mutants coding for truncated forms of TEF-1 and TEF-5 were generated and were tested in transient transfections. These studies reveal a HeLa (human cervical carcinoma) cell-specific potent transcriptional activator in the form of a truncated TEF-1.

Further work on the characterization of remote sequences in the upstream as well as downstream flanking sequences of the GH/CS gene locus was done. Structural analysis of hypersensitive sites I and II, which are located ~15 kilobases upstream of the GH-N gene and form part of the GH/CS locus control region, led to the suggestion and identification of the involvement of the pituitary-specific transcription factor, growth hormone factor-1 (GHF-1)/Pit-1 in the pituitary-specific enhancer activity associated with these regions. Characterization of far-downstream sequences of the GH/CS gene loci revealed the location of two ubiquitously expressed genes: BAF60b (a gene coding for a subunit protein of SWI/SNF protein complex) and TRIP-1 (thyroid hormone receptor interacting protein-1). The pattern of this arrangement of genes, GH-BAF60b-TRIP-1, appears to be evolutionarily conserved among several species of vertebrate animals. A model is proposed to signify the presence of these ubiquitously expressing genes downstream of the GH/CS genes.

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ANOVA	Analysis of Variance
AP-1	Activator protein-1
APS	Ammonium persulfate
bp	base pairs
BSA	Bovine serum albumin
BLAST	Basic local alignment search tool
cDNA	Complementary DNA
c/EBP	CCAAT/enhancer-binding protein
CAT	Chloromphenicol acetyl transferase
CMV	Cytomegalo virus
CNBr	Cyanogen bromide
CS-A	Chorionic somatomammotropin-A
CS-B	Chorionic somatomammotropin-B
CS-L	Chorionic somatomammotropin-L
CRE	c-AMP responsive element
CREB	CRE binding protein
CSEF-1	CS enhancer factor-1
CTF-1	CCAAT-binding transcription factor
dNTP	deoxyribonucleotide triphosphate
DBD	DNA binding domain
DMEM	Dulbecco's modified Eagles medium
DMS	Dimethyl sulfate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraaceticacid
FBS	Fetal bovine serum
GH	Growth hormone
GH-N	Growth hormone-Normal
GH-V	Growth hormone-Variant

GHF-1	Growth hormone factor-1
GH-R	Growth hormone receptor
GTFs	General transcription factors
GR	Glucocorticoid receptor
HAT	Histone acetyltransferase
HGMW	Human genome management workshop
HS	Hypersensitive site
HEPES	N-2[hydroxyethyl]piperazine-N'-[2-ethanesulfonicacid]
IAC	Isoamyl alcohol-Chloroform
IGF-1	Insulin-like growth factor
IPTG	Isopropyl β -D-thiogalactopyranoside
JAK	Janus Kinase or Just another kinase
kb	Kilo bases
kDa	Kilo Dalton
LCR	Locus control regionmRNA Messenger RNA
MAR	Matrix attachment region
MOPS	3-[morpholone] propane-sulfonic acid
NF-1	Nuclear factor-1
ODN	Oligodeoxynucleotide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-CMF	Calcium and magnesium free PBS
PCR	Polymerase chain reaction
PCV	Packed cell volume
PIC	Preinitiation complex
PL	Placental lactogen
PMA	Phorbol-12-myristate-13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PRL	Prolactin
PRL-R	Prolactin receptor

PSF	P-sequence specific factor
PSF-A	P-sequence specific factor-A
PSF-B	P-sequence specific factor-B
RNA	Ribonucleic acid
RNAPII	RNA polymerase II
RT	Reverse transcriptase
SCN4A	Sodium channel α -subunit gene
SDS	Sodium dodecyl sulfate
Sp-1	Promoter specific factor-1
SSC	Sodium chloride and sodium citrate buffer
STAT	signal transducers and activators of transcription
tRNA	Transfer RNA
TAF	TBP-associated factor
TBP	TATA-binding protein
TE	Tris-EDTA buffer
TEF-1	Transcriptional enhancer factor-1
TEMED	N, N, N', N'-tetramethylethylenediamine
TF	Transcription factor
TPA	12-O-tetradecanoylphorbol-13-acetate
TR	Thyroid hormone receptor
TRE	TPA responsive element
TRIP-1	thyroid hormone receptor interacting protein-1
TSH- β	Thyrotropin- β
USF	Upstream stimulatory factor
UTR	Untranslated region

CHAPTER 1

1.1. GENERAL INTRODUCTION

The main focus of this piece of work is to present more insights into molecular regulatory mechanisms involved in the transcription and regulation of members of the human (h) growth hormone/chorionic somatomammotropin (GH/CS) gene family. More emphasis is given to placental growth hormone genes as transcriptional regulatory mechanisms for the pituitary-specific growth hormone (GH-N) gene are well studied.

This thesis deals with multiple possible mechanisms involved in control of GH/CS gene expression. The regulatory sequences present inside and outside the GH/CS gene locus and their possible sequence-specific binding proteins are dealt with separately. The thesis is written with a broad “introductory chapter” detailing a historical perspective of growth hormone, brief introductions to each of the genes/proteins of the GH/CS family, their differential expression patterns and their physiological importance. In addition, the process of transcriptional regulation as well as some of the transcription factors involved in growth hormone gene expression are introduced. All general methodologies used in this study are included in the “Materials and Methods” section and some specific details are dealt with in individual subsections. With regard to Chapters detailing results, these are presented as a combination of “Results and Discussion”. Most of the experiments were repeated at least twice, and representative results are presented in the thesis. A chapter titled “Final Comments” was written in an effort to combine all the results and ideas generated during the assembly of this thesis, and ends with the “Future perspectives” of this research work. Some of the references included in the thesis were not available in the library and were collected from other references or reviews. These references were included with a view to recognize the work of these authors.

Finally, “*reSEARCH*” is an endless process, and we always end up with inconclusive work which paves the way for a further search. This thesis details several

pieces of such work and I have tried to bring an essence of completion to each and every aspect by proposing a next step.

1.2. GROWTH HORMONE - HISTORICAL BACKGROUND

According to religious beliefs, the world was first populated by a race of giants who were the product of divine and human mating. An early view was that modern man represents a degraded form of this original stronger and more noble race (reviewed by Daughaday, 1992). It is not an exaggeration to state that men and women of unusual height (gigantic or dwarf) have always attracted attention from the earliest days of human existence.

In 1886, Dr Pierre Marie, a French neurologist, described pituitary enlargement in acromegalic patients and postulated that the pituitary plays an important role in the pathogenesis of this disease (reviewed by Daughaday, 1992). This observation laid the foundation for studies of pituitary physiology. The importance of the pituitary gland with regard to linear growth came from observations made by Dr. Harvey Cushing at the Johns Hopkins Hospital in the early 1900s (as reviewed by Strobl and Thomas, 1994). In addition, bovine pituitary gland extracts were shown to promote growth in normal rats (Evans and Long, 1921 and as reviewed by Strobl and Thomas, 1994). However, it was the hormone prolactin, which was identified as a lactogen, that was thought to be the somatogen, because of the impure nature of the pituitary extracts (as reviewed by Cooke and Liebhaber, 1995). Bovine growth hormone (bGH) was first purified to homogeneity by Li and Evans (1944) and human growth hormone (hGH-N) followed later (Li and Papkoff, 1956; Li, 1957; Raben, 1957 references from the review by Strobl and Thomas, 1994). Although, bGH was somatogenic for rats, only primate growth hormones stimulated longitudinal growth in primates, and this was attributed to the biochemical differences between the primate and non-primate growth hormones (Li, 1957).

Although prolactin like substances were extracted from placentas by many investigators in the late 1950's, their nature was revealed only in the early 1960's (reviewed by Tyson, 1972). Chorionic somatomammotropin (CS) was initially identified as placental lactogen (PL) (Josimovich and MacLaren, 1962) and/or chorionic growth

hormone-prolactin (CG-P) (Kaplan and Grumbach, 1964). In 1967, the name “chorionic somatomammotropin” was coined to signify the origin and known biological properties of the PL or CG-P (Tyson, 1972).

Amino acid sequencing of hGH-N (Catt et al. 1967; Li et al. 1969a; Sherwood, 1967), prolactin (Jacobs and Niall, 1975; Shome and Parlow, 1977), hCS (Catt et al. 1967; Li et al. 1971; Niall et al. 1971; Sherwood, 1967) and ovine prolactin (Li et al. 1969b,c) led to the identification of common structural features among these proteins, and this strongly suggested that these genes originated from a common ancestral gene. Furthermore, these polypeptides also share biological functions and show cross-immunoreactivity (Niall et al. 1973). Thus, these genes were grouped into a superfamily.

1.3. THE GROWTH HORMONE AND PROLACTIN GENE SUPERFAMILY

The growth hormone (GH) and prolactin (PRL) genes appear to have evolved from a common ancestral gene in many species of vertebrate animals. In primates, the family consists of GH, GH-like chorionic somatomammotropins (CS) and PRL genes, whereas in non-primates the family is GH, PRL and PRL-like placental lactogens (Table. 1.1). Based on the amino acid and gene organization, two other members, proliferin and somatolactin, were included in the GH/PRL gene family (Linzer and Nathans, 1985; Ono et al. 1990; Takayama et al. 1991). The GH, PRL and somatolactin genes are exclusively expressed in the pituitary gland. There are four members of the GH/CS gene family which are expressed during pregnancy in the placenta (Parks, 1989). The proliferin gene is expressed in proliferating murine fibroblasts and placenta (Linzer and Nathans, 1985; reviewed by Wallis et al. 1992). Recent studies report the presence of two GH-N gene alleles in the genomes of sheep and goats (Wallis et al. 1998). However, a variation was observed among strains of sheep for the presence of duplicated GH genes, and it is not known whether this duplication is equivalent in sheep and goat (Wallis et al. 1998).

Table. 1.1. GH/PRL gene superfamily

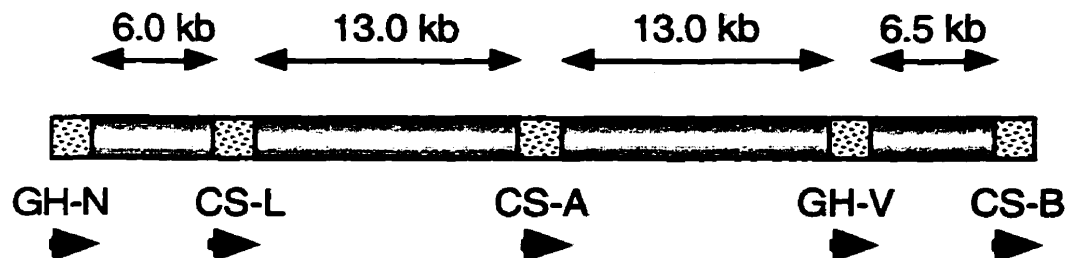
<u>Gene</u>	<u>Species</u>	<u>Tissue of expression</u>
<u>GH/GH-like</u>		
GH-N	Most vertebrates	Anterior pituitary
GH-Variant	Human (Primates)	Placenta
CS-A/-B/-L	Human (Primates)	Placenta
GH2-N	Sheep	?
GH2-Z	Sheep	?
gGH2	Goat	?
gGH3	Goat	?
<u>GH/PRL-like</u>		
Somatolactin	Teleost fish	Pituitary
<u>PRL-like</u>		
Prolactin	Most vertebrates	Anterior Pituitary
PL-I	Mouse, rat	Placenta
PL-II	Mouse, rat, hamster	Placenta
PL-I variant	Rat	Placenta
Proliferin	Mouse	Placenta, some proliferating cells
PRP	Mouse	Placenta
PLP-A/-B/-C	Rat	Placenta
PL	Cow, Sheep	Placenta
PRP-I to VI	Cow	Placenta
PL, placental lactogen; PLP, Prolactin-like protein; PRP, prolactin-related protein.		

1.4.1. STRUCTURAL FEATURES OF GH/CS GENE FAMILY

The GH gene is specific to vertebrate animals. With the exception of primates, most animal species possess only one GH gene. Primates have a cluster of physically-linked five genes on the same chromosome. In humans, the GH/CS genes are located on the long arm of chromosome 17q22-24 and span 48 kilo bases (kb) (Barsh et al. 1983; Chen et al. 1989; George et al. 1981; Hirt et al. 1987; Owerbach et al. 1980). These five genes, from 5' to 3', are pituitary growth hormone (GH-N or GH1), chorionic somatomammotropin-Like (CS-L or CSHL1 or CS5 or PL-1), CS-A (CSH1 or CS1 or PL-4), GH-Variant (GH-V or GH2), and CS-B (CSH2 or CS2 or PL-3), in that order (Fig. 1.1). However,

according to HGMW-approved gene nomenclature, these genes are now to be represented as GH-N, CS-L, CS-A, GH-V and CS-B. Throughout the study this nomenclature was used.

Fig. 1.1. The human GH/CS gene locus on chromosome 17. All five genes are shown with arrow heads indicating the transcriptional orientation of the genes. The intergenic distances are also shown. Scale 1 inch = 10 kb



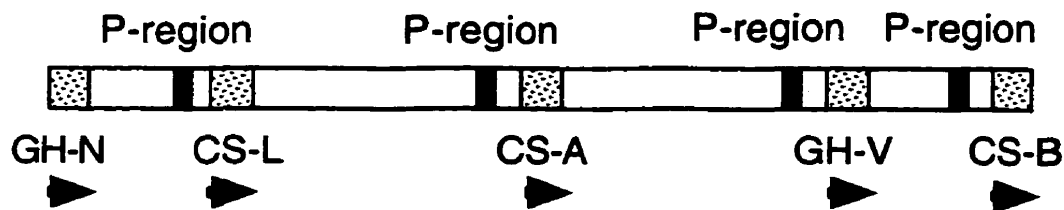
The entire nucleotide sequence (66 kb) of the GH/CS gene cluster has been described (Chen et al. 1989). These genes are arranged in the same transcriptional orientation and are highly homologous, sharing 91-99% nucleotide sequence identity both in their primary transcripts and immediate 5'-flanking regions (Chen et al. 1989; Hirt et al. 1987; Seeburg, 1982). Furthermore, these five genes also share common genomic structure in terms of exon and intron organization. Each gene consists of five exons and four introns with a primary transcript of about 1.6 kb (Barrera-Saldana et al. 1982; Chen et al. 1989; DeNoto et al. 1981; Hirt et al. 1987; Miller and Eberhardt, 1983; Seeburg, 1982). Despite their sequence similarity, the GH/CS genes are expressed in different tissues. The GH-N gene is expressed efficiently in the somatotrophs of the anterior pituitary gland (Nelson et al. 1988; Pelletier et al. 1978; Simmons et al. 1990) and the CS/GH-V genes are expressed in syncytiotrophoblasts of the placenta (McWilliams and Boime, 1980; Parks, 1989). GH-N/CS-L and GH-V/CS-B genes are separated by intergenic distances of 6.0 to 6.5 kb, whereas CS-L/CS-A and CS-A/GH-V genes are interspersed with 13 kb of DNA

(Fig. 1.1, Chen et al. 1989).

Chen et al (1989), while presenting the sequence data for the GH/CS gene locus, identified some interesting regions, including both highly repetitive as well as conserved domains within the locus, and suggested these regions might be involved in the differential and tissue-specific regulation of GH/CS genes. The following is a list of putative regulatory regions including those discussed by Chen et al. (1989):

1. Approximately 21% of the GH/CS locus contains 48 copies of the 300 bp *Alu* middle repetitive DNA elements. It was thought that the repeat sequences are responsible for the duplication of the human GH-N gene.
2. Another repetitive region with a highly conserved sequences is the "P-region". A 1.0 kb element which was localized to a region 1.7-2.1 kb upstream of the CS-L, CS-A, GH-V and CS-B genes, and is absent from the 5' upstream sequences of the GH-N gene (Fig. 1.2). As this sequence is primarily associated with the placental GH genes, the region was named the "P-region". It was suggested that this P-region may contribute to the exclusive placental expression of the placental GH genes.

Fig. 1.2. P-sequence region is located upstream of the placenta-specific CS/GH-V genes. All five GH/CS genes are shown. The P-region is shown as filled dot boxes. Scale 1 inch = 10 kb



-
3. A 30-50 bp long poly-purine (A_nG) was identified upstream of CS-L, CS-A and CS-B genes. These poly-purine sequences were suggested to affect the chromatin structure

(Gross and Garrard, 1988 as reviewed by Chen et al. 1989).

4. A *KpnI* repeat element (~200 bp) located in the 5'-flanking sequences of the CS-B gene, was suggested to contribute to the reduced expression of the CS-B gene.

5. Another characteristic element includes a 50 bp CCCCA-rich sequence located 650 bp upstream of the transcription start site of the GH-V gene. A shorter version of this sequence is also located in a similar location upstream of the CS-L gene, suggesting their possible role in the significant lower expression of these genes compared to CS-A and CS-B genes, and

6. Although not identified by Chen et al (1989), the CS-L, CS-A and CS-B genes are also characterized by the presence of another 1.0 kb sequence with 95% homology, approximately 2.2 kb downstream of these genes which has been characterized as an enhancer region (Walker et al. 1990).

1.4.2. STRUCTURAL FEATURES OF THE PROLACTIN GENE

The human prolactin (PRL) gene is unique in the haploid genome and is located on chromosome 6 (Owerbach et al. 1981). The PRL gene is also similar to GH/CS genes in terms of genomic organization with five exons (Cooke et al. 1980; 1981). Rodent and ruminant animal species contain a family of PRL-related genes (reviewed by Wallis et al. 1992). Many of these genes are exclusively expressed in the placenta (Table. 1.1).

1.5. EVOLUTION OF THE GH/PRL GENES

The GH and PRL genes were grouped into a common family based on their protein sequence similarity, shared biological functions and immuno-crossreactivity. Comparisons of mRNA sequences for rat GH (Seeburg et al. 1977) and PRL (Cooke et al. 1980; Gubbins et al 1980), human GH (Martial et al. 1979; Roskam and Rougeon, 1979) and CS

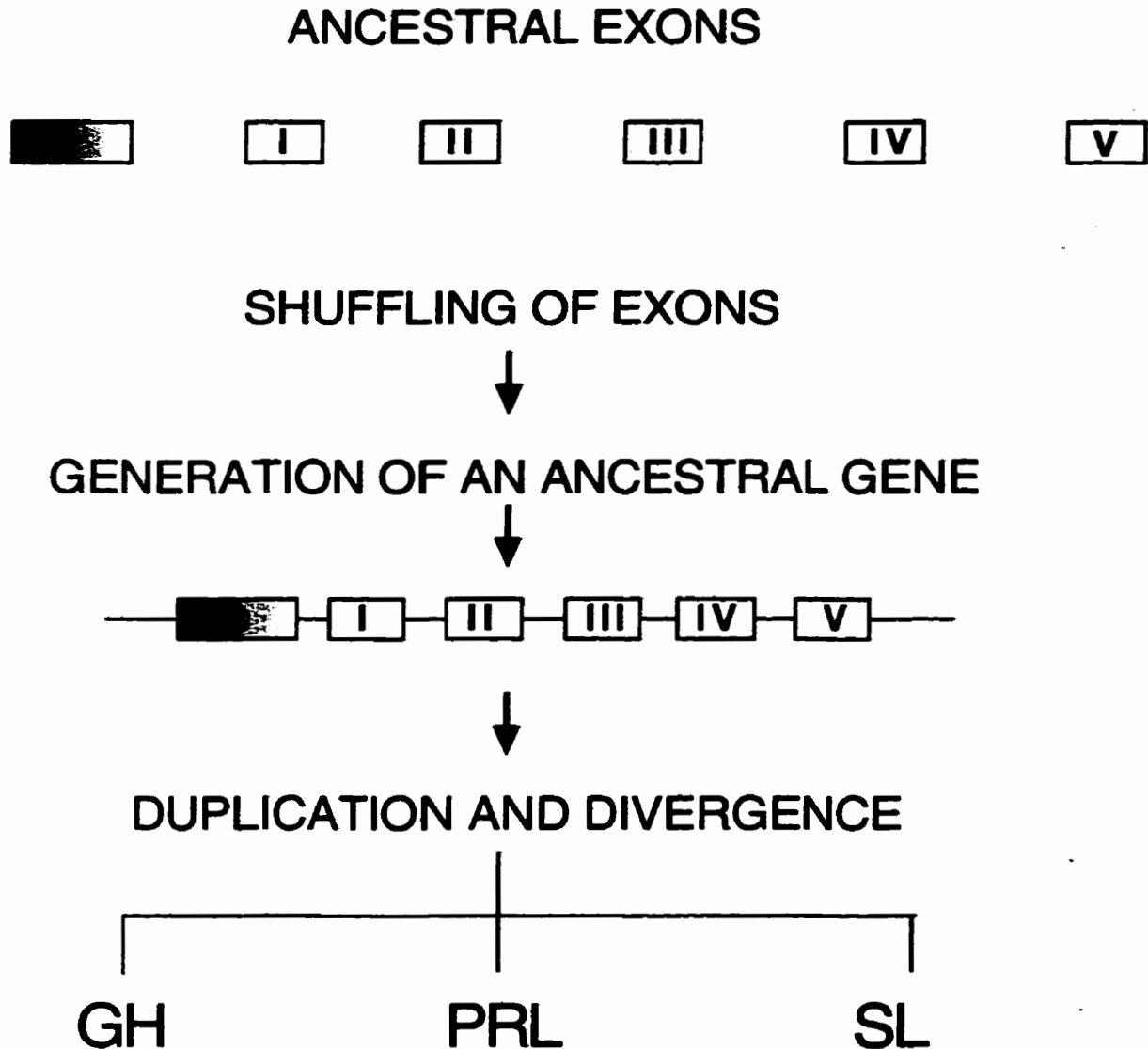
(Shine et al. 1977), as well as bovine GH (Miller et al. 1980a) and PRL (Miller et al 1980b; Nilson et al. 1980), not only confirmed earlier grouping, but also strongly suggested that these genes might have evolved from a common ancestral gene. In humans, GH-N and PRL share 35% homology at the amino acid level (Bewley et al. 1972; Shome and Parlow, 1977), whereas these genes show 41% homology at the mRNA level (Hirt et al. 1987). It was estimated that the GH-N and PRL genes could have duplicated from an ancestral gene over 350 million years ago (Barsh et al. 1983; Cooke et al. 1980; Miller and Eberhardt, 1983). This duplication resulted in two separate precursors, one for PRL and the other for the GH/CS genes in humans. These precursors then segregated onto two different chromosomes, hence, the GH/CS and PRL genes are present on chromosome 17 and 6, respectively, in humans (Chen et al. 1989; Owerbach et al. 1981). This segregation event appears to have occurred in all species of vertebrate animals.

The notion that a common ancestral gene existed for the GH and PRL genes was further supported with the identification of the somatolactin (SL) gene in the pituitaries of teleost fish, including salmon and cod (Ono et al. 1990; Takayama et al. 1991). Amino acid sequence revealed that somatolactin is 24% identical to the GH-N and PRL hormones of the fish (Ono et al. 1990). Interestingly, this gene also has a five-exon structure which is similar to GH-N and PRL gene organization. It was suggested by Takayama et al (1991) that somatolactin may be the ancestral gene or have originated from an ancestral gene, which preceded the divergence of GH-N and PRL genes (Fig. 1.3). These findings also support the suggestion that duplication of GH-N and PRL occurred before the divergence of fish and tetrapods (Cooke et al. 1980; 1981). However, the function of somatolactin is still not known.

From the above observations, it is evident that the GH/PRL and somatolactin genes originated from an ancestral gene. Since the transcription factor GHF-1/Pit-1 is important for expression of all these genes, the conservation of this factor in the pituitary in all species of vertebrate animals also signifies a close relationship between these genes (Cohen et al. 1996; Takayama et al. 1991).

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Fig. 1.3. Evolutionary model for growth hormone (GH), prolactin (PRL) and Somatolactin (SL) genes. An ancestral gene was formed by shuffling of five prototype exons and a regulatory element. Subsequent duplication and divergence of the ancestral gene generated GH, PRL and SL genes before the divergence of vertebrates.



1.6. DUPLICATION OF THE PRIMATE GH PRECURSOR GENE

After the evolution of primates from other mammals, the primate GH precursor gene, in a peculiar event involving three duplications and a gene non-reciprocal cross-over, gave rise to five different GH/CS genes, which bear high sequence homology (Barsh et al. 1983; Hirt et al. 1987; Seeburg, 1982; Selby et al. 1984). It is believed that the CS genes arose after divergence of rodents and primates 80 million years ago (Moore et al. 1982) and as recently as 10-15 million years (Barsh et al. 1983; Chen et al. 1989). As stated earlier, this duplication is peculiar to primates with the exception of caprine species (Wallis et al. 1998). During the evolutionary process, the primate GH precursor gene acquired the *Alu* middle repeat sequences and it was proposed that the presence of these sequences might predispose the locus to duplication events, resulting in the GH-N, CS-L, CS-A, GH-V and CS-B genes (Chen et al. 1989). Furthermore, during the duplication process, other sets of repetitive sequences such as P-region and CS-enhancer sequences were inserted into the GH/CS locus (Chen et al. 1989). It was suggested that the CS-A gene is the most recent in origin among the GH/CS genes (Chen et al. 1989).

Although duplication of the GH gene and the presence of the CS genes in the rhesus macaques was suggested earlier (Parks, 1989), it was Golos and co-workers (Golos et al. 1993) that were the first to isolate and characterize the five GH/CS genes from the rhesus monkey pituitary and placenta. These genes also follow a similar pattern of expression as that of the hGH/CS genes. However, the chromosomal localization and genome organization of the monkey GH/CS genes are unknown, and so the extent of the similarity between the monkey and human genomes is yet to be determined.

Interestingly, it was speculated that the GH/CS gene duplication occurred more rapidly than was necessary for a similar duplication process of the human beta-like globin gene family (Barsh et al. 1983). However, the major advantage and requirement of the GH/CS gene duplication in primate species is still not clear, as the physiological role and essentiality of the CS hormones are still unknown.

1.7. GH/CS GENE EXPRESSION AND THE POLYPEPTIDE PRODUCTS

The GH-N gene is expressed primarily in the somatotroph in the anterior pituitary gland, whereas the GH-N related CS/GH-V genes are exclusively expressed in the placental villi syncytiotrophoblasts. According to sequence information available, the GH-N, CS-L, CS-A, GH-V and CS-B genes will encode a similar size heterogenous primary transcript of 1650 nucleotides which is further processed into a mature mRNA 800 nucleotides in length (Chen et al. 1989; DeNoto et al. 1981). It was observed that multiple forms of mRNA from GH-N (Cooke et al. 1988a; DeNoto et al. 1981), CS-A/CS-B (MacLeod et al. 1992), GH-V (Cooke et al. 1988a, b; MacLeod et al. 1991) and CS-L (Misra-Press et al. 1994) arise due to alternative splicing. The GH/CS transcripts were shown to have a poly (A) tail of 80 nucleotides at the 3'-end (Boime and Boguslawski, 1974; Boime et al. 1975) and 60- and 100 bp untranslated regions (UTR) in their 5'- and 3'- ends (Seeburg, 1982).

1.7.1. The pituitary GH-N

The GH-N gene encodes predominantly, a protein of 22 kDa in the pituitary, consisting of a 191 amino acid polypeptide (Li et al. 1971; Niall et al. 1971). The biologically active conformation of hGH-N is maintained by two disulfide bridges (Li et al. 1971; Niall et al. 1971). However, 10% of the GH-N transcripts undergo alternate splicing, resulting in a 20 kDa protein that has an amino acid sequence identical to 22 kDa hGH except for a 15 amino acid internal deletion from residues 32 to 46 (Chapman et al. 1981; Lewis et al. 1978; Lewis et al. 1980; Singh et al 1974). Both 22 and 20 kDa GH-Ns are capable of stimulating growth in transgenic mice (Stewart et al. 1992). However, the 20 kDa form does not bind to GH receptors with high affinity in human liver (Strobl and Thomas, 1994).

1.7.2. The placental CS-A and CS-B

The CS-A and CS-B genes express essentially an identical protein (Barrera-Saldana et al. 1983). However, the CS-A and CS-B mRNA and their corresponding cDNAs bear nucleotide differences, which were utilized to differentiate the levels of expression of these genes in the term placenta. It is now well established that there is a switch in the relative levels of these gene transcripts from early pregnancy (8 weeks), where both CS-A and CS-B levels are equal, to term where CS-A levels are six times higher than CS-B (Barrera-Saldana, 1998; Chen et al. 1989; MacLeod et al. 1992; Martinez-Rodriguez et al. 1997). CS is the major polypeptide hormone produced in placenta accounting for 10-20% of the total placental protein production (Seeburg et al. 1977; Barrera-Saldana et al. 1982). Synthesis of CS messenger RNA is generally considered as a function of gestation (Boime et al. 1976). The structure of the polypeptide is similar to GH-N protein. Alternatively spliced forms of mRNA for CS-A and CS-B genes were also reported (MacLeod et al. 1992). In these low abundance splice variants, intron 4 is retained resulting in a transcript size of 1250 nucleotides (MacLeod et al. 1992).

1.7.3. The placental GH-V

The GH-V gene was initially assumed to be a pseudogene (Seeburg, 1982). Frankenne et al. (1987) were the first to provide evidence for GH-V expression in the placenta. Later the GH-V cDNA was isolated from a placental library (Chen et al. 1989; Igout et al. 1988). GH-V is also expressed as a 22 kDa protein in the placenta (Igout et al. 1989). An alternative form of the GH-V transcript, GH-V2, which represented 30% of total GH-V transcripts, was also reported (Cooke et al. 1988a, b; MacLeod et al. 1991). GH-V2 retains intron 4 resulting in a 1250 nucleotide transcript, and the size of the protein was predicted to be 26 kDa (Cooke et al. 1988a, b; MacLeod et al. 1991). During pregnancy, GH-N in the maternal circulation is replaced by GH-V and the GH-N completely disappears by midpregnancy (Frankenne et al. 1990; Hennen et al. 1985; Jara et al. 1989; Mirlesse et al. 1993).

1.7.4. The placental CS-L

The CS-L gene is a pseudogene (Hirt et al. 1987). Although the CS-L specific mRNA was detected in the placenta (Chen et al. 1989), the corresponding CS-L protein is yet to be identified. The CS-L gene undergoes a complex set of alternative splicing events generating at least five major splice variants (Misra-Press et al. 1992). It was suggested that the majority of processed CS-L mRNA is non-functional (Misra-Press et al. 1992). It is intriguing to observe that the promoter for CS-L is as active as the CS-A and CS-B promoters *in vitro* (Selvanayagam et al. 1984). Both CS-L and GH-V genes are expressed at very low levels relative to CS-A/-B genes in the placenta (Chen et al. 1989).

1.8. PHYSIOLOGICAL IMPORTANCE OF PITUITARY GH-N

The functional significance of pituitary GH-N is very obvious. Being a vertebrate hormone, the GH-N is required for the linear growth of an individual. However, GH-N is not an essential hormone for life itself, because a complete deletion of the GH-N gene results in only short stature for an individual. Furthermore, GH-N is not required during pregnancy and is not involved in normal intrauterine growth and development of the fetus (Curran et al. 1998; Gluckman et al. 1981). However, GH-N is present in the fetal circulation from the 12th week of gestation, and by midpregnancy plasma concentrations are in the 100 ng/ml range and then fall late in gestation (Cornblath et al. 1965). Despite the abundance of GH-N in the circulation, the hormone is not thought to play a major role in fetal growth because pituitary aplasia and congenital hypopituitarism do not cause severe growth retardation (Goodman et al. 1968; Lovinger et al. 1975). It is also known that GH-N can not cross the placental barrier (Fholenhag et al. 1994). In contrast, the role of GH-N in postnatal growth of an individual is well known.

GH-N regulates growth through effects on tissue differentiation, cell proliferation, and protein synthesis (reviewed by Strobl and Thomas, 1994). These effects bring either

hypertrophy, hyperplasia, or both in target tissues like bone, cartilage and skeletal muscle. Furthermore, GH-N can exert its effects directly or through the actions of a mediator, insulin-like growth factor-I (IGF-I), which is primarily produced in the liver. GH-N also elicits many metabolic effects such as stimulation of lipolysis, protein synthesis and modulation of carbohydrate metabolism and fluid electrolyte balance. Growth hormone has an anti-insulin diabetogenic effect.

Previously, it was thought that GH-N stimulates the expression of IGF-I from liver, and via IGF-I, promotes linear growth by stimulating the prechondrocytes in the epiphyseal plate. However, later it was proved that the GH-N can act directly or stimulate local production of IGF-I at epiphyseal plate as well as increase somatic growth (Isaksson et al. 1987). GH-N also stimulates the differentiation of preadipocytes to adipocytes (Hauner et al. 1992). In children and adults, GH-N initiates lipolysis (reduces the fat deposits) and increases lean body mass. GH-N also stimulates many genes including IGF-I (reviewed by Isaksson et al. 1985), c-fos and c-jun (Slootweg et al. 1991), serine protease inhibitor (Yoon et al. 1987) and serum response factor and Elk-1 (Liao et al. 1997). Regulation of expression of these genes by GH-N occurs through GH-receptor signaling. GH-N is also expressed in lymphocytes (Lytras et al. 1993; Weigent et al. 1988) suggesting a role for GH-N in immunological responses. However, GH-N-deficient patients do not exhibit gross deficiencies in immunological responses (Spadoni et al. 1991). GH-N also has a role in normal human reproduction (Reviewed by Katz et al. 1993).

1.9. PHYSIOLOGICAL SIGNIFICANCE OF PLACENTAL CS/GH-V

Although the physiological importance of GH-N is known, the absolute requirement of placental GH/CS during pregnancy has not been established. However, there are many suggestions regarding the role of CS/GH-V proteins in fetal growth and metabolism. Very high levels of CS in the maternal versus fetal circulation strongly suggests a higher tendency of CS secretion into the maternal circulation than into the fetal

circulation. However, detection of CS in the fetal circulation suggests a possible anabolic role for CS (Hill et al. 1988). CS, like GH-N, will also increase insulin secretion, impair glucose tolerance and cause retention of nitrogen during pregnancy (reviewed by Hill, 1992). CS is also lipolytic and this may be a rescue mechanism in maternal starvation (Williams and Coltart, 1978). The fetal tissues express CS-specific-receptors and CS is able to stimulate amino acid uptake and glycogenesis in fetal tissues (Freemark et al. 1988; Freemark and Comer, 1989; Hill et al. 1985; 1986; 1988). CS protein secretion is not pulsatile like that of GH-N, however, spontaneous variations in the levels of CS hormones during pregnancy were reported (Vigneri et al. 1975). The physiological relevance of this nature is not known.

The physiological role of placental growth hormone (GH-V) is poorly understood. The GH-V does not cross the placenta/fetal barrier and is not detected in the fetus or in cord blood of the newborn (Frankenne et al. 1988). As stated earlier, GH-V completely replaces pituitary GH-N in the maternal circulation during pregnancy by the 25th week (Frankenne et al. 1990; Hennen et al. 1985; Jara et al. 1989; Mirlesse et al. 1993). GH-V is also nonpulsatile (Erikson et al. 1989), unlike GH-N which is highly pulsatile. The level of GH-V in the maternal circulation plateaus by the 35th week of pregnancy and rapidly disappears with the onset of labor (Mirlesse et al. 1993). Recombinant GH-V (22 kDa) had similar somatogenic effect on rabbit and rat cells as that of GH-N, but had a different lactogenic activity on the rat (Igout et al. 1995).

Both CS and GH-V were shown to have somatogenic and lactogenic properties (Friesen, 1966; Hill et al. 1985; 1988; MacLeod et al. 1991; Nickel et al. 1990a; Tyson, 1972 and references within). Specific-receptors for these hormones in fetal tissues were identified (Freemark et al. 1988; Freemark and Comer, 1989; Hill et al. 1985; 1986; 1988), suggesting their role in fetal growth. However, the actual physiological significance of these hormones is yet to be determined.

1.10. GROWTH HORMONE RECEPTOR AND SIGNALING

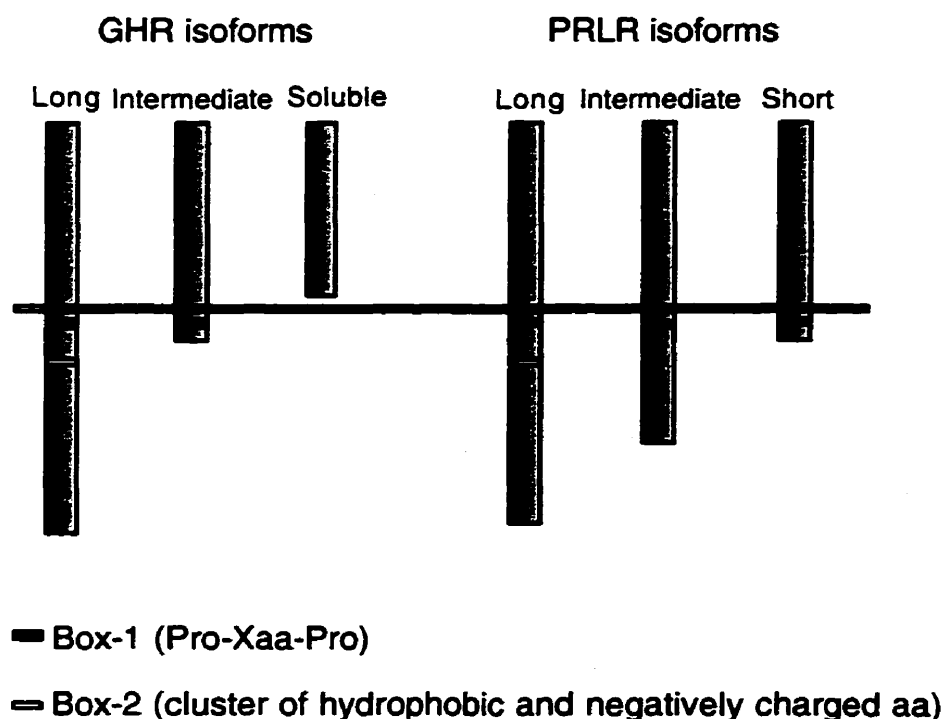
To exert its biological functions, GH-N has to bind to receptors on the target cells. The cDNA encoding the growth hormone receptor (GH-R) was cloned by Leung et al. (1987). The human GH-R is a single polypeptide receptor with a unique transmembrane domain (reviewed by Goffin and Kelly, 1996). The PRL receptor (PRL-R) was also cloned and found to be similar to the GH-R (Boutin et al. 1988; 1989). The GH-R and PRL-R belong to the class I type of cytokine-receptor superfamily (reviewed by Moutoussamy et al. 1998 and references therein). The Class I receptors are characterized by four conserved Cys residues in the amino-terminal part of their extracellular domain and the presence of a conserved WSXWS (Trp-Ser-Xaa-Trp-Ser) motif in the C-terminal part. In several species of animals, the extracellular domain of the GH-R exists as a soluble receptor in the circulation and is known as GH-binding protein. The GH-N/GH-R complex had been crystallized and the complex contained a molecule of GH interacting with a homodimer of GH-R.

Once GH-N binds to the receptor, rapid induction of phosphorylation of several cytoplasmic proteins occurs. Although GH-R is not a tyrosine kinase receptor, the phosphorylation is initiated by the activation of specific-receptor associated kinase, JAK2, which belongs to a family of kinases known as *Janus kinases* or “*just another kinase*” (JAKs). Furthermore, the GH-R activated JAK2 phosphorylates a set of transcription factors known as *signal transducers and activators of transcription* (STATs). GH-N activates STAT1, STAT3 and STAT5 (both a and b isoforms). STATs are phosphorylated on their Tyr and Ser residues by JAKs and phosphorylated forms are translocated into the nucleus, where they modulate the expression of GH-N target genes. STATs bind to DNA elements with a consensus sequence of TTCCNGGAA.

It was largely thought that IGF-I is the mediator of GH-N physiological function. However, it is still not clear how GH-N and GH-R signaling induces transcription of the IGF-I gene.

Prolactin (PRL), which belongs to GH/PRL hormone superfamily, also binds to a class I type of cytokine receptor. In most species, GH-N binds to somatogen receptors and PRL binds to lactogen receptors (Ray et al. 1990). However, hGH-N is an exception, since it binds to both somatogen and lactogen receptors (Shiu et al. 1973). Although, it was originally thought that the binding of GH-N to its receptor was restricted to liver, it was found that the GH-R is expressed in a wide variety of cells (reviewed by Kelly et al. 1993). Similarly, the PRL receptors (PRL-Rs) are widely distributed in several cells and tissues of mammals (reviewed by Kelly et al. 1993). At least three forms of both GH-N and PRL receptor proteins were identified (Fig. 1.4). Although distinct receptors for CS were identified in fetal tissues (Freemark et al. 1988; Freemark and Comer, 1989; Hill et al. 1985; 1986; 1988), their functional role is yet to be characterized. GH-V was also shown to bind to both somatogenic and lactogenic receptors, however, with different affinities than that of GH-N (Ray et al. 1990).

Fig. 1.4. Structure of growth hormone and prolactin receptor family.



An interesting observation was also made with the genes encoding GH-N and PRL receptors. Both the GH-R and PRL-R genes co-localize to chromosome 5p13-14, suggesting that these proteins may be encoded by a cluster of related genes that evolved from a common ancestral gene, in a manner consonant with that of their ligands (Arden et al. 1990; Barton et al. 1989).

It was reported that the GH-R receptor can also translocate into the nucleus, but the functional significance of this is yet to be elucidated (Lobie et al. 1994 a, b). This observation is similar to that observed with other cytokines and growth factors (reviewed by Jans and Hassan, 1998).

1.11. GENE DELETIONS IN THE HGH/CS GENE CLUSTER

A deletion of the GH-N gene can be recognized immediately by looking at the appearance of an individual. Several cases of GH-N gene deletion in a 7.5 kb GH-N gene deletion or a 45 kb hGH/CS gene deletion were reported (Akinci et al. 1992; Braga et al. 1986; Cacciari et al. 1994; Ghizzoni et al. 1994; Mullis et al. 1992). Although rare, several cases of complete to very low maternal concentrations of CS/GH-V hormones were reported and in all these cases the babies born were healthy and the pregnancy progressed without any obvious complications (Alexander et al. 1982; Alsat et al. 1997; Barbieri et al. 1986; Borody and Carlton, 1981; Frankenne et al. 1986; Gaede et al. 1978; Giampietro et al. 1984; Hubert et al. 1983; Nielsen et al. 1979; Sideri et al. 1983; Simon et al. 1986; some of these references are from Rygaard et al. 1998). Furthermore, analysis of the genomic DNA from some of these individual cases revealed various types of deletions in the GH/CS gene cluster (Alsath et al. 1997; Nielsen et al. 1979; Parks et al. 1985; Rygaard et al. 1998; Simon et al. 1986; Wurzel et al. 1982). Recently, a case with a complete lack of the CS-A/-B and GH-V genes but with intact GH-N and CS-L genes resulted in a severely growth retarded but otherwise normal baby (Rygaard et al. 1998). This is the only clinical report where severe growth retardation was reported. In another case with a similar type of

deletion, the pituitary GH-N gene was shown to be expressed in the placenta (Alsat et al. 1997). However, Rygaard et al (1998) did not discuss GH-N gene expression. In both cases, no CS-L transcript in the placenta or CS-L protein in the maternal circulation was detected (Alsat et al. 1997; Rygaard et al. 1998).

1.12. TRANSCRIPTIONAL REGULATION OF THE GH-N GENE

1.12.1. General principles involved in transcriptional regulatory mechanisms

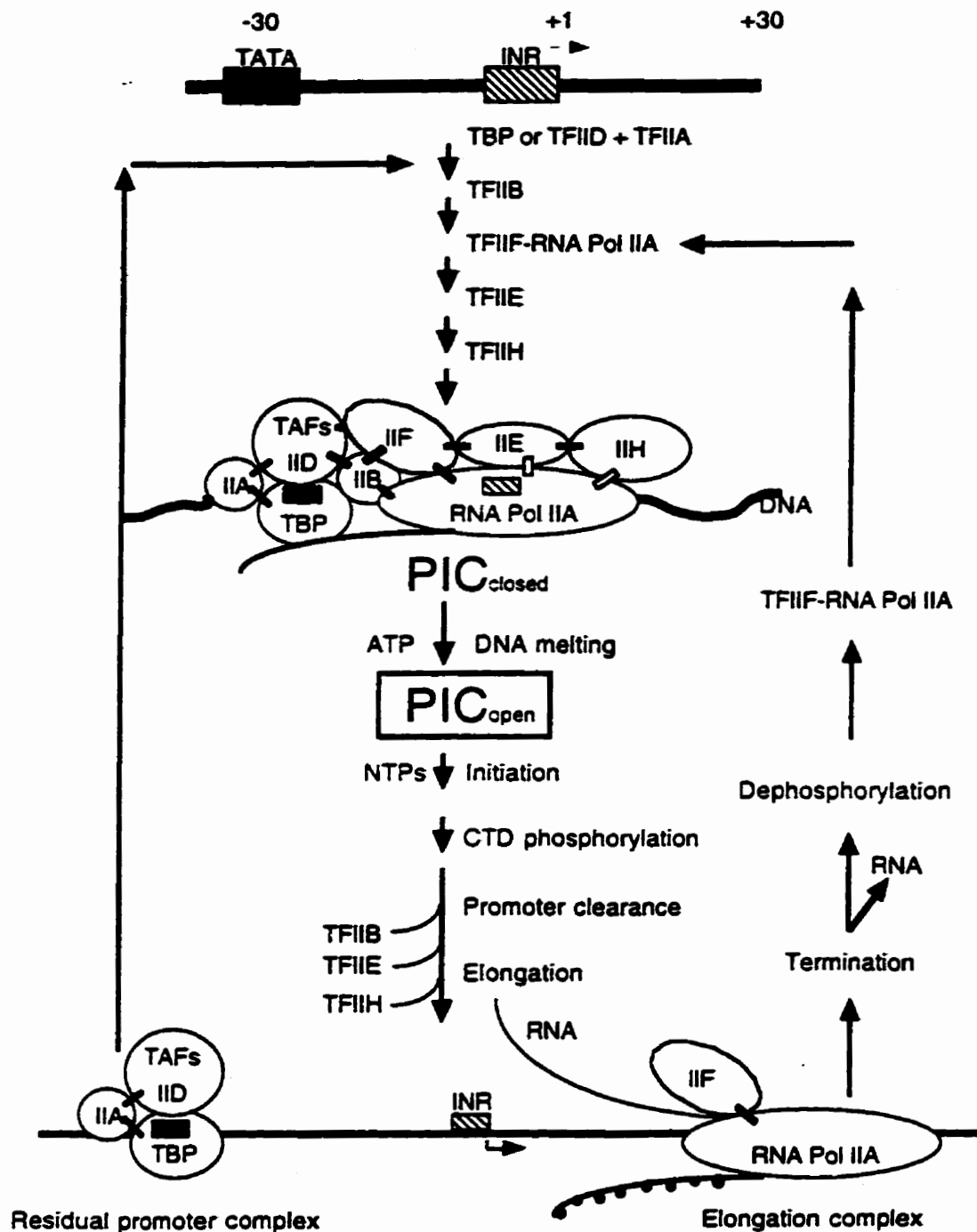
During the evolution of multicellular organisms, different cell types and tissues acquired some characteristics and peculiarities that allow them to carry out specialized functions. In general terms, a function or characteristic is the result of the action of a protein or set of proteins, which in turn is/are the products of genes. Therefore, these cells or tissues express a gene or set of genes in a cell or a tissue-specific manner as well as in spatial and temporal manner. Although all cells in an individual possess the same genetic material, there are several regulatory mechanisms including transcriptional, post-transcriptional, translational and post-translational which can play an important role in the expression and function of a particular gene. With the recent developments in molecular biology, it is now evident that the expression of a particular gene is primarily regulated at the transcriptional level.

The genes which code for a protein are transcribed by RNA polymerase II (RNAPII), and the regulatory mechanisms involved in this process are well studied. Generally, the information necessary to regulate the expression of a particular gene is present in the sequences immediately upstream of the transcriptional initiation site of a gene. These sequences are classified into core-promoter and enhancer/silencer regions. Analysis of these sequences for many genes revealed various regulatory elements. Further studies on these regulatory sequences led to the identification of both general and cell- or tissue-specific transcriptional mechanisms involved in the expression of a gene.

Core-promoter elements are 'minimal DNA sequences required for accurate transcription initiation by RNAPII in reconstituted cell-free systems' (Roeder, 1996). Most of the core-promoter sequences contain a TATA box (consensus TATAa/tAa/t) located 30 bases upstream of the transcription initiation site. Furthermore, an initiator element (Inr, consensus YYANt/aYY) located near the transcription initiation site was also shown to be important for transcription initiation of some genes (reviewed by Roeder, 1996). However, the core-promoter of some genes contain both initiator sequences and the TATA box, whereas some promoters contain only the initiator sequence or the TATA box, and some contain neither of these two elements. In spite of these variations in the core-promoter sequences, transcription from all these promoters is initiated by the same set of general transcription factors (GTFs) which include RNAPII, TFIID (which is a complex of TATA-binding protein, TBP, and TBP associated factors known as TAFs), and TFIIB, TFIIIE, TFIIF, TFIIH. These factors bind in a sequential manner to the core promoter elements (specifically to the TATA box) and initiate accurate transcription (Fig. 1.5). However, recent results indicate that the RNAPII exists as a "holoenzyme" complex which is comprised of some of the above mentioned GTFs, a group of transcriptional mediator proteins, chromatin remodeling factors, coactivators and corepressors (reviewed by Bjorklund and Kim, 1996).

In contrast, the enhancer/silencer regulatory elements are recognized by sequence-specific-DNA binding proteins or transcriptional activators. These elements are either proximal or distal promoter elements depending on their distance from the transcription initiation site of the particular gene. Several proximal promoter regions are characterized by the presence of GC-rich, AP-1 and CCAAT elements which are important for activated transcription of a gene in a cell. Many of the tissue-specific genes also possess these elements as well as elements which are recognized by the tissue-and sequence-specific activator factors. Examples of some ubiquitous activator proteins are the GC-box binding Sp1 (Briggs et al. 1986), AP-1 (Angel et al. 1987; Lee et al. 1987), NF-1/CTF-1 (Jones et al. 1987), c/EBPs (Landshultz et al. 1988), whereas GHF-1/Pit-1 (Bodner et al. 1988; Ingraham et al. 1988) is a classic example of a tissue-specific activator protein.

Fig. 1.5. Model for preinitiation complex (PIC) formation and initiation of transcription of a typical RNA polymerase II dependent protein coding gene. (Redrawn from the review article by Roeder, 1996).



Identification of most of the elements involved in transcription was based on *in vitro* studies using naked DNA. Although, these studies are necessary to identify the gene promoter sequences/elements and their respective regulatory factors, many times the information obtained is insufficient to understand the underlying stringent transcriptional controls. At present, many investigators are unraveling the transcriptional mechanisms involved at the level of DNA in its native chromatin conformation in the nucleus.

Transcription from class II genes involves complex protein-protein interactions among general (basal) transcription factors and sequence-specific transcriptional activators to bring a synergistic effect. Regulation of these transcriptional events involved in the initiation process are an area of intensive study. Except for the housekeeping genes, which are constitutively expressed in a cell, the initiation of transcription of the class II genes occurs in response to both developmental and environmental signals. Results from many laboratories had demonstrated that the transcriptional activators interact with TAFs, and this interaction enhances the recruitment and/or stabilization of TFIID on the promoter. Furthermore, there is evidence that another set of proteins, known as 'coactivators and corepressors' are also involved as adaptors between activators and basal transcription factors.

There are many components of the nucleus that are involved in the transcriptional process. They include chromatin structure, modifications of nucleosomes, nuclear matrix, enhancers and the locus control regions associated with the gene.

1.12.1.1. Interplay between chromatin structure and transcription

In an eukaryotic cell, the DNA is compacted into chromatin structure. The primary component of chromatin structure is the nucleosome which is a complex of histone proteins (H1 and core histones H2A, H2B, H3 and H4) and DNA. Chromatin structure is always in a dynamic state. An inactive gene in the ground state exists as a transcriptionally repressed chromatin structure that may be folded or compacted and inaccessible to transcription

factors, as well as resistant to nuclease treatments. In contrast, an active gene possesses a more open state, nucleosome structure is altered due to modifications of the core histones, histone H1 may be depleted and the DNA is accessible to transcription factors as well as sensitive to nucleases (reviewed by Paranjape et al. 1994). The location of nucleosomes *in vivo* is typically at specific positions along the DNA (reviewed by Simpson, 1991) and alterations in the positioning of nucleosomes are responsible for transition of inactive chromatin to a transcriptionally competent state. Recent studies have shown that an important function of promoter- and enhancer-binding factors is to relieve chromatin mediated repression of transcription. Furthermore, at the chromatin level, activated transcription of a gene is several thousand fold higher when the DNA is presented in a chromatin structure as opposed to being “naked” (Kornberg and Lorch, 1995).

1.12.1.2. Current principles of activation and repression of transcription

It is clear now that hyperacetylation of the NH₂-terminal tails of histones H3 and H4 is one of the major nucleosome alterations that occurs in transcriptionally active chromatin domains (Davie and Hendzel, 1994). The enzymes catalyzing histone acetylation and deacetylation were shown to be coactivators and corepressors. These findings are consistent with the notion that alterations in nucleosome conformation allow increased accessibility of the target gene promoters to the transcriptional machinery (reviewed by Bjorklund and Kim, 1996; Davie, 1998; Kingston et al. 1996; Kornberg and Lorch, 1995; Maldonado and Reinberg, 1995; Nikolov and Burley, 1997; Paranjape et al. 1994). Many transcriptional coactivators which act as histone acetyltransferases (HATs), include yeast GCN5 (Brownell et al. 1996), p300/CBP (Bannister and Kouzarides, 1996; Ogryzko et al. 1996), PCAF (Yang et al. 1996; Blanco et al. 1998), ACTR (Chen et al. 1997), TAF_{II}250 (Mizzen et al. 1996) and SRC-1 (Spencer et al. 1997) have been shown to interact with sequence-specific transcription factors and thus target specific genes.

Recently, corepressors such as mSin3 were identified in the complexes formed with histone deacetylases (HDAC1 and HDAC2), nuclear hormone receptor corepressors (N-

CoR and SMRT) and sequence-specific transcription factors (reviewed by Torchia et al. 1998). Although, it is known that CBP/p300 proteins interact with several transcription factors, some of the examples of transcription factors which are either activators or repressors, or both, forming complexes with HATs and/or HDACs, include the Mad family of transcription factors (Ayer et al. 1995; Schreiber-Agus et al. 1995), CREB (Kurokawa et al. 1998; Korzus et al. 1998), STAT-1 (Kurokawa et al. 1998; Korzus et al. 1998), yeast *gcn4* and herpes virus VP16 (Utley et al. 1998), NFAT (Garcia-Rodriguez and Rao, 1998), AP-1 (Lee et al. 1998), GHF-1/Pit-1 (Xu et al. 1998), Myo-D (Puri et al. 1997), p53 (Gu et al. 1997), Ets-1 (Yang et al. 1998), NF-Y (Currie, 1998) and nuclear hormone receptors (Heinzel et al. 1997; Chen et al. 1997; Kurokawa et al. 1998; Korzus et al. 1998; Nagy et al. 1997; Torchia et al. 1997). Some of these transcriptional activators which form complexes with HATs and HDACs are also important for cell and tissue development and differentiation.

The erythroid-specific transcription factor EKLF interacts with HATs such as CBP and p300. It undergoes post-translational acetylation suggesting a mechanism by which EKLF is able to alter chromatin structure and induce beta-globin expression within the beta-like globin cluster (Zhang and Bieker, 1998). A p300/CREB binding protein-related protein p270 was identified as a component of mammalian SWI/SNF complexes, suggesting the presence of both chromatin remodeling and histone-modifying enzymes at transcription sites (Dallas et al. 1998). Recently, analysis of the PCAF histone acetylase complex revealed the presence of polypeptides which were identical to TAFs (Ogryzko et al. 1998). Patients with dermatomyositis have a high malignancy rate. It was recently reported that the dermatomyositis-specific autoantigen Mi2 β exists as a protein complex which contains both histone deacetylase and nucleosome remodeling activities (Zhang et al. 1998). These observations emphasize the importance of these protein complexes in gene expression both in normal and disease conditions.

1.12.1.3. The role of the nuclear matrix and cytoskeleton in gene expression

Initial studies had suggested that the nuclear matrix provides the scaffolding structure for the chromatin. However, it appears more appropriate to regard the nuclear matrix as a dynamic entity. The nuclear matrix divides the chromatin into subnuclear domains where the replication and transcription occurs. Nuclear matrix is also involved in gene localization, the concentration and targeting of regulatory factors and the processing of RNA transcripts (reviewed by Cardoso and Leonhardt, 1998; Schul et al. 1998; Stein et al. 1996, 1998; Stenoien et al. 1998). There is strong evidence to support a linkage between the cytoskeletal structure, chromatin and nuclear architecture in the signaling pathways that modulate gene expression both in normal and abnormal cellular conditions (reviewed by Holth et al. 1998).

Studies of the nuclear matrix have led to the identification of topoisomerase I as a major component of the matrix. Furthermore, the nuclear matrix proteins bind to specific DNA regions known as matrix attachment regions (MARs). It seems that transcription factors, co-activators (HATs) and co-repressors (HDACs) are also associated with MARs (Holth et al. 1998). The histone acetyltransferase activity is also associated with the nuclear matrix (Hendzel et al. 1994). However, it is yet to be determined whether it is the general transcriptional regulatory mechanism by which MARs localize and target regulatory factors for gene activation or silencing.

1.12.1.4. Unification of all nuclear processes involved in gene expression through protein-protein interactions

RNAPII is the key player in gene expression. It is evident that RNAPII is not only involved in transcription but also in the post-transcriptional processing of the transcript. RNAPII forms complexes with the splicing (McCracken et al. 1997), CAP (Cho et al. 1997) and polyadenylation (Hirose and Manley et al. 1998) machinery. These complexes

are found concentrated in nuclear speckles which are sites for RNA processing (reviewed by Schul et al. 1998).

These observations strongly suggest that all nuclear processes are unified by forming complexes through protein-protein interactions. The whole cellular architecture including the cytoskeletal and nuclear matrix and their dynamic nature seems to play an important role in gene expression (Holth et al. 1998). However there are many fundamental questions, including identification of cellular and biological controls that aid dynamic modifications in cellular architecture resulting in the intracellular trafficking of regulatory factors to effect gene expression, that are yet to be answered.

In this context, it is interesting to note that GHF-1/Pit-1, which is one of the tissue-specific factors identified, also forms complexes with coactivators and corepressors (Xu et al. 1998). GHF-1/Pit-1 is essential for the pituitary-specific expression of growth hormone and prolactin genes.

1.12.1.5. The role of the enhancers in gene expression

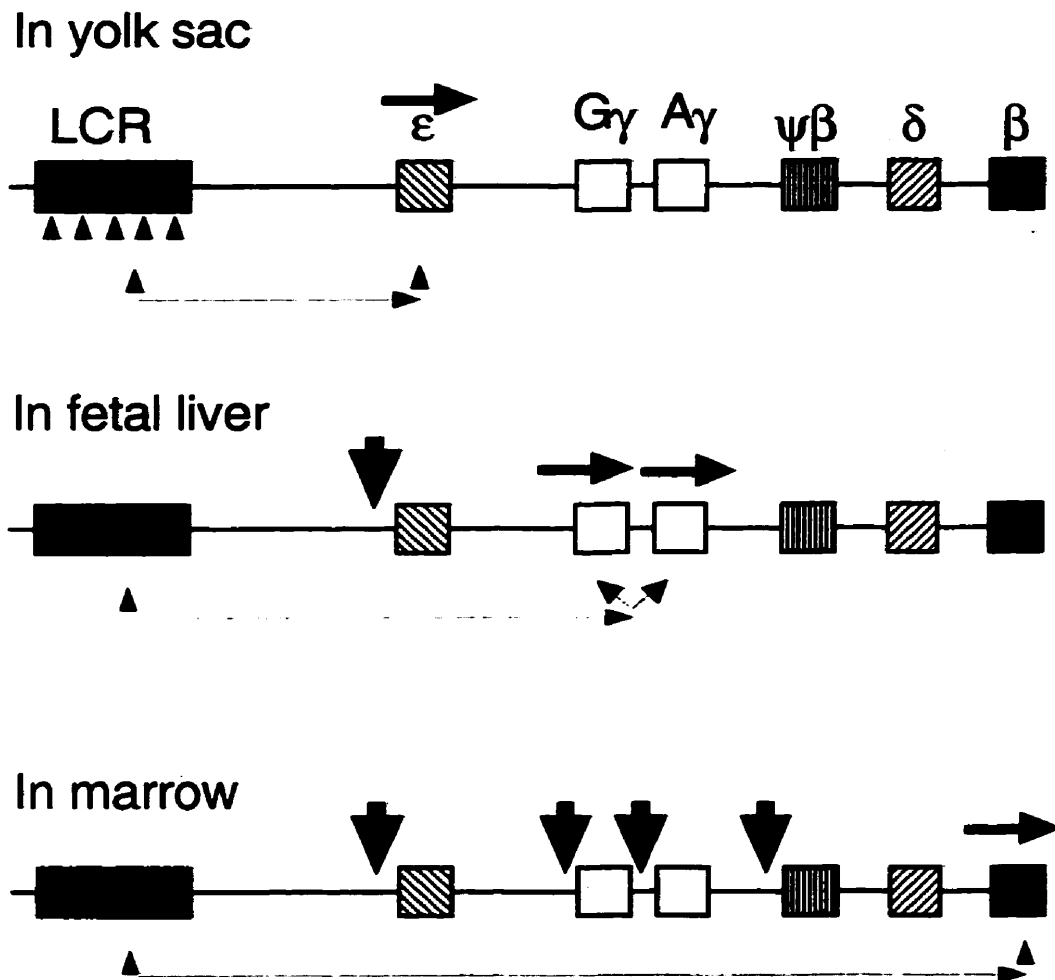
Enhancers were originally defined as cis-acting DNA-elements that enhance the transcription of a gene in a manner that is independent of their orientation and distance relative to the transcription initiation site. Occasionally, as the *Drosophila cut* gene, these enhancers can be located as far as 85 kb upstream of a gene promoter. In contrast, some enhancers lie within the gene such as the immunoglobulin *Hμ* core enhancer which lies within the second intron of the transcription unit (reviewed by Blackwood and Kadonaga, 1998). Another interesting phenomenon known as “transvection” was observed in *Drosophila* with *yellow* gene enhancers. In transvection, upon chromosome pairing, an active enhancer on one chromosome activates transcription from the homologous promoter on the other chromosome. (Ed Lewis coined the term ‘transvection’ in 1954; Morris et al. 1998).

In spite of this, the nature of enhancer activity is still not clear. It was suggested that enhancers either uniformly increase the amount of transcription in a cell in a continuous manner, or increase the probability of transcription but not the amount of transcription (reviewed by Blackwood and Kadonaga, 1998). Enhancers may function through the complex protein-protein interactions between coactivators and sequence-specific DNA binding proteins, bound to both enhancer sequences and promoter elements, and brought together by DNA-looping.

1.12.1.6. The role of locus control regions in gene expression

The identification and functional role of far-upstream sequences on gene expression was first reported through studies of a 15 kb region associated with the human β -globin gene cluster (Fig. 1.6; Grosveld et al. 1987). This cis-acting transcriptional regulatory element is known as a locus control region (LCR) (reviewed by Grosveld et al. 1998). It is becoming increasingly evident that a LCR is required for the tissue- and developmental-specificity of the transcription of genes in the β -globin gene cluster. The globin LCR is characterized by the presence of five DNase I hypersensitive sites (HSs). In stable transformation assays, the LCR confers position independent and copy number-dependent expression to genes with which it is linked. Each HS was characterized and the LCR was found to contain a plethora of recognition sites for sequence-specific transcription factors including GATA-1, NF-E2/AP-1, Sp1/EKLF/CACBP, LCRF1/Nrfl, YY1, SSP, USF, TAL1/SCL (reviewed by Hardison et al. 1997). The mechanism by which the LCR functions remains to be determined although two models were proposed for the activation of gene transcription after chromatin activation of a locus (Grosveld et al. 1998). In one model the sole function of distal elements is to generate a favorable chromatin structure which allows the promoter to bind transcription factors in a stochastic fashion. The second model involves an interaction between the LCR and individual β -globin promoters, interacting with one gene at a time (Grosveld et al. 1998). A multicomponent LCR for the GH/CS genes was identified recently (Jones et al. 1995). However, the sequence of the LCR and factors recognizing these regions are yet to be determined.

Fig. 1.6. Human β -globin switching and model showing locus control region involvement in activation of β -globin genes at different stages. Small arrow heads indicated the hypersensitive sites (HSs) 1-5 in LCR and large arrow heads in the upstream regions of the genes indicate interaction of silencing factors to these regions (not drawn to scale). A model is presented indicating interaction between LCR and individual genes at different stages of development.



1.12.2. PITUITARY-SPECIFIC EXPRESSION OF THE GROWTH HORMONE GENE

1.12.2.1. Role of GHF-1/Pit-1

The founding members of the GH/PRL gene superfamily, the GH-N and PRL genes are predominantly expressed in the pituitaries of vertebrate animals from fish to human (Takayama et al. 1991). However, these two genes are expressed in two different cell types in the pituitary. Therefore, both the GH and PRL genes are excellent models to study transcriptional regulatory mechanisms involved in both cell- and tissue-specific expression. Many laboratories were involved in the characterization of pituitary-specific regulatory elements present in both the proximal and distal upstream sequences of these genes. Initial studies with the rat GH-N and PRL (Nelson et al. 1986) as well as the human GH-N (Cattini et al. 1986; Lefevre et al. 1987) promoter sequences resulted in the suggestion that these sequences can support pituitary-specific expression. The physical evidence for the presence of pituitary-specific elements in the hGH-N promoter was first provided by Lefevre et al (1987). This observation and identification of pituitary-specific AT-rich elements led to the identification and isolation of GHF-1/Pit-1, a transcription factor belonging to a family of proteins which contain a POU-homeodomain (Bodner et al. 1988; Ingraham et al. 1988). The GHF-1/Pit-1 factor is necessary for transcription of both GH-N, PRL and thyrotropin- β (TSH- β) gene promoters (Bodner et al. 1988; Day et al. 1990; Ingraham et al. 1988; Mangalam et al. 1989; Steinfelder et al. 1991). GHF-1/Pit-1 was also shown to be absolutely required for the determination of the pituitary cell lineage as well as development of the pituitary gland (Dolle et al. 1990; Li et al. 1990; Simmons et al. 1990). Furthermore, the pituitaries from the Snell and Jackson dwarf mice, which bear mutations in the GHF-1/Pit-1 gene, are characterized by the absence of GH, PRL and TSH- β gene expression as well as hypoplasia of the somatotrophs, lactotrophs and thyrotrophs (Cohen et al. 1995; Li et al. 1990; Pfaffle et al. 1992; Radovick et al. 1992). However, in the Snell dwarf mouse, both GHF-1/Pit-1 independent and GHF-1/Pit-1 dependent TSH- β gene expression and thyrotrophs proliferation was observed (Lin et al.

1994).

During mouse embryonic development, GHF-1/Pit-1 transcripts are initially detected in the neural tube and neural plate on embryonic day (e) 10 to 11. By e15, the GHF-1/Pit-1 transcripts are present in all five cell types of the pituitary gland (Dolle et al. 1990; Simmons et al. 1990). However, GHF-1/Pit-1 protein is only detected in the somatotrophs, lactotrophs and thyrotrophs, preceding GH-N and PRL gene expression (Dolle et al. 1990; Simmons et al. 1990). Both the POU-S and POU-homeodomain of GHF-1/Pit-1 are necessary for high-affinity DNA binding as well as Pit-1-Pit-1 interactions on the GH-N and PRL genes (Ingraham et al. 1990; reviewed by Andersen and Rosenfeld, 1994). GHF-1/Pit-1 is also important for thyrotropin- β -subunit (TSH- β) gene expression (Steinfelder et al. 1991). Recently, a novel, tissue-specific, paired-like homeodomain transcription factor, termed Prophet of Pit-1 (Prop-1) was shown to be important for initial pituitary lineage determination and an apparent failure of this step resulted in the Ames (df) dwarfism (Sornson et al. 1996).

The rat GH promoter contains two GHF-1/Pit-1 sites, at a proximal (-96/-70) and distal (-134/-106) location; the hGH-N promoter also contains two GHF-1/Pit-1 sites (Lefevre et al. 1987; Nelson et al. 1988). The human and rat PRL genes contain at least eight GHF-1/Pit-1 sites, of which four sites are present in the proximal promoter sequences and the other four GHF-1/Pit-1 sites are present in a distal enhancer region (Ingraham et al. 1988; Peers et al. 1990). Expression of GHF-1/Pit-1 in heterogeneous cell lines is sufficient to partially transactivate the PRL and GH-N promoters (Fox et al. 1990; Ingraham et al. 1988; Mangalam et al. 1989; data/results in this thesis).

GHF-1/Pit-1 binds to the DNA as a dimer (Ingraham et al. 1990) as well as a heterodimer (Voss et al. 1991). However, under certain conditions GHF-1/Pit-1 binds preferentially as a monomer (Rosenfeld, 1991). Furthermore, GHF-1/Pit-1 was shown to exhibit an unique promoter spacing requirement for activation and synergism (Smith et al. 1995). It was also reported that GHF-1/Pit-1 stimulates transcription *in vitro* by influencing

pre-initiation complex assembly (Sharp, 1995)

The GHF-1/Pit-1 gene autoregulates its own expression as a consequence of GHF-1/Pit-1 binding sites in its own promoter (Chen et al. 1990; McCormick et al. 1990). Furthermore, a tissue-specific enhancer, in the GHF-1/Pit-1 gene, containing four GHF-1/Pit-1 sites confers enhancer activity (Rhodes et al. 1993). GHF-1/Pit-1 is also upregulated by cAMP and this is thought to be one of the mechanisms by which cAMP regulates GH-N gene expression (McCormick et al. 1990). Phosphorylation of the GHF-1/Pit-1 and subsequent enhanced or lowered GHF-1/Pit-1 binding activity is dependent on the sequences in the GHF-1/Pit-1 DNA elements (Fischberg et al. 1994; Kapiloff et al. 1991; Okimura et al. 1994; Steinfeld et al. 1992). However, it was also shown that GHF-1/Pit-1 undergoes a M-phase-specific phosphorylation, performed by a cell cycle-regulated protein kinase, which inhibits GHF-1/Pit-1 from binding to DNA (Caelles et al. 1995). Furthermore, it was shown that activin inhibits GHF-1/Pit-1 binding to the GH-N promoter by increasing phosphorylation and decreasing the stability of GHF-1/Pit-1 (Gaddy-Kurten and Vale, 1995; Struthers et al. 1992). There is evidence that the GHF-1/Pit-1 gene is downregulated by AP-1 and Oct-1 (Delhase et al. 1996) and repressed by NF-1 (Rajas et al. 1998).

1.12.2.2. Other transcription factors involved in the expression of GH-N

Initial studies with rat and human GH-N promoter sequences suggested that the information for pituitary-specific activity of these promoters resides in the proximal promoter sequences. However, in these studies it was revealed that the two GHF-1/Pit-1 binding sites alone may not confer full promoter activity (Nelson et al. 1986; Cattini et al. 1986). Furthermore, in a transgenic mouse, transgenes under the influence of these two GHF-1/Pit-1 cis-active regions (-145/+1 of rat GH promoter) were expressed at extremely low levels in the pituitary gland, although the sequences supported pituitary-specific expression (Lira et al. 1993). However, addition of 35 base pairs of 5'-flanking information, contributing other elements including a thyroid hormone/retinoic acid response

element, resulted in much higher levels of transgene expression. Sequences located upstream (-310 bp) of this segment contributed to a further 5- to 10-fold activation (Lira et al. 1993). Thus, these results suggest that other transcription factors along with the GHF-1/Pit-1 are necessary to activate the GH-N promoter synergistically in a pituitary-specific manner (Bradford et al. 1997; Lira et al. 1993).

In the case of rat GH-N and PRL genes other transcription factors, along with the GHF-1/Pit-1, were shown to be important for transcription. These include, Zn-15 (Lipkin et al. 1993), thyroid/retinoic acid receptor (Brent et al. 1989; Lira et al. 1993; Schaufele et al. 1992; Ye et al. 1988), estrogen receptor (Kim et al. 1988; Nowakowski and Maurer, 1994; Peers et al. 1990; Simmons et al. 1990), glucocorticoid receptor (Treacy et al. 1991) Sp-1 (Schaufele et al. 1990), CCAAT/Enhancer-binding Protein alpha (C/EBPalpha; Schaufele, 1996), and ets-1 (Bradford et al. 1997; Howard and Maurer, 1995). Furthermore, binding elements for these factors are present in the promoter sequences. Although cAMP was shown to enhance GH-N gene expression, a consensus CRE was not identified in the rat GH-N promoter. Furthermore, synergism reported between the thyroid hormone receptor and GHF-1/Pit-1 (Schaufele et al. 1992) was not confirmed by other investigators and the thyroid hormone stimulation of rat GH-N was shown to be independent of GHF-1/Pit-1 (Suen and Chin, 1993). The hGH-N gene promoter is also activated by the ras oncogene (Petersenn et al. 1997) and this effect was similar to that observed with the rat PRL promoter (Bradford et al. 1996). However, the hGH-N was not stimulated by ras in placental JEG-3 cells (Petersenn et al. 1997).

Like the rat GH-N gene, the human GH-N gene follows a similar pattern in that minimal GH-N promoter sequences with two GHF-1/Pit-1 sites is not sufficient for efficient activity *in vitro* (Cattini et al. 1986). Several transcription factor binding sites were observed within 400 bp of the hGH-N promoter (Table. 1.2). Although the transcription factor Zn-15 was shown to be important for GH-N gene expression, the equivalent transcription factor is yet to be characterized from the human pituitary and more importantly, the Z box to which the Zn-15 binds has variations in the hGH-N promoter

(Lipkin et al. 1993). A consensus thyroid hormone receptor (TR) binding sequence is not present in the hGH-N promoter (Lipkin et al. 1993), despite the report that TR binds to defined regions in the hGH-N gene (Barlow et al. 1986). However, in stably or transiently transfected cells, thyroid hormone negatively regulated the hGH gene (Cattini et al. 1986a; Zhang et al. 1992). Two glucocorticoid receptor binding regions were identified in the hGH-N gene. One of high affinity is located in the first intron (Eliard et al. 1985; Moore et al. 1985; Rousseau et al. 1987; Slater et al. 1985) and one of low affinity in the 5'-flanking sequences (Dana and Karin, 1989; Rousseau et al. 1987). Furthermore, these sites were shown to stimulate transcription. However, it was also shown that glucocorticoids increase GH-N secretion (Mulchahey et al. 1988) as well as stabilization of GH-N mRNA (Paek and Axel, 1987). Regulation of the GH-N gene expression by glucocorticoids was also studied in transgenic mice (Selden et al. 1989). The GH-N gene also possesses Sp-1 (Lemaigre et al. 1989a; b; 1990), NF-1, AP-2 (Courtois et al. 1990) and upstream stimulatory factor (USF) (Lemaigre et al. 1989a; b) binding sites, and all these sites were shown to stimulate GH-N gene transcription *in vitro*. However, a synergism between GHF-1/Pit-1 and any of these factors is yet to be reported.

Table. 1.2. Location of various transcription factors' binding sites in proximal promoter sequences of the hGH-N gene

<u>FACTOR</u>	<u>POSITION (nucleotide positions)</u>
GHF-1/Pit-1 (proximal)	-95/-66
GHF-1/Pit-1 (distal)	-130/-107
Sp1	-140/-116
AP-2	-290/-262; -168/-142
NF-1	-290/-262
USF	-267/-253
GR (high affinity)	+115/+86
GR (low affinity)	-224/-208
TR	-159/-144; -70/-50; -62/-48

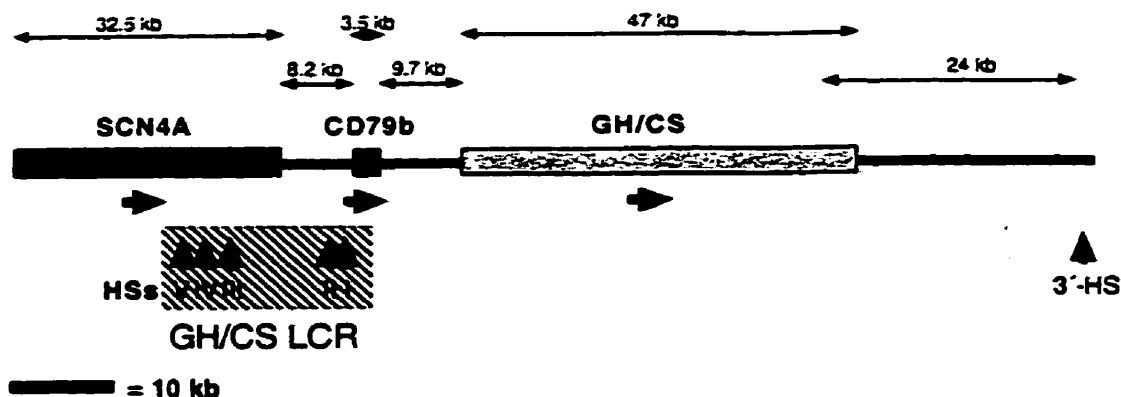
1.13. REQUIREMENT FOR A LOCUS CONTROL REGION FOR PITUITARY-SPECIFIC EXPRESSION OF THE HUMAN GH-N GENE

In vitro transfection studies clearly indicated that pituitary somatotroph-specific expression of rat GH-N or hGH-N can be achieved with 200 to 250 bp of 5'-flanking sequences of the transcription start site (Casanova et al. 1985; Cattini et al. 1986; Crew and Spindler, 1986; Nelson et al. 1986). In order to study the role of these sequences *in vivo*, it is necessary to integrate these regulatory sequences along with reporter genes into cellular chromatin. Two methods that are popularly used are generation of either stable cell lines or transgenic mice. Even before the identification of the GH gene regulatory sequences, attempts were made to target the expression of reporter genes using the 5'-flanking sequences of the rat GH-N gene or the hGH-N gene in transgenic mice, but these studies resulted in no expression of the target gene in the transgenic mouse pituitary (Hammer et al. 1984; Palmiter and Brinster, 1986). It was reasoned that vector sequences in the transgene fragments injected were interfering with the expression of the reporter gene (Lira et al. 1988). However, further studies with reporter genes (hGH-N gene) fused to the rat GH-N gene 5'-flanking sequences (devoid of vector sequences) as transgenes revealed that even -180 bp of the 5'-flanking sequences were sufficient to confer the pituitary somatotroph-specific expression of the target gene (Lira et al. 1988). However, a low level of reporter gene expression was observed in the thyrotrophs and lactotrophs which are generally GH-N negative (Lira et al. 1989). Furthermore, different levels of the reporter gene expression were observed among transgenic mice generated with different lengths of rat GH-N gene 5'-flanking sequences (-1.7 kb to -145 bp) (Lira et al. 1993). It was reasoned that combinatorial interactions involving both cell-specific and general transcription factors determine the quantitative level of GH-N gene expression in somatotrophs (Lira et al. 1993). The higher levels of reporter gene expression observed in the -1.7 kb rat GH-N transgenic mouse could be due to the presence of a TRE at nucleotide -180 bp and a CRE at nucleotide -1256, as well as the formation of an unusual secondary structure (cruciform and Z-DNA) within the -1.7 kb flanking sequences (Thomas et al. 1990 as reviewed by

Lira et al. 1993).

However, when transgenics were generated using either 2.6 kb of human genomic sequences containing the GH-N structural gene, along with 0.5 kb of the hGH-N gene 5'-flanking sequences (which *in vitro* conferred somatotroph-specific expression; Cattini et al. 1986; Lefevre et al. 1987) or a 43 kb human genomic fragment including the GH-N, CS-L and CS-A structural genes as well as 5.0 kb of GH-N 5'-flanking sequences as transgenes, none of the transgenes were expressed in the pituitary (Hammer et al. 1984; Palmiter and Brinster, 1986). Recently, similar transgenic studies using either the 2.6 kb of human GH-N genomic sequences or a human genomic fragment containing the GH-N as well as 7.5 kb of GH-N 5'-flanking sequences were performed, but the expression of the target gene (hGH-N) in transgenic mouse pituitaries appeared to be inconsistent (Jones et al. 1995). Thus, these studies suggested that the proximal promoter (-0.5 kb) and/or more distal sequences (-7.5 kb) 5'-upstream of the hGH-N gene are insufficient to establish appropriate expression *in vivo* (Jones et al. 1995). These observations also suggest the requirement of more information and possibly a locus control region (LCR) for hGH-N gene expression in the pituitary. Subsequently, further studies led to the identification of a multicomponent LCR (Jones et al. 1995). Therefore, in contrast to the rat GH-N gene, the hGH-N gene seems to require a more complex transcriptional regulatory mechanism. The LCR of GH/CS genes has some similarities with that of the β -globin gene in the presence of five HSs and in possessing enhancer activity (Fig. 1.7; Jones et al. 1995). Among these HSs, the HSs I and II are pituitary-specific, act as enhancers and are located in immediate 5'-flanking sequences of the B-lymphocyte-specific CD79b gene or 15 kb upstream of the GH-N gene transcription initiation site (Bennani-Baiti et al. 1998a; b; Jones et al. 1995). The HS IV is placenta-specific whereas HSs III and V are present in both the pituitary and placenta tissues, and these HSs are located in the intronic sequences of the muscle-specific SCN4A gene (Bennani-Baiti et al. 1995; Jones et al. 1995). The role of the placenta-specific HS IV is not yet characterized. However, at present the sequence information and the factors responsible for generation of HSs as well as the LCR function are yet to be elucidated.

Fig. 1.7. The GH/CS gene locus control region and location of the 3'-hypersensitive site. The five HSs I-V of the GH/CS gene LCR and location of the 3'-HS are indicated by vertical arrow heads. The GH/CS upstream genes and the approximated distances in kilobases (kb) are shown. Transcriptional orientation of SCN4A, CD79b and GH/CS genes are indicated by horizontal arrow heads.



1.14. GH GENE TRANSCRIPTION IN VIRAL INFECTIONS

de la Torre and Oldstone (1992) reported an interesting phenomenon with regard to the pituitary-specific expression of the GH-N gene. Lymphocytic choriomeningitis virus (LCMV), a noncytolytic virus, can persistently infect a rat pituitary cell line. Although the infected cells remain free of structural damage, the virus markedly interferes with GH-N transcription, but only minimally interferes with PRL transcription. It was suggested that the virus interferes with GHF-1/Pit-1. However, it is unclear how the LCM virus selectively disrupted the GH-N transcription machinery.

1.15. REGULATION OF PLACENTA-SPECIFIC CS/GH-V GENE EXPRESSION

1.15.1. Possible role for GHF-1/Pit-1

Although the CS genes are highly expressed, little is known about the placenta-specific transcriptional regulatory mechanisms for the GH-N gene-like CS/GH-V genes. With the recent identification of the GHF-1/Pit-1 gene expression in human placenta and placental cells (Bamberger et al. 1995; Schanke et al. 1997), it is possible that similar transcriptional mechanisms may be operating in placenta as was seen in the pituitary. The CS-L, CS-A, GH-V and CS-B genes are expressed at different levels in the placenta (Chen et al. 1989). The CS-A/-B genes are expressed at higher (> 1000 times) levels than the CS-L/GH-V genes (Chen et al. 1989). The sequences contained in 0.5 kb of the CS/GH-V 5'-flanking DNA resemble (> 90%) the equivalent region of the GH-N gene, but show differential GHF-1/Pit-1 binding activities with rat pituitary nuclear proteins (Lemaigre et al. 1989; Nickel et al. 1991). Furthermore, it was noticed that this differential GHF-1/Pit-1 binding might be responsible for the differential expression of the CS-A and GH-V genes as observed in the placenta *in vivo* (Nickel et al. 1990). When the intact hGH-V and hCS-A genes (containing 0.5 kb of 5'-flanking sequences as well as the structural genes) were stably integrated into rat pituitary (GC) cells, expression of the CS-A gene was more than that of the GH-V gene (Nickel et al. 1990). This suggested that the subtle sequence differences in the 5'-flanking region of these genes could be responsible for differential expression *in vivo*. Although GHF-1/Pit-1 was identified in placenta and placental tumor cells, GHF-1/Pit-1 binding to the CS/GH-V promoter elements in placenta is yet to be assayed.

1.15.2. Other transcription factors involved in CS/GH-V gene expression

The placenta-specific expression of the CS and GH-V genes is also modulated by thyroid hormone (Nickel and Cattini, 1991; Tansey and Catanzaro, 1991). The CS promoter absolutely requires the Sp-1 element for maximal and enhancer-stimulated promoter activity in placental and non-placental cells (Fitzpatrick et al. 1990; Jiang et al. 1995; Tansey and Catanzaro, 1991). The role of other elements such as NF-1, AP-2 and USF in the transcriptional regulation of CS/GH-V genes is not known. Furthermore, an

enhancer element with multiple TEF-like sites was shown to be essential for the high placenta-specific stimulatory activity of the CS-B gene (Jacquemin et al. 1994a; b; Jiang and Eberhardt, 1997; Rogers et al. 1986; Walker et al. 1990).

1.15.3. Role of enhancer elements in CS/GH-V gene expression

Analysis of the 3'-flanking sequences of the CS-B gene revealed the presence of an enhancer element (Rogers et al. 1986). Although, homologous sequences were associated with the CS-A and CS-L genes, these were shown to possess only weak stimulatory activity *in vitro*. The actual role of these enhancer sequences in CS gene transcription is yet to be established *in vivo*. Recently, the placenta-specific expression of the CS-A gene was studied in a transgenic mouse placenta (Jones et al. 1995). The transgenics were generated using a 15 kb genomic DNA fragment containing CS-A structural gene with 5.4 kb of 5'- and 7.2 kb of 3'-flanking DNA which contains the enhancer sequences. Although the CS-A gene was expressed in mouse placenta, the expression was not coordinated in a copy number dependent manner (Jones et al. 1995). At present it is not known whether this phenomenon was due to repeat induced heterochromatin formation as was reported with other transgenes (Garrick et al. 1998) or if it was due to the inability of the regulatory flanking sequences of the CS-A gene to overcome the integration event. However, the authors concluded that the latter possibility is the reason for uncoordinated expression of the CS-A gene in the mouse placenta (Jones et al. 1995).

1.15.4. Possible role of chromatin structure in CS/GH-V gene expression

Identification of placenta-specific HSs, one 5'-HS (HS IV of the GH/CS gene LCR) and the other 3'-HS (24 kb downstream of the CS-B gene) (Cooke and Liebhaber, 1995; Jones et al. 1995) raises the possibility that these HSs might play a role in creating a favorable chromatin structure to influence CS/GH-V expression in the placenta. Similarly, with a view to identify regulatory elements responsible for this placental-specific expression of CS/GH-V genes, Jimenez et al (1993) mapped and compared the DNase I

HSs present at the hGH gene cluster in two placental cell lines, BeWo that expresses the hGH-V and hCS genes, and JEG-3 that does not express the hGH-V and hCS genes. This comparison revealed a very similar pattern of placenta-HSs, suggesting that the sites detected are established before the onset of transcription. These results indicate that the transition to an active hGH/CS locus in placental cells requires multiple alterations in chromatin structure, and provide a framework for the molecular analysis of the regulatory elements and mechanisms mediating such processes.

1.16. THE MAJOR PARADIGM ASSOCIATED WITH THE HGH/CS GENES

Although the GH/CS genes are highly related structurally and functionally, the CS/GH-V genes are not expressed in the pituitary. A possible repressor mechanism was identified for restriction of the expression of the CS/GH-V genes in the pituitary (Nachtigal et al. 1993). Part of the present work was to identify the factor (s) binding to these repressor elements.

Another enigma associated with the CS/GH-V genes is the physiological importance of these hormones during pregnancy. Furthermore, it is yet to be determined why the GH-N gene is not expressed in the placenta. Interestingly, the hGH-N gene sequences code for another protein (Labarriere et al. 1995). The hGH-N promoter sequences from -294/-177 contain a potential TATA box (-229/-223) as well as an USF site (-266/-253), and was found to be active as a promoter in cell-free transcription assays (Courtois et al. 1992). Further studies revealed that this segment acts as a promoter for a transcript that codes for a protein of 107 residues (Labarriere et al. 1995). This transcript was observed only in pituitary and placental cells. The corresponding protein acts as a potent transcriptional activator (GH gene-derived transcriptional activator, GHDTA) in these cells (Labarriere et al. 1995). This could be one of the reasons multiple placenta-specific HSs were observed in the vicinity of the GH-N gene promoter and may be responsible for inhibition of GH-N gene transcription in the placenta (Jones et al. 1995).

The essential role of GHDTA and the role of these HSs is yet to be determined.

1.17. RATIONALE AND OBJECTIVES OF THE RESEARCH PRESENTED IN THIS THESIS

Transcriptional regulation is the primary controlling mechanism in gene expression. Although, it was proposed initially that sequences immediately upstream of the transcription start site of a particular gene are sufficient for regulating gene expression in a temporal and spatial manner, present studies suggest that mechanism of control is far more complex. There is increasing evidence that a complex network of DNA-protein and protein-protein interactions, as well as modifications of chromatin structure (in terms of euchromatin and heterochromatin) play an important role in gene expression. If a single gene is controlled in such a manner, then the complexity magnifies to almost unimaginable levels to control a family of physically linked and highly homologous genes such as hGH/CS genes. The primate GH/CS genes offer a good model system to study this complexity at the transcriptional level involving two different tissues. The major hypothesis of this thesis is that **human GH/CS genes are controlled by a complex network of transcriptional mechanisms which involve DNA sequences not only within the locus but located remotely upstream or downstream of the locus.**

The transcriptional regulatory mechanisms involved in the expression of the placental members of the human GH/CS gene family are poorly understood. Our laboratory has a longstanding interest in developing an understanding of these mechanisms. Despite the high sequence homology among the GH/CS genes, the GH-N gene is expressed in the pituitary and the CS/GH-V genes are expressed in the placenta. On the basis of sequencing analysis, several putative regulatory sequences or regions were identified in the GH/CS gene locus (~66,000 bp), and have been suggested to play a role in the differential expression of the GH/CS genes. Such sequences or regions include the **P-sequences** which are specific to placental members of the GH/CS gene family as well as the CCCAACC-rich sequences (**C-sequences**) which are present in the upstream sequences of the GH-V/CS-L genes. Furthermore, an **enhancer region(s)** which is specific to CS-

L, -A and -B genes was shown to play a role in CS-B gene expression. Part of this thesis deals with the partial characterization of P- and C- sequences as well as enhancer regions and the possible DNA-binding factors recognizing the elements in these regions.

Previous work from our laboratory had identified a pituitary-specific repressor mechanism associated with P-sequences. Two elements, **PSF-A and PSF-B**, were identified and were implicated in the repression mechanism. **Identification, purification and characterization of the proteins recognizing these elements** constitutes the first and a major objective of the thesis work presented. The rat anterior pituitary tumor GC cell line was used as the source of the PSFs. Various purification methods including ion-exchange and DNA-affinity chromatography were applied to purify the PSFs to near homogeneity. The UV-crosslinking and southwestern blotting were performed to determine the size of the PSF proteins. Attempts were made to develop model systems to study the functional importance of the suggested interaction between the pituitary-specific factor, GHF-1/Pit-1 and PSF proteins.

A second objective was to **study the role of C-sequences in the differential regulation of the GH-V/CS-L genes in placenta**. The functional and structural characterization of the C-sequences was done using transient transfections, gel mobility and nuclease protection assays.

Previous work from several laboratories including ours emphasized the importance of the **enhancer** sequences on CS gene expression in the placenta. The enhancers are present in the 3'-flanking sequences of the CS genes and it was shown recently that these CS enhancers contain multiple recognition elements for TEF-1 or TEF-like proteins. Furthermore, the CS enhancers act as **silencers** in rat pituitary GC cells *in vitro*. TEF-1 or TEF-like factors are ubiquitous in nature. Thus, these observations suggest that the TEF proteins form tissue-specific complexes with other regulatory proteins resulting in enhanced or repressed gene expression. Thus, the third objective of this thesis was devoted to the **functional characterization of TEF factors**. Expression vectors containing

different deletion mutants of TEF cDNAs were constructed, and tested for their ability to influence the transcriptional activity of different promoters in a tissue-/cell- specific manner.

In contrast to these sequences that lie in relatively close proximity to the transcription initiation site, recent studies revealed the importance of **far upstream LCRs** in the regulation of a gene or a family of genes. These regions are characterized by DNase I hypersensitive sites (HSs). Recently, analysis of the GH/CS gene sequences led to the identification of five far upstream HSs and a downstream HS. These HSs were either pituitary-specific (HSs I and II) or placenta-specific (HSs IV and the downstream HS). The upstream HSs (HSs I, II, III and V) constitute a LCR for the GH/CS genes. Furthermore, these HSs were mapped to the inter or intragenic regions of the adjacent SCN4A and CD79b genes (Fig. 1.7). These genes are physically linked to GH-N gene in several species of animals including human and rat. However, the sequence information and the factors responsible for LCR activity are not known. Furthermore, the GH/CS downstream HS is also not characterized. Thus a fourth objective of this thesis was an attempt to **isolate and characterize far-upstream and far-downstream sequences of the GH/CS gene locus containing both the GH/CS LCR and downstream HS.** This was done

- a) using P1 clones to obtain DNA fragments containing HSs I and II as well as III to V,
- b) The AT-rich sequences of the HS I and II were tested for their ability to bind GHF-1/Pit-1 antibodies raised against GHF-1/Pit-1 in "supershift" assays to assess a possible role of GHF-1/Pit-1 in GH/CS LCR function,
- c) transient transfections to characterize the functional role of the placenta-specific HS IV, and
- d) DNA blotting, PCR and sequencing to characterize sequences the region downstream of the GH/CS genes and containing placenta-specific HS.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals, reagents and equipment

Most of the chemicals and reagents, restriction enzymes and other molecular biology reagents, cell culture products, radioactive materials and kits used in this study were obtained from Amersham Canada Ltd, Oakville, ON; Amicon Div, Beverly, MA; Anachemia science, Winnipeg, MB; Bio/Can Scientific, Mississauga, ON; BioRad, Mississauga, ON; Boehringer Mannheim Canada, Laval, QC; Fisher Scientific, Nepean, ON; Gibco-BRL, Burlington, ON; ICN Biomedical Canada, Mississauga, ON; Invitrogen Carlsbad, CA; Mandel Scientific, Guelph, ON; Millipore Canada Ltd, Mississauga, ON; New England Biolabs, Mississauga, ON; Pharmacia Biotech, Baie d'Urfe, QC; Qiagen Inc, Mississauga, ON; Rose scientific Ltd, Edmonton, AB; Sigma-Aldrich Canada Ltd, Oakville, ON.

Centrifuges (Beckman, J2-21; IEC-HNS; IEC-CL; HBI microfuge and Hermle microcentrifuge) were obtained from Beckman Instruments Canada Inc, Mississauga, ON; Mandel Scientific, Guelph, ON; Fisher Scientific, Nepean, ON).

PCR machine (PTC-100, Programmable thermal cycler) was purchased from MJ research Inc, Watertown, MA.

Laminar flow hood (SterilGARDhood) is from Baker company, Sanford, ME. Water jacketed cell culture incubators were from Fisher Scientific, Nepean, ON. Hybridization incubators (Model 2000 microhybridization incubator) were purchased from Robbin Scientific Corp. Sunnyvale, CA.

All oligonucleotides used in this study were synthesized by the University Core

2.2. Cell lines and cell culture

Several cell lines of different tissue and species origin were used in these studies. They include: rat anterior pituitary tumor GC (Tashjian et al. 1968); human choriocarcinoma BeWo (Pattillo et al. 1968), JAR (Pattillo et al. 1971) and JEG-3 (Kohler and Bridson, 1971); human cervical epithelial adenocarcinoma HeLa (Gey et al. 1952) and African monkey kidney, SV40 transformed, COS-1 (Gluzman, 1981) cell lines. The GC, HeLa, JEG-3 and COS-1 cells were grown in Dulbecco's modified Eagles medium (DMEM), JAR cells were maintained in RPMI 1640 medium and BeWo cells were grown in Ham's F12 medium. All media were supplemented with 8-15% FBS and 50 U/ml penicillin, 50 µg/ml streptomycin and 500 µM glutamine. All cell lines were grown on 100 mm disposable sterile tissue culture dishes in a 100% humidified atmosphere with 5% carbon dioxide/95% air at 37° C. The growth media were changed every 24-48 h. Cells were visualized using inverted microscope to determine confluency of the cell monolayer.

Depending on the cell line and the number of cells represented at 80-100% confluency, cells from a single 100 mm culture dish were subcultured to four to five 100 mm dishes. Harvesting of cells was done by washing the monolayer with 10 ml of prewarmed, sterile calcium and magnesium free phosphate buffered saline (PBS-CMF) and subsequent incubation at 37 °C in 1 ml of 0.05% (v/v) trypsin/0.53 mM ethylenediaminetetraaceticacid (EDTA) for 1 to 2 minutes. After observing detachment of the cells from the dish, 2 to 3 ml maintenance medium was added to the the dish, cells were collected by repeated manual pipetting using a sterile Pasteur pipette and transferred to a sterile 15 ml polystyrene tube. Cells were then pelleted by centrifugation at 3000 rpm for 2 to 3 minutes at room temperature using HN clinical centrifuge. The supernatant was aspirated into a conical flask containing bleach, and the cell pellet was resuspended in 5 ml of culture medium and distributed as 1 ml aliquot to 5 culture dishes containing 9 ml culture medium.

For storage and frozen stock purposes, the cells were grown to 80% confluency, and harvested as described earlier. The cell pellet was resuspended in freezing medium containing 10% (v/v) dimethyl sulfoxide (DMSO) in FBS, to a final concentration of $1-2 \times 10^6$ cells per ml and were aliquoted as 2 ml per a Corning cryogenic vial. The cells were left at room temperature for 5 minutes and further incubated on ice for 20 minutes before transferring to a -70°C freezer.

Culturing of cells from frozen stocks was done by quickly thawing the cells by immersing the vial in 37°C water bath and transferred to the culture dish containing 9 ml of culture medium. After allowing cells to attach to the culture dish (approximately 8 h after seeding), the culture medium is replaced with 10 ml fresh medium.

2.3. Preparation of nuclear protein extracts

Crude nuclear protein extracts from different cell lines were prepared according to the method described by Dignam et al (1983). In detail, the cells being maintained as monolayers on 100 mm culture dishes were harvested as done in the earlier section. Cells from each dish was seeded to a 150 mm dish containing 18 to 20 ml of culture medium. A minimum of 20-25 dishes (150 mm) were maintained for preparation of nuclear protein extract. Once the cells reached 80% confluency, monolayers were washed with 15 ml of PBS-CMF and the cells were harvested with 4 ml of PBS-CMF/1 mM EDTA by scraping the cells with a rubber policeman. The cells were pooled into 50 ml polystyrene tubes and were pelleted as described earlier. The number of cells and packed cell volume (PCV) of the pellet was recorded. The cell pellets were frozen and stored at -70°C if not processed immediately.

The following steps of nuclear extract preparation was done at 4°C . The cells were resuspended in 5 X PCV of ice cold hypotonic buffer "A" (10 mM HEPES (N-2[-hydroxyethyl]piperazine-N'-[2-ethanesulfonicacid]) -KOH pH 7.9, 1.5 mM MgCl_2 , 10

mM KCl, 0.5 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF)) and were incubated for 10 minutes on ice for swelling. After centrifugation at 1000 x g for 10 minutes, the supernatant was carefully pipetted out, and the pellet was resuspended in two packed cell volumes of buffer "A" and transferred to a Dounce homogenizer. Using either "hard/pestle A" (for JEG-3 or JAR cells) or "loose/pestle B" (for GC and HeLa cells), the cells were homogenized (at least 15-20 strokes) until the free nuclei along with disrupted cell membranes were observed under microscope. The homogenate was transferred to a 30 ml Oak Ridge tubes. The nuclei were pelleted with an initial centrifugation at 1000 x g for 10 minutes and careful aspiration of supernatant, followed by 24,000 x g for 20 minutes. The nuclear pellet was resuspended in buffer "C" (20 mM HEPES-KOH pH 7.9, 25% v/v glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT and 1 mM PMSF) at the rate of 3 ml/ 10⁹ cells and homogenized in Dounce homogenizer using either pestle "A" or "B". The suspension was transferred to a Oak Ridge tube and mixed on nutator for 30 minutes. The nuclear suspension was centrifuged at 14,000 rpm for 30 minutes and the supernatant was collected and dialyzed in a Spectrapor dialysis tubing (6-8,000 Dalton cut-off) against two changes of at least 50 volumes of buffer "D" (20 mM HEPES-KOH pH 7.9, 20 % v/v glycerol, 100 mM of KCl, 0.2 mM EDTA, 0.5 mM DTT, and 1 mM PMSF) for 3-5 h. The material was collected from the tubing into a Oak Ridge tube and centrifuged at 24,000 x g for 30 minutes and the nuclear protein extract (supernatant) was aliquoted in 50-100 µl volume and quick frozen in a dry ice-ethanol bath and stored at -70 °C. Protein concentrations were determined by Bradford assay (BioRad) using BSA as the standard (Bradford, 1976).

A rapid method of nuclear extract preparation from small numbers of cells was done according to the method detailed by Andrews and Faller (1991). All centrifugations were done for 30 seconds and were performed at room temperature; between the steps, the samples were placed on ice. The cells (5×10^5 to 10^7) were scraped into 1.5 ml microfuge tube, pelleted and resuspended in 400 µl of cold buffer A (10 mM HEPES-KOH pH 7.9; 1.5 mM MgCl₂; 10 mM KCl; 0.5 mM DTT; 0.2 mM PMSF) by flicking the tube. The cells were allowed to swell on ice for 10 minutes, and then mixed for 10 sec. Samples were

centrifuged for 10 seconds, and the supernatant fraction was discarded. The nuclear pellet was resuspended in 20-100 μ l (according to starting number of cells) of cold buffer C (20 mM HEPES-KOH pH 7.9; 25% glycerol; 420 mM NaCl; 1.5 mM MgCl₂; 0.2 mM EDTA; 0.5 mM DTT; 0.2 mM PMSF) and incubated on ice for 20 minutes for high-salt extraction. Cellular debris was removed by centrifugation for 2 minutes at 4 °C and the supernatant fraction was stored as nuclear extract at -70 °C. Before using in gel-mobility shift or nuclease protection assays, the nuclear extract was diluted by adding three volumes of buffer D (20 mM HEPES-KOH pH 7.9, 20 % v/v glycerol, 100 mM of KCl, 0.2 mM EDTA, 0.5 mM DTT, and 1 mM PMSF).

2.4. Procedures used in the purification of PSFs from GC nuclear extracts

2.4.1. Heparin agarose fractionation

The nuclear extracts were fractionated using heparin agarose either in mini scale using Costar Spin-X centrifuge filter units (fitted with 0.45 μ M of cellulose acetate filters) or in large-scale using 50 ml tubes. All the following steps were performed at 4 °C in cold room.

2.4.1.1. Mini scale fractionation of GC nuclear extract

Approximately, 200 μ l (settled bed volume) of heparin agarose (HA) resin was equilibrated with buffer D that was used in nuclear extract preparations. The slurry was taken into a 15 ml polypropylene tube (Corning) and centrifuged for 2 minutes at 2,000 x g in a clinical centrifuge. The supernatant was discarded and the resin was mixed with 5 ml of buffer D on a nutator for at least 30 minutes. Repeated centrifugation and treated again with buffer D. Approximately 6-8 mg of nuclear proteins were incubated with 200 μ l of pre-equilibrated heparin agarose-resin in a microfuge tube and mixed on a nutator for 1 h. After incubation, the tube was centrifuged for 1 minutes at 15,000 x g in a microfuge. The supernatant was removed and stored as flow-through. The resin was added with 200 μ l of

buffer D, mixed and transferred to the upper chamber of a Spin-X column and again mixed on a nutator for 10 minutes. The column was centrifuged for 30 s and the filtrate from the lower chamber was collected as 0.1 M KCl wash. The resin was mixed with 200 μ l of buffer D containing 0.2 M KCl on a nutator for 15 minutes. After repeating the centrifugation, the 0.2 M eluate was collected from the lower chamber. Furthermore, the nuclear proteins were eluted from the resin by centrifugation essentially as described above, using 200 μ l of buffer D containing increasing concentrations of KCl from 0.3 M to 1.0 M. All eluates (and 0.1 M KCl wash) were collected and stored at -70 °C until further use or dialyzed against buffer D on 0.025 μ M Millipore filters floating in a 150 mm petridish with 100 ml of buffer D. Dialysis was done for 1 h and dialyzed samples were stored at -70 °C until further use.

2.4.1.2. Large scale fractionation

A similar procedure was followed for large scale preparations. The amounts of buffers used for elutions are detailed in the results and discussion section in Chapter 3. Finally, the eluates were placed in Spectrapor dialysis tubing (6-8 K MW cut off) and dialyzed against two changes of 50 volumes buffer D.

Protein estimation in each sample was done using Bradford (1976) assay and the PSF binding activity in HA fractions were detected by nuclease protection assay using 5-10 μ g of nuclear protein from each fraction.

2.4.1.3. Phosphocellulose and hydroxylapatite column purification

GC nuclear extracts were also fractionated using either phosphocellulose or hydroxylapatite resins essentially as described in mini scale fractionation of nuclear extract with HA resin.

2.4.2. Preparation of DNA-affinity columns

DNA-affinity columns were prepared using PSF-B, PSF-A or D8 double stranded oligodeoxynucleotides (ODNs) representing the corresponding binding sites. Designing of ODNs, subsequent preparation of ODNs and resin for affinity column were done essentially according Kadonaga (1991). The following are different steps in the preparation of a DNA-affinity column.

2.4.2.1. 5'-Phosphorylation of the ODNs

Approximately 250 µg of sense or antisense ODNs representing each of the binding element was dissolved in 32.5 µl of TE and mixed together in a 1.5 ml microfuge tube. To the ODN solution, 10 µl of 10X T4 polynucleotide kinase buffer (500 mM Tris.Cl, pH 7.6, 100 mM MgCl₂, 50 mM DTT, 1 mM EDTA and 1 mM spermidine) and was incubated at 88 °C for 2 minutes, 65 °C for 10 minutes, 37 °C for 10 minutes and then at room temperature for 5 minutes. These steps allow the ODNs to anneal together and to form concatemers because of the 5'-GATC overhangs. To the ODN solution, 3 µl of 100 mM ATP pH 7.0, 1.0 µl of DTT, 10.5 µl of water and 5 µCi of [γ -³²P]ATP, 100 units (10 µl, 10 units/µl) T4 polynucleotide kinase and incubated at 37 °C for 2 h to phosphorylate the 5'-ends of the ODNs. After incubation, to the ODN solution 50 µl of 10 M ammonium acetate and 100 µl of water were added, mixed and heated to 65 °C for 15 minutes to inactivate the kinase. After cooling the ODN solution to room temperature, the ODNs were precipitated with 750 µl of ethanol, mixed by inversion and DNA was pelleted by centrifugation for 15 minutes at 15,000 x g. The DNA pellet was dissolved in 225 µl of TE by mixing, extracted once with 250 µl of phenol:IAC (24:1 of chloroform and isoamyl alcohol) and once with 250 µl of IAC, each time mixing for a minute and centrifuging for 5 minutes. Finally, the DNA was precipitated with the addition of 25 µl of 3 M sodium acetate pH 5.2, and 750 µl of ethanol. The DNA was pelleted and washed once with 75% ethanol and dried under vacuum.

2.4.2.2. Ligation of ODNs

The DNA from earlier step was dissolved in 65 µl of water and added with 10 µl of 10X linker-kinase buffer (660 mM Tris.Cl, pH 7.6, 100 mM MgCl₂, 150 mM DTT, and 10 mM spermidine). To this solution, 4 µl of 100 mM ATP pH 7.0, 1 µl of 1.0 M DTT and 32 units (4 µl, 8 units/µl) of T4 DNA ligase were added. The ligation reaction mixture was made up to 100 µl with the addition of water and incubated for the ligation at room temperature for 16-20 h. The extent of ligation was determined by loading 0.5 µl of the reaction mixture in a 3% agarose gel and subsequent electrophoresis.

2.4.2.3. Preparation of ligated DNA for coupling to Sepharose CL-2B

The ligation reaction (100 µl) was extracted once with 1 volume of phenol:IAC and once with IAC. The DNA was mixed with 33 µl of 10 M ammonium acetate and precipitated with 133 µl of isopropanol by incubating at -20 °C for 20 minutes. The DNA was pelleted by centrifugation at 15,000 x g for 15 minutes at 4 °C. The pellet was dissolved in 225 µl of TE, added with 25 µl of 3 M sodium acetate, pH 5.2, and precipitated with 750 µl of ethanol. DNA was pelleted as described earlier and the pellet was washed twice with 750 µl of 75% ethanol and dried under vacuum. Finally the DNA was dissolved in 50 µl of glass distilled water (at this step, use of TE as solvent was not recommended as it was observed that TE might interfere with coupling reaction, Kadonaga, 1991).

2.4.2.4. Preparation of Sepharose CL-2B for coupling

Approximately 10-15 ml (settled bed volume) of Sepharose CL-2b was extensively washed with glass distilled water (at least 500 ml) in a coarse sintered glass funnel. The moist resin (10 ml) was transferred to a 25 ml graduated cylinder and made to 20 ml with the addition of water. The slurry was transferred to a 150 ml beaker in a water bath equilibrated to 15 °C placed over a magnetic stirrer in a fume hood. In the fume hood, 1.1 g

of cyanogen bromide (CNBr) was dissolved in 2 ml of N,N-dimethylformamide in a 25 ml of Erlenmeyer flask. Carefully, the CNBr solution was added to the stirring slurry, drop wise and over a minute in the fume hood. After adding the entire CNBr solution to the Sepharose slurry, the slurry was mixed immediately with 18.8 ml of 5 M NaOH, drop wise over 10 minutes. Again, immediately after this step, 100 ml of ice cold water was added to the slurry, poured into a 60 ml coarse-sintered glass funnel and the resin was slowly filtered by applying suction by taking care not to dry the resin while suction filtering. The resin was washed four times with 100 ml of ice cold water followed by twice with 100 ml ice cold 10 mM potassium phosphate pH 8.0 (1 M KH_2PO_4 was used to adjust 1 M of K_2HPO_4 to pH 8.0). Five ml of the above CNBr-activated resin was transferred to a 15 ml polypropylene tube and made to a thick slurry with 2 ml of 10 mM potassium phosphate pH 8.0. The DNA was mixed immediately with the slurry, placed on a nutator and mixed overnight at room temperature, to allow the DNA to couple to the resin. Next day, the resin was transferred to a 60 ml coarse-sintered glass funnel and washed twice with 100 ml of water, followed by a wash with 100 ml of 1 M ethanolamine-HCl pH 8.0 (15 ml of ethanolamine (FW 61.08) in 200 ml of water and pH was adjusted to 8.0 with HCl and volume was made to 250 ml). At this stage the DNA coupling to the resin was monitored by comparing the level of radioactivity with the resin and with the wash. The resin was transferred to a 15 ml polypropylene tube, added with 1 M ethanolamine-HCl, pH 8.0. to form a smooth slurry and mixed on the nutator for 4 to 16 h at room temperature. The resin was washed with 100 ml of 10 mM potassium phosphate pH 8.0, 100 ml of 1 M potassium phosphate pH 8.0, 100 ml of 1 M potassium chloride, 100 ml of water and finally with 100 ml of column storage buffer (10 mM Tris.HCl, pH 7.6; 1 mM EDTA; 300 mM NaCl and 0.04% sodium azide). The resin was stored in this buffer at 4 °C.

2.4.3. Purification of partially purified GC nuclear extract on DNA affinity columns

Heparin agarose purified GC nuclear protein fractions positive for PSF binding

activity were further purified using either PSF-B, PSF-A or D8 affinity resins. All steps were done at 4 °C. For small scale purifications 5.0 ml cryovials and for large scale purifications 15 or 50 ml polypropylene tubes were used to let the PSF proteins to bind to the DNA-resin.

The DNA-affinity resin was equilibrated with buffer Z (0.1 M KCl, 25 mM HEPES-KOH, pH 7.6; 6.25 mM MgCl₂; 0.1 M KCl; 20% (v/v) glycerol; 0.1% (v/v) NP-40 and 1 mM DTT). Required amounts of resin (200 µl for small scale and 1 ml for large scale purifications) was incubated with 10 volumes of buffer Z/0.1M KCl for 10 minutes on a nutator and centrifuged at 10,000 x g for 10 minutes and the supernatant was carefully removed. This step was repeated one more time and the resin was collected.

The protein fractions were incubated with non-specific competitor DNA, poly [dIdC] at the rate of 1.25 µg of poly [dIdC] to 100 µg of nuclear protein for 10 minutes and the insoluble DNA-protein complexes were pelleted by centrifugation at 12,000 x g for 10 minutes (1.5 ml microfuge tubes for small quantities or Oak Ridge centrifuge tubes for large quantities of nuclear protein were used for this step) .

In case of small scale purifications, 3 mg/2 ml of partially purified GC nuclear protein was incubated with 200 µl of DNA-affinity resin in a 5 ml cryovial (Nunc), and mixed on a nutator for 1 h. After incubation, the tube was briefly centrifuged at 2,000 x g for 5 minutes and the supernatant was collected as flow-through. The resin was then washed with 1.0 ml buffer Z/0.1 M KCl by repeating mixing and centrifugation as done in the earlier step. The supernatant was stored as 0.1 M wash. Subsequently elutions were performed with successive additions of 200 µl of buffer Z containing 0.2, 0.3, 0.4, 0.6, 0.8 and 1.0 M KCl. The resin was mixed with the elution buffers for 15 minutes on the nutator and centrifuged as done earlier. The supernatant fractions were collected as their respective salt eluted fractions. To identify the PSF binding activity in these fractions, the fractions were dialyzed against 100 ml of buffer Z/0.1 M KCl for 1 h on a 0.025 µM Millipore filter discs floating on the buffer in a 150 mm petridish. After 1 h, the fractions

were collected and 12.5 μ l of each fraction was used for the nuclease protection assay as described earlier. However, in the footprint assay, initial DNA/protein binding reaction was done without the addition of poly [dIdC] and $MgCl_2$.

For large scale purification on the DNA affinity resin, 1.0 ml the resin was loaded onto a BioRad 2.0 ml Poly-Prep column and equilibrated with buffer Z/0.1 M KCl as was done as detailed in earlier section. After 1 h incubation to allow the binding of the GC nuclear protein material to the DNA affinity resin, the column was fixed to a stand and height was adjusted in such a way that the flow rate due to gravity from the column was 15 ml/h. The flow-through was collected and the resin was washed four times with 2 ml of buffer Z/0.1 M KCl. Proteins were eluted from the resin with successive addition of 1.0 ml portions of buffer Z containing 0.2, 0.3, 0.4, 0.6, 0.8 and 1.0 M by maintaining the flow rate at 15 ml/h. Elution with buffer Z/1.0 M KCl was repeated one more time. The fractions were collected, placed in separate dialysis tubings (Spectrapor dialysis tubing 6-8 K MW cut off) and dialyzed against 100 volumes of buffer Z/0.1 M KCl at least for 3 h. Footprint reactions were done to determine the PSF binding activity in the purified fractions as described earlier.

2.4.4. Regeneration of the DNA-affinity resin

The DNA-affinity resin can be reused a maximum of 10-15 times without a change in the binding capacity of the resin (Kadonaga, 1991). The following steps were done at room temperature to regenerate the DNA affinity resin.

Five ml of regeneration buffer (10 mM Tris.HCl pH 7.8; 1 mM EDTA; 2.5 M NaCl; 1% (v/v) NP-40) was added to the resin and mixed with a narrow siliconized glass rod and allowed the buffer flow out of the column. The treatment was repeated with regeneration buffer one more time and the resin was equilibrated twice with 15 ml of column storage buffer with freshly added sodium azide and stored in this buffer at 4 °C.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Separation of proteins depending on their molecular size was accomplished by fractionation of proteins in a denaturing gel according to the method described by Laemmli (1970). Either standard size (14 cm x 14 cm) or mini size (6 cm x 8 cm) gel was used in this study. After assembling the glass-plates and spacers of required thickness (most of the times 1 mm spacer was used), required amount of separating gel solution (for 10% gel- 5 ml of 30% (w/v) acrylamide/0.8% (w/v) bisacrylamide stock, 3.75 ml of 4 x separating gel buffer (1.5 M Tris.Cl pH 8.8/0.4%SDS), 6.25 ml of water, 50 µl of 10% (w/v) ammonium persulfate (APS) and 10 µl of N, N, N' N'-tetramethylethylenediamine (TEMED), swirled gently to mix. The acrylamide solution was then poured between the glass plates sandwich to a height so that there is at least 1-2 cm of stacking gel for standard gels or 0.5 cm for mini gels between the bottom of the well and top of the separating gel. A layer of water saturated-isobutyl alcohol was gently layered over the separating gel and the gel was allowed to polymerize for 2 -12 h. The water saturated-isobutyl alcohol solution was discarded and the top of the separating gel was rinsed with 1 x Tris.Cl/SDS pH 8.8 solution. A 4% polyacrylamide stacking gel (0.65 ml of 30% (w/v) acrylamide/0.8% (w/v) bisacrylamide stock, 1.25 ml of 4 x stacking gel buffer (0.5 M Tris.Cl pH 6.8/0.4% SDS), 3.05 ml water, 25 µl 10% APS and 5 µl of TEMED) was layered over the separating gel and appropriate teflon comb was inserted into the stacking gel. The gel was allowed to polymerize for 1 h at room temperature. Once the gel was ready, the comb was removed gently, and the wells were washed with water and the gel assembly was inserted into the tank containing running buffer (3 g of Tris base, 14.4 g of glycine, 1 g of SDS in 1 L). The upper chamber was also filled with sufficient amount of running buffer.

Protein samples were prepared by adding 1/5 th volume of 6 x protein sample preparation buffer (7 ml of 4 x stacking gel buffer, 3 ml of glycerol, 1 g of SDS, 0.93 g DTT, 1.2 mg of bromophenol blue), heated for 3-5 minutes at 100 °C and loaded onto the gel. Prestained-protein-molecular-weight standards were treated according to supplier's

instructions and loaded along with the protein samples.

The gel was run at 10 mA (constant current), until the bromophenol blue tracking dye enters the separating gel. Then the current was increased to 15 mA. The electrophoresis was stopped once the dye reached the bottom of the gel. At this stage, the gels were stained with silver stain or prepared for transfer onto a nitrocellulose or a PVDF membrane.

For protein sequencing purpose, the separating gel was preran for a minimum of 3 h and then the stacking gel was layered over it.

2.5.1. Silver-staining of SDS-PAGE gels

The gels were handled with care and wearing gloves. The gel was placed in a glass container containing 100 ml of fixing solution (50% (v/v) methanol, 10% (v/v) acetic acid, 40% water) and agitated slowly for 30 minutes on a shaker. The fixing solution was replaced with destaining solution (5% methanol, 7% acetic acid and 88% water) and treated for 30 minutes. The destaining solution was poured out and the gel was covered with 50 ml 10% glutaraldehyde and agitated slowly for 10 minutes in fume hood. The gel was washed several times with water for 2 h and finally the gel was soaked in 100 ml of 5 µg/ml of DTT solution for 30 minutes. After discarding the DTT solution, 100 ml of 0.1% silver nitrate solution was added to the gel and agitated for 30 minutes. The silver nitrate solution was poured out and the gel was washed with small amounts of water and small amounts of carbonate developing solution (0.5 ml 37% formaldehyde/L and 3% (w/v) sodium carbonate). Then the gel was soaked in 100 ml of carbonate developing solution and agitated slowly until desired staining was achieved. The staining reaction was stopped by adding 5 ml of 2.3 M citric acid to the 100 ml carbonate developing solution for 10 minutes and agitated slowly. The gel was washed several times with water for 30 minutes and the gel was dried using BioDesignGelWrap.

2.6. Protein (Western) blotting

The gels after electrophoresis was equilibrated with transfer buffer (dissolved 3.1 g of Tris base, 14.4 g of glycine in 800 ml of water and made to 1000 ml with 200 ml of methanol) for 30 minutes at room temperature. Nitrocellulose or PVDF membrane as well as two pieces of Whatman 3 paper was cut to the gel size. The nitrocellulose membrane was treated with water, whereas the sequi-grade PVDF membrane was treated briefly with 100% methanol and then the membranes were equilibrated with transfer buffer for 15 minutes. The gel, membrane and the filter papers were assembled in the following order. From the cathode side of the plastic transfer cassette, placed the sponge, gel, membrane, two Whatman filter papers, and the sponge, in that order. The cassette was closed and inserted into the transfer tank containing the transfer buffer. Transfer of proteins from the gel to membrane was done by applying 100 V (constant voltage) for 1-2 h at 4 °C. After the transfer the membrane was either used for southwestern blotting or stained with Coomassie brilliant blue R-250 and sent for microsequencing.

2.7. Southwestern blotting

For south western blotting, mini gels were used for protein separation. After the transfer, the nitrocellulose membrane was placed in a container containing buffer H (25 mM HEPES-KOH pH 7.9, 1 mM MgCl_2 , 10 mM KCl, and 1 mM DTT) and washed briefly. The membrane was then treated with 30 ml of buffer H containing 6 M guanidium hydrochloride or urea and incubated at 4 °C for 5 minutes with gentle shaking. This incubation was continued for another 5 minutes with 30 ml of fresh buffer H containing 6 M guanidium hydrochloride or urea. The membrane was then sequentially treated for 5 minutes each time with buffer H containing 3 M, 1.5 M, 0.75 M, 0.375 M guanidium hydrochloride or urea, and finally the membrane was washed with 2-3 times with buffer H. The membrane was blocked with buffer H containing 5% Carnation nonfat milk powder for 1 h at 4 °C with gentle shaking and then was washed twice with buffer H containing 0.25% Carnation nonfat milk powder. For Southwestern hybridization, the membrane was

incubated with 10 ml buffer H containing 0.25% Carnation nonfat milk powder, 10 µg/ml sonicated denatured calf thymus DNA, 1×10^6 cpm/ml of probe. The membrane was sealed in a hybridization bag and incubated for 1-2 h at 4 °C with gentle shaking. After incubation, the membrane was washed with buffer H containing 0.25% Carnation nonfat milk powder for 5 minutes and washings were repeated four more times. The membrane was dried and exposed to X-ray film.

2.8. Elution-renaturation of PSF-binding proteins separated by SDS-polyacrylamide gel electrophoresis

This method was applied to estimate the size of the PSF factors in the GC nuclear extracts. Approximately, 2.0 mg of heparin-agarose fractionated 0.4 M KCl fraction, which had shown very high PSF binding activity, was concentrated 100 µl using a Centricon concentrator. The protein solution was placed in the concentrator and centrifuged at 5,000 x g until the volume of the protein solution was reduced to 100 µl (approximately it took 30 minutes). This material was treated with 25 µl of 6 X sample preparation buffer and incubated at 37 °C for 10 minutes. The proteins were separated on a SDS- 10% PAGE gel as detailed under SDS-polyacrylamide gel electrophoresis method.

Elution and renaturation of proteins from the gel was done according to the method described by Ossipow et al. (1993). After electrophoresis, the gel lane with nuclear proteins was sliced into 8 equal size of pieces with a razor blade. Each gel piece was placed into a 1.5 ml microfuge tube containing three volumes (per weight of the polyacrylamide gel slice; 300 µl/100 mg of gel piece) of elution-renaturation buffer (1% Triton X-100; 20 mM HEPES pH 7.6; 1 mM EDTA; 100 mM NaCl; 5 mg/ml of BSA; 2 mM DTT; 0.1 mM PMSF; 0.1% aprotinin) and homogenized with a small plastic homogenizer. After a four hour incubation at 37 °C, the residual polyacrylamide was sedimented in an microfuge (15,000 x g for 15 minutes) and the supernatant (approximately 75 µl) was transferred into a new microfuge tube. Nuclease protection assay was performed with 15 µl of each fraction without the addition of poly [dIdC].

In a repetition of above experiment, a mini PAGE gel was used instead of standard gel to fractionate the GC nuclear proteins and the lane was sliced into 11 equal size pieces. Elution and renaturation of proteins were performed as done above.

2.9. Screening of cDNA clones encoding DNA-binding proteins

Complementary DNA expression libraries prepared from rat pituitary GC (λ -ZAP), human placental (λ Uni-ZAP) and mouse fibroblast (λ gt11) were screened with either a PSF-B (3-binding sites) fragment, radiolabelled by Klenow end filling or a PSF-B ligated concatemerized ODNs, radiolabelled by nick translation method for double strand DNA (Vinson et al. 1988). *E.coli* strains XL Blue (for ZAP library) and Y1090 (for gt11 library) were used as hosts.

2.9.1. Uniform labeling of DNA by nick translation

Concatemerized and ligated PSF-B ODNs that were used in the preparation of affinity column were used as double strand DNA for nick translation. Approximately 500 ng of DNA was mixed with 2.5 μ l of 500 μ M dNTPS-dATP, 2.5 μ l of 10 x *E.coli* DNA polymerase I buffer (500 mM Tris.Cl pH 7.5, 100 mM MgCl₂, 10 mM DTT, 0.5 mg/ml of BSA), 10-20 μ l of 3000 Ci/mmol [α -³²P]dATP (100-200 μ Ci), 1 μ l of DNase I (0.1 ng), 1 μ l of *E.coli* DNA polymerase I (10 U)). The total reaction volume was made to 25 μ l with water and incubated at 14 °C for 30 minutes. The reaction was stopped by adding 1 μ l of 0.5 M EDTA, 3 μ l of 10 mg/ml tRNA and 100 μ l of TE. The reaction mixture was extracted once with phenol:IAC and the aqueous phase was purified on a Sephadex G-50 column. The 1 μ l of labeled DNA was counted and the remainder was used as probe to screen the libraries.

2.9.2. Preparation of nitrocellulose replicas

The hosts were grown overnight in Luria broth (LB; 10 g of bactotryptone, 5 g of

yeast extract and 5 g of NaCl in 1 L) supplemented with 0.2 % maltose and 10 mM Mg SO₄, overnight at 37 °C. For each plating, 0.5 ml of overnight bacterial culture was mixed with 3-5 x 10⁵ plaque forming units of the library in a tube and incubated at 37 °C for 15 minutes to allow the phage to adsorb to bacteria. To the tube, 9 ml of top agarose (0.7% agarose in LB, sterile, melted and cooled to 50 °C) was added, mixed and immediately poured onto 150 mm LB agar plates. Plates were incubated for 3 h at 42 °C till tiny plaques appeared and then moved to 37 °C incubator. Meanwhile 132 mm nitrocellulose filters were impregnated with IPTG by immersing the filters in 10 mM IPTG solution for 30 minutes and air dried at room temperature for 1 h. These filters were overlayed onto each plate and the plates were further incubated at 37 °C for 6-8 h. The plates were cooled for 10 minutes at 4 °C and the filter positions were marked with needle holes. The filters were carefully lifted, placed in a deep dish containing BLOTTO (5% Carnation nonfat milk powder, 50 mM Tris.Cl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) and incubated for 1 h at room temperature. Filters were transferred to a dish containing binding buffer (500 ml/10 filters, BLOTTO without milk powder) and incubated for 5 minutes at room temperature. This washing was repeated twice and the filters were left in the binding buffer for overnight at 4 °C.

2.9.3. Screening of nitrocellulose filter replicas

Heat denatured calf thymus DNA was added to the binding buffer to 5µg/ml final (25 ml of binding buffer/10 filters) and the radiolabelled probe was added at 1-2 x 10⁶ cpm/ml. The filter replicas were added to the probe-binding buffer and incubated with gentle shaking for 1 h at room temperature. The filters were washed 4 times (7.5 minutes per wash, 30 minutes total) at room temperature with 500 ml binding buffer/10 filters. Filters were blot dried, covered with plastic wrap and exposed to X-ray film with an intensifying screen at -70 °C. X-ray films were developed and viewed for positive signals.

Modifications in binding conditions were also introduced in later screenings.

Buffer-D that was used in nuclear extract preparations was used by replacing the Tris based binding buffer.

2.10. Growth and maintenance of bacteria, plasmid DNA purification and construction of plasmid vectors

Growth and maintenance of the bacteria, preparation of plasmid DNA (either small or large scale) used in this study were done according to standard protocols as described in Ausubel et al (1994).

All ligation reactions were performed in 20 µl reaction volumes using 50 -100 ng of plasmid vector and 100-300 ng of insert (depending on the size of the insert) in the presence of 4 U of ligase. Incubations for ligase were done at room temperature for 16 h.

2.10.1. Preparation of competent bacterial cells

Competent bacterial cells were prepared using CaCl₂ method (Maniatis et al. 1982). An aliquot of *Escherichia coli* from the bacterial glycerol stock (strain DH1 or DH5α) was inoculated into 5 ml of LB and grown at 37 °C for 16 h in a shaker incubator. From this overnight culture, 2 ml was inoculated into 100 ml of LB and the bacteria were grown at 37 °C in a shaker incubator till the optical density was about 0.6 at A₆₀₀. The bacteria were transferred into a sterile centrifuge tube and pelleted at 3000 x g for 10 minutes. The pellet was resuspended in 34 ml of sterile, ice cold 0.1 M CaCl₂ and left on ice for 1 h. The bacteria were pelleted again as mentioned above, and the pellet was gently resuspended in 10 ml of ice cold 0.1 M CaCl₂ containing 15% (v/v) glycerol. The bacteria were left in this solution for 12 h on ice and aliquoted as 100 µl into 1.5 ml microfuge tubes and stored at -70 °C.

2.10.2. Transformation of bacteria with plasmid DNA

The competent bacterial cells were thawed on ice for 15 minutes. For transformations 20-100 ng of plasmid DNA or ligation reaction mixture in 10-20 µl volume was added to the bacteria, mixed with gentle flicking of the tube and left on ice for 45 minutes. Then the bacteria were exposed to 42 °C for 2 minutes, added with 1 ml of LB and placed in 37 °C incubator shaker with gentle shaking (100 rpm) for 1 h. Either 100-200 µl of the culture was plated onto 1.5% agar-LB plates containing required amount of the antibiotic marker (most of the plasmids used in this study have ampicillin resistant gene and 100 µg/ml of ampicillin was used both in LB and LB agar plates). The plates were incubated at 37 °C overnight.

2.10.3. Small scale preparation of plasmid DNA

Plasmid DNA from bacteria was prepared according to alkali lysis method. Once the individual colonies are seen on the plate, each colony was picked up by a sterile tooth prick, inoculated into 2 ml of LB containing ampicillin and grown overnight in 37 °C shaker incubator. Approximately 1.5 ml of the bacterial culture was transferred into a 1.5 ml microfuge tube and centrifuged briefly for 45 seconds at 15,000 x g. The supernatant medium was aspirated into bleach and the pellet was resuspended in 100 µl of solution I (25 mM Tris.Cl, pH 8.0, 50 mM glucose and 10 mM EDTA) and kept on ice for 5 minutes. Alkali lysis of bacteria and denaturation of DNA were done with the addition of freshly prepared 200 µl of solution II (0.2 M NaOH, 1% SDS) and further incubated on ice for 5 minutes. Selective circular plasmid DNA renaturation was accomplished by the addition of 150 µl of neutralization solution III (3 M sodium acetate, pH 5.2). After mixing by inverting the tube several times and the tube was incubated for 15 minutes on ice. The bacterial debris along with chromosomal DNA was pelleted by centrifugation at 15,000 x g for 10 minutes and the supernatant (~400 µl) was transferred to a fresh tube. This aqueous solution was extracted once with 1:1 of phenol:IAC and the plasmid DNA in aqueous phase was precipitated with 1 ml of ice cold ethanol. The DNA was pelleted by centrifugation at

15,000 x g for 15 minutes and the DNA pellet was washed once with 70% ethanol. DNA pellet was dried under vacuum and was dissolved in 20-40 µl of TE (10 mM Tris.Cl pH 8.0, 1 mM EDTA).

Large-scale plasmid DNA purification was done using Qiagen plasmid preparation kit. P1 plasmid DNA was also prepared according to suppliers instructions.

2.11. Electrophoretic Mobility Shift Assay (EMSA)

Gel mobility shift assays were performed as described by Baldwin (1990). Different types of probes (either end-labeled ODNs or Klenow end filled DNA fragments) were incubated with various nuclear protein extracts prepared from different cell lines or tissues. DNA-protein interactions were carried out by incubating nuclear protein extracts (2-15 µg/reaction) with 0.1 to 1 ng of radiolabelled (~ 10,000 cpm) DNA probe in binding buffer (20 mM HEPES-KOH pH 7.9, 20 % v/v glycerol, 100 mM of KCl, 0.2 mM EDTA, 0.5 mM DTT) along with 2 µg of poly dIdC and 6.25 mM of MgCl₂, on ice for 15 minutes. For competitions, the competitor ODNs were added to the binding reaction along with the DNA probes. The DNA-protein complexes were resolved through a 4% non-denaturing gels. The gel was pre-run for 60-90 minutes at 150V in 0.5X TBE (1X TBE- 0.089 M Tris-base, 0.089 M boric acid, 0.002 M EDTA). After loading the binding reactions into the wells, the gel was run at 25 mA/gel. A tracking dye mixture containing 0.125% bromophenol blue and 0.125% xylene cyanol was also loaded into a separate well along with the samples. Depending on the size of the probe used, the gels were run until the bromophenol blue reached the desired length. The gels were dried and the complexes were assessed by autoradiography.

2.12. Nuclease (DNase I) protection assay

Most of the DNA fragments, in pUC19 plasmid, used to detect the DNA- protein interaction by nuclease protection assays, were isolated as *EcoRI-HindIII* fragments.

Depending on the size of the fragment to be labelled and to get 100-200 ng of the fragment, sufficient amounts of plasmid DNA were digested with *EcoRI*. The *EcoRI* site was radiolabelled by filling reaction using [α - 32 P]dATP, 250 μ M each of dTTP, dCTP and dGTP, and the large (Klenow) fragment of DNA polymerase I for 2 h at 37 °C. Subsequently, the radiolabelled fragment was released with *HindIII* and purified from agarose gels (1-2% agarose gels depending on the size of the fragment), by running the fragment into a well made in the gel and filled with 4X TBE. The DNA was extracted once with 1:1 a mixture of phenol and IAC and then precipitated with 2 volumes of 95% ethanol after the addition of 300 mM of sodium acetate pH 5.2. The DNA was pelleted, the dried pellet was dissolved in water as 1 ng/ μ l and 1 μ l was counted in a beta counter to determine the amount of radioactivity. Radiolabelled DNA (10,000 cpm or 0.1-0.25 ng) was incubated with or without nuclear proteins (5-25 μ g) in a 20 μ l reaction volume for 15 minutes on ice. Each reaction contained radiolabelled DNA, 1 μ g of poly dIdC, 6.25 mM MgCl₂, require amount of nuclear extract and the volume was made to 20 μ l by adding buffer-D used in nuclear extract preparations. After incubation, the DNA was digested with DNase (0.1 to 0.2 U/reaction; Promega) contained in 24 μ l reaction mix of 5 mM CaCl₂ and 10 mM MgCl₂ for 60-90 seconds at 26 °C. The reaction was stopped by the addition of 160 μ l of a buffer containing 100 μ l of 2X proteinase K buffer (200 mM Tris.HCl pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% (v/v) SDS), 2 μ l of yeast tRNA (10 μ g/ μ l), 10 μ l of proteinase K (10 μ g/ μ l) and 48 μ l water). Samples were extracted once with phenol:IAC, and then precipitated with ethanol. The pelleted DNA was resuspended in 5.0 μ l of sequencing gel loading buffer (90% (v/v) formamide, 1X TBE, 100 mM NaOH, 0.1% (w/v) bromophenol blue and xylene cyanol), heated at 90 °C for 3-5 minutes, placed on ice and then loaded onto a sequencing gel. The samples were fractionated in a 7 M urea-6% polyacrylamide gel (11.4 g of acrylamide, 0.6 g of bis-acrylamide, 100 g of urea and 20 ml of 20 X TBE in 100 ml) which was pre-run at 45 W for 30 minutes and run with samples till the bromophenol blue migrated to the required distance. The gel was taken off the plate onto a Whatman 3 filter paper, dried under vacuum at 80° C and the protected regions were assessed by autoradiography.

2.13. Chemical (Maxam-Gilbert) sequencing

The radiolabelled DNA used in the nuclease protection assays was used in the chemical sequencing method (Ausubel et al. 1994). DNA equivalent of 10-50,000 cpm/10 µl was distributed in to four 1.5 ml microfuge tubes labelled as "G", "G+A", "T+C" and "C". To the G tube Two hundred µl of dimethyl sulfate (DMS) reaction buffer (50 mM sodium cacodylate pH 8.0; 1 mM EDTA) was added to the G tube, and 10 and 5 µl of water was added to the T+C and C tubes. An additional 5 µl of 5 M NaCl was added to the C tube. Base specific modifications were performed by adding 1 µl of DMS to the G tube, 25 µl of formic acid to G+A tube and 30 µl of hydrazine to T+C and C tubes. Each reaction was done at 25 °C for different timings (4 minutes for G, 5 minutes for G+A, 8 minutes for T+C and C). The reactions were stopped by adding 50 µl of DMS stop buffer (1.5 mM sodium acetate pH 7.0; 1.0 M β-mercaptoethanol; filter sterilized and added with 100 µg/ml of tRNA) to the G tube. Whereas the other reactions were stopped with 200 µl of hydrazine stop buffer (300 mM of sodium acetate pH 7.0; 0.1 mM EDTA, filter sterilized and added with 25 µg/ml tRNA). The DNA in all tubes was precipitated with 750 µl of chilled 100% ethanol, immersed in a bath of dry ice/ethanol for 5 minutes and was pelleted by centrifugation at 15,000 x g for 5 minutes. The pellet was washed twice with 70% ethanol and the trace amounts of any reagents were completely removed by reprecipitating the DNA. The strand scission at modified bases was performed by incubating the DNA with 70 µl of 10% piperidine for 30 minutes at 90 °C. The piperidine was removed by vacuum drying and furthermore, the traces of piperidine was removed by evaporating the sample twice from 20 µl of water. The samples were resuspended in 10 µl of formamide loading buffer (80% formamide; 10 mM NaOH; 1 mM EDTA; 0,1% xylene cyanol; 0.1% bromophenol blue) and heated for 2-3 minutes at 90 °C and loaded onto sequencing gels along with the footprinted DNA samples.

2.14. Transient transfer of plasmid DNA into cells

Gene transfer into eukaryotic cell lines was done using calcium phosphate/DNA

precipitation method (Howley et al. 1983). Generally cells were seeded at the density of either 0.5×10^6 (for GC and HeLa) or 1.0×10^6 (for JEG-3 and JAR) 24 hours prior to transfections. Usually a 250 μ l mixture of 10 -15 μ g of plasmid DNA (hybrid CAT gene constructs) and 250 mM of CaCl_2 was added drop by drop to 250 μ l of a 2 x HBSS (280 mM NaCl, 50 mM HEPES (FW 238.3) and 1.5 mM Na_2HPO_4 , dibasic) inspirated with a steady stream of sterile air, in a 15 ml polystyrene tube (Falcon). The mixture was briefly mixed and left at room temperature for 30 minutes to allow to form the DNA/ CaPO_4 precipitate.

Meanwhile, the cells to be transfected were fed with DMEM supplemented with 8% FBS as 9 ml per 10 cm dish. The DNA precipitate was briefly mixed, and was distributed uniformly across the entire culture dish by gentle swirling. The DNA precipitate was left in contact with cells for 16-24 hours. After this incubation time, the cells in the dish were washed twice with 5 ml of PBS, and refed with the appropriate medium (GC, HeLa and JEG-3 cells were fed with DMEM-8% FBS; whereas JAR cell was fed with RPMI-10% FBS). Most of the times transfection of cells with the test plasmids (hybrid CAT gene constructs) and appropriate expression vectors were done in triplicate unless otherwise stated. Amounts of reporter and expression constructs were detailed in the respective sections in the results and discussion.

For treatment with either forskolin (Sigma), 8-Bromo-cyclic adenosine monophosphate (8-Bromo-cAMP; Sigma)) or phorbol-12-myristate-13-acetate (PMA; Sigma), the transfected cells were washed with 10 ml of PBS 24 h after transfection and refed with 5 ml of the respective medium or medium containing either 10 μ M of forskolin, 1 mM of 8-Bromo-cAMP or 100 nM of PMA.

2.15. Stable introduction of plasmid DNA into eukaryotic cell chromatin

Stable introduction of Pit-1 expressing plasmid DNA into JEG-3 and JAR cells was essentially performed as described by Cattini et al (1986a). A vector containing the gene

coding for neomycin resistance (pKOneo) under the control of simian virus (SV) 40 promoter was used to select the stably transformed cells. Approximately 10 µg CMV-Pit-1 plasmid DNA was precipitated along with 1 µg of pKOneo, and transfections were performed as described earlier. After leaving the plasmid DNAs on the cells for 24 h, the cells were fed with appropriate growth medium containing 0.5 mg/ml of G-418 (Geneticin; Gibco-BRL). The cells were fed once in two days with this medium until discrete colonies appear to grow. Once the individual colony reaches approximate size of 2-3 mm, each colony was harvested from the plate by placing a sterile Whatman 3 filter paper soaked in trypsin solution. Each filter disc containing cells was then placed into separate wells in a 24-well culture dish containing 1 ml of the above medium. The cells were allowed to grow to optimum number, then harvested and grown in larger dishes. Once sufficient number of dishes with cells were cultured, frozen stocks of cells were prepared as described earlier or the cells were continued to grow for further analysis such as detection of the stably integrated gene.

2.16. Chloromphenicol acetyl transferase (CAT) assay

Cells transiently transfected with reporter (hybrid CAT gene constructs) plasmid DNA were harvested 48 h after the addition of the DNA to the cells. The monolayers were washed once with 5 ml of PBS-CMF and lifted from the dish by the addition of 4 ml of PBS-CMF containing 1 mM EDTA. The cells were transferred to a 15 ml tube (Corning) and pelleted by centrifugation at 1500 x g for 3 minutes at room temperature. The cell pellet was resuspended in 300-500 µl (depending on size of the cell pellet) of 100 mM Tris.HCl pH 7.8/0.1% (v/v) Triton X-100 (TT buffer), transferred to a 1.5 ml microfuge tube and incubated for 15 minutes on ice. The cell/nuclear debris was sedimented by centrifuging at 15,000 x g for 15 minutes at 4 °C. The supernatant was transferred to a new 1.5 ml microfuge tube. An aliquot (1-2 µl) from the supernatant was used to determine the protein concentration using Bradford (1976) assay. The cell extracts were either used immediately for the CAT assay or stored at -20 °C until use.

Analysis of CAT activity was determined by a two-phase fluor diffusion assay (Neumann et al. 1987). Protein extracts (50-100 µg) were made to a final volume of 200 µl with the addition of TT buffer in a 1.5 ml microfuge tube. The samples were heated to 70 °C for 15 minutes to inactivate the acetyltransferase inhibitors present in the cell extracts. The samples were cooled to room temperature for 10 minutes and transferred to a 7 ml scintillation counting vial containing 75 µl a ³H-acetyl coenzyme A cocktail (0.5 µCi of ³H-acetyl coenzyme A/1 mM chloromphenicol/300 mM Tris.HCl pH 7.8). The mixture was shaken well and in dark, 3 ml of organic-phase counting, ScintiLene cocktail (Fisher) was slowly and carefully layered onto the aqueous mixture. The tubes were capped and covered with aluminum foil to avoid exposure to light, and incubated for 30 minutes at 37° C. The rate of conversion of chloromphenicol to ³H-acetylated chloromphenicol was measured using a Beta counter. At least 5 complete cycles of 1 minute counting per tube was performed to measure the CAT activity. The CAT activity was calculated by plotting the cpm per minute by regression analysis to find the slope, and then determining the cpm per minutes per mg of protein.

2.17. Luciferase assay

In all transfections, a luciferase expressing CMVp.Luc construct was transferred into the cells along with hybrid CAT gene constructs and the luciferase expression was assayed. For luciferase assay, 20 µl of above cell extracts was taken into a 12 x 75 mm borosilicate glass tubes, and the luciferase activity was measured in a photon counting luminometer (ILA911 Luminometer, Tropix Inc, Bedford, MA) in the presence of 100 µl of luciferase substrate (Promega Luciferase assay system) solution per sample.

2.18. Measurement of protein concentrations in samples (Bradford assay)

Protein concentrations of nuclear protein and cell extracts were measured using the Bradford assay (Bradford, 1976). Bovine serum albumin (BSA)-Fraction V was used as standard. For the assay, 1-5 µl of the protein extract was taken into 12 x 75 mm

borosilicate glass tube and the volume was made to 800 μ l with water. Similarly, BSA (1 mg/ml) was added to the glass tubes at the concentration of 2, 4, 6, 8 and 10 μ g and the volume was made up to 800 μ l with water. All samples are briefly mixed and 200 μ l of Bradford's reagent (BioRad) was added to all samples including standards, were thoroughly mixed by mixing and were kept at room temperature for 10-15 minutes. Meanwhile the spectrophotometer (Hitachi U-100) was warmed up at 595 nm wavelength. The protein concentrations of the samples were measured by transferring the solution to a cuvette and reading the optical density at 595 nm. Calculation of protein concentrations were done by plotting the values against the BSA standard.

2.19. Isolation of total RNA

Total RNA from cells or tissue was extracted according to Chomczynski and Sacchi (1987). The monolayers were washed once with 10 ml of ice cold PBS and added with 1.8 ml of GITC solution (4 M guanidinium isothiocyanate; 25 mM sodium citrate pH 7.0; 0.5% (v/v) sarcosyl; 100 mM β -mercaptoethanol). The cells were scraped into a 15 ml polypropylene centrifuge tube (Falcon) using rubber policeman. To this cell lysate, 180 μ l of 2 M sodium citrate pH 4.0, 1.8 ml of phenol saturated with water and 360 μ l IAC (24:1 of chloroform and isoamyl alcohol) were added and vigorously mixed. This was maintained on ice for 30 minutes, and the phases were separated by centrifugation at 12,000 x g for 15 minutes at 4° C. The aqueous (upper) phase was collected and added to a new tube and mixed with 1 volume of isopropanol and the RNA was precipitated by centrifugation at 12,000 x g for 15 minutes at 4 °C. The pellet was resuspended in 400 μ l of TE and then transferred to a 1.5 ml microfuge tube and added with 400 μ l of phenol saturated with water, 40 μ l of 2 M sodium acetate pH 4.0 and 80 μ l IAC. The tube was vigorously mixed and then centrifuged at 15,000 x g for 10 minutes at 4° C. The supernatant was precipitated with 1 volume of isopropanol and the RNA was pelleted by centrifugation at 15,000 x g for 10 minutes at 4 °C, the pellet was washed once with 70% ethanol, dried under vacuum and then resuspended in 100-200 μ l of sterile distilled water. To dissolve the pellet, the RNA was heated at 65 °C for 10 minutes. Quantification of RNA

was done spectrophotometrically reading absorbency at A₂₆₀ and quality of RNA was determined by measuring the ratio of absorbancies at A₂₆₀ and A₂₈₀. Generally the RNA was stored as 50-100 µg aliquots mixed with 2.5 volumes of ethanol with 300 mM of sodium acetate pH 4.0.

Total cellular RNA from tissues was also prepared essentially as described above. Approximately, 1 g of the tissue was minced well in 9 ml of GITC solution. This was distributed in 1.8 ml aliquots and then RNA extracted as described above.

2.20. Fractionation of total RNA

Total RNA was subjected to size fractionation in a formaldehyde gel according to the method described by Maniatis et al (1982). A 1.5% (w/v) agarose gel was made by dissolving 3.75 g of agarose in 205 ml of MOPS solution pH 7.0 (20 mM 3-[N-morpholino] propane-sulfonic acid, 5 mM sodium acetate, 1 mM EDTA). When the agarose solution was cooled down to 50 °C, 45 ml of 37% (v/v) formaldehyde (2.2 M final concentration) was added and mixed. The gel was poured in a fume hood and left to solidify.

Meanwhile, the RNA samples from -70 °C were taken out and pelleted. The RNA pellet was resuspended in 40 µl of RNA reaction mixture (2 µl of a 10X MOPS solution, 7 µl of 37% (v/v) formaldehyde, 20 µl of de-ionized formamide, 9 µl of sterile water and 2 µl of 0.4 µg/µl ethidium bromide). The samples were heated at 65 °C for 20 minutes to dissolve the RNA pellet and mixed with RNA loading dye (50% (v/v) glycerol, 1 mM EDTA, 0.4% (w/v) bromophenol blue, 0.4% (w/v) xylene cyanol). The samples were loaded into the gel and electrophoresed in MOPS solution at 25-30 V 12-16 h. The gels were photographed to mark the migration of 28S (4.8 kb) and 18S (1.8 kb) ribosomal RNA (Maniatis et al. 1982). This information was used to determine the approximate size of the RNA detected from hybridization and subsequent autoradiography.

2.21. RNA (Northern) transfer and hybridization

Transfer RNA from the agarose gel to a nitrocellulose membrane was done according to the method of Maniatis et al (1982). A glass trough was filled with 300-500 ml of 20X SSC (3 M sodium chloride and 0.3 M sodium citrate) and overlaid with a glass plate and a Whatman 3 filter paper. The filter paper was let to soak with 20X SSC by capillary action. The gel was marked to identify the top and bottom, and laid on the top of the filter paper, followed by a layer of nitrocellulose membrane (cut to the exact size of the gel and presoaked in 6X SSC), 3 layers of Whatman 3 filter papers and 10 -15 cm thickness of stack of paper towels. A weighted glass plate was put on the top of the paper towels and transfer was then allowed to proceed for 16 h. After the transfer the membrane was soaked briefly in 6X SSC, air dried, and then baked for 40 minutes at 80 °C under vacuum. The blot was store at -20 °C until use.

DNA fragments were used as probes to detect the specific-RNA transcripts in the blots. Probes were routinely labelled using random priming kit (Prime-a-Gene kit, Promega), to a specific activity greater than 1×10^5 cpm/ μ g of DNA. Either [α - 32 P]dATP or -dCTP (Amersham) was used to label the DNA. The cDNA probes used in these studies were hGH-N (Martial et al. 1979) and Pit-1 (Ingraham et al. 1988) .

The blot with RNA was inserted into a hybridization chamber and incubated at 42 °C for 3-12 h, with 20 ml of prehybridization containing 50% formamide, 5X SSC, 5X Denhardts solution (50X = 1% (w/v) Ficoll, 1% (w/v) polyvinyl pyrrollidone, 1% (w/v) BSA), 50 mM sodium phosphate pH 6.5, 0.1% SDS, 100 μ g/ml yeast tRNA and 25 μ g/ml salmon sperm DNA. After preincubation, the solution was discarded, and replaced by 10-15 ml hybridization solution (50% (v/v) formamide, 5X SSC, 5X Denhardts solution, 50 mM sodium phosphate pH 6.5, 10 μ g/ml yeast tRNA, 25 μ g/ml salmon sperm DNA and 10% (w/v) dextran sulfate). Radiolabelled DNA probes were denatured by heating to 95 °C and added immediately to the hybridization solution. Hybridization was done for 16-20 h at 42 °C and then the membranes were washed 3 times for 15 minutes in 0.1X SSC, 0.1%

(v/v) SDS at 65 °C and exposed to Kodak XAR-2 X-ray film using intensifying screens (DuPont) at -70 °C.

2.22. DNA (Southern) transfer and hybridization

Genomic DNA (5-10 µg) was digested with appropriate restriction enzymes and fractionated in a 1% agarose gel. Gel electrophoresis was performed at 30-40 V for 12-16 h. Once the tracking dyes had migrated to the desired distance in the gel, the gel was photographed and top or bottom of the gel was marked. The gel was then subjected to treatments to denature the DNA in the gel. The gel was placed in a glass trough containing sufficient amount of denaturation buffer (1.5 M NaCl; 0.5 M NaOH) for 30 minutes with gentle shaking. The buffer was replaced with a fresh one and the treatment was continued for another 30 minutes. Subsequently, the gel was treated with a neutralization buffer (3 M NaCl; 0.5 M Tris.Cl pH 7.0) similarly as done earlier.

Transfer of DNA onto nitrocellulose membrane and hybridization of DNA with different probes was similar to that was described with RNA transfer and hybridization.

2.23. Polymerase chain reaction (PCR)-DNA sequencing

Double strand DNA sequencing was performed using the fmol sequencing kit (Promega). Approximately 1 µg of template (plasmid containing DNA of interest) DNA was used for sequencing. The sequencing reaction mixture was prepared by mixing 1 µl (1 µg) of DNA with 2 µl (20 ng) of primer, 10 µl of 5 x DNA sequencing buffer, 1 µl of [α - 32 P]dATP and 0.5 µl 92 U) Taq DNA polymerase and water was added to make the volume to 16 µl. Four 0.5 ml microfuge tubes were labeled as A, C, G and T and from the kit, 2.0 µl of ddATP, ddCTP, ddGTP and ddTTP mixture was added to their respective 0.5 ml tube. To each of these tubes, 4.0 µl of the sequencing reaction mixture was added and the tubes were placed in the thermal cycler. The PCR conditions were an initial step of 95 °C for 4 minutes and sequencing reaction was performed for 30 cycles each cycle consisting of

a denaturation step of 92 °C for 1 minutes, an annealing step of 55 °C for 30 seconds and an extension step of 72 °C for 1 minute. The reactions were stopped by adding 3 µl of stop solution and treated at 95 °C for 3 minutes and loaded on to a 7 M urea-6-8% polyacrylamide sequencing gels and electrophoresed at 45 W constant power supply. Once the the tracking dyes reach the desired length from the top of the gel, the gel was removed from the plates using a Whatman 3 paper, dried and exposed to an X-ray film.

2.24. Statistical analysis

The data from CAT assays were analyzed using the statistical program Instat 2.03 (GraphPad Software). Analysis of variance (ANOVA), Student's "t" and analogous nonparametric tests such as alternative Welch, nonparametric Mann-Whitney, paired and unpaired tests were used for determination of statistical significance. The data were considered significant only when the probability of variance among groups upon comparison was less than 5% (<0.05).

CHAPTER 3

3.1. PARTIAL PURIFICATION AND CHARACTERIZATION OF THE PROTEINS RECOGNIZING THE PSF ELEMENTS

3.1.1. General introduction to purification of the DNA-binding proteins

Many investigators are studying DNA-binding proteins because of their involvement in cellular processes such as replication, recombination, transcription and bacteriophage and viral integration (Kadonaga, 1991). The advent of recombinant DNA technology, and isolation of several biologically important genes has led to a common interest in how these genes are regulated at the transcriptional level and especially, in a temporal or tissue-specific manner in response to environmental and developmental signals. Subsequently, characterization of the DNA sequences responsible for this regulation necessitated the identification, isolation and characterization of the proteins recognizing these regulatory DNA sequences. With the further understanding of the mechanisms involved in the transcriptional regulation of the genes, it is now evident that the factors that recognize the proximal promoter elements, such as general transcription factors, as well as the proteins that bind to distal sequences ("action at a distance", Hochschild, 1991) are also important in gene expression.

To understand the role of these factors in biological processes and to study their biochemical properties, it is necessary to purify the proteins to homogeneity. The major difficulty in the isolation of the DNA-binding proteins is their characteristic very low abundance in the cells (with levels as low as 0.001% of cellular protein; Kadonaga and Tjian, 1986). However, investigators are still able to identify these factors using various methods to isolate and purify the DNA-binding proteins.

3.1.2. Methods applied in isolation and purification of DNA-binding proteins

1. The majority of the DNA binding proteins were identified by conventional protein purification methods coupled with sequence-specific DNA-affinity chromatography (Table. 3.1). In this method, the nuclear protein extracts would be subjected to series of fractionations by column chromatography, such as gel filtration and/or ion-exchange chromatography. These partially purified proteins would be further purified to near homogeneity by using their specific recognition sequences bound to an agarose resin as in DNA-affinity chromatography (Kadonaga and Tjian, 1986). Once the factor is identified and purified to homogeneity, the protein is subjected to microsequencing. From this sequence information, oligodeoxynucleotides (ODNs) would be synthesized and used as probes to screen cDNA libraries to identify the gene encoding the DNA-binding protein.

Table: 3.1. Some important mammalian sequence specific DNA-binding proteins purified by DNA affinity technique

<u>Factor</u>	<u>Source</u>	<u>Reference</u>
Sp-1	HeLa	Briggs et al. 1986
CTF/NF-1	HeLa	Jones et al. 1987
AP-1	HeLa	Lee et al. 1987; Angel et al. 1987
AP-2	HeLa	Mitchel et al. 1987; Imagawa et al. 1987
NF-kB	Namalwa	Kawakami et al. 1988
	HeLa	Baeuerle and Baltimore. 1989
PUF-1/GHF-1/ Pit-1	GH3/GC	Cao et al. 1988; Castrillo et al. 1989; Mangalam et al. 1989
TEF-1	HeLa	Davidson et. 1988
SRF/CBF	HeLa	Prywes and Roeder. 1987; Boxer et al. 1989
UBF-1	HeLa	Bell et al. 1988

2. The second most popular method of isolating a DNA-binding protein is by screening a cDNA expression library (southwestern screening) using the specific binding site as a probe (Table. 3.2; Singh et al. 1988).

Table: 3.2. Some important mammalian sequence specific DNA-binding proteins isolated from a cDNA expression library screened with a specific binding site (oligonucleotide)

<u>Factor</u>	<u>Binding site/probe</u>	<u>Organism</u>	<u>Reference</u>
MBP-1	GGGGATTCCCC	Human	Singh et al. 1988
Oct-2	ATGCAAAT	Human	Clerc et al. 1988
Oct-1	ATGCAAAT	Human	Sturm et al. 1988
Pit-1	GATTACATGAATATTCATGA	Rat	Ingraham et al. 1988
NF-E1	CCTCCATC	Human	Park and Atchison, 1991
TEF-1	GTGGAATGT	Human	Xiao et al. 1991
CREB	TGACGTC	Human	Hoeffler et al. 1988

3. A variation of the above method is the one-hybrid system of screening cDNA expression libraries using yeast genetics as a tool (Wang and Reed, 1993). The olfactory neuronal transcription factor Olf-1 was the first factor to be identified by this method. The yeast provides the natural eukaryotic environment and is capable of modifying the protein post translationally. These modifications may be important for the interaction between the protein and the DNA.

4. Tissue-specific variants of a known DNA-binding protein or transcription factors with highly conserved DNA binding domains can be identified by screening libraries with these conserved regions as DNA probes or by polymerase chain reaction using primers designed to these conserved regions to isolate the variant cDNA. Some of the homeodomain containing transcription factors, tissue-specific members of GATA and TEA-DNA binding domain-containing transcription factors were identified by this method.

5. The DNA-binding proteins not only bind to their specific DNA sequence, but also interact with other DNA-binding proteins to exert their influence on transcription. This principle can be utilized to identify factors interacting with a known DNA-binding protein in a yeast two-hybrid system of screening cDNA libraries (Fields and Song, 1989).

3.1.3.1. Role of the PSF-A and PSF-B recognition factors in the transcriptional regulation of the human growth/chorionic somatomammotropin gene family

The human growth hormone (GH) gene family presents an unique model to study tissue-specific regulation of highly homologous genes, which are transcribed specifically in two different tissues. The GH-N gene, probably the most ancestral gene of the family, is specifically expressed in the somatotroph cell lineage of the pituitary (Nelson et al. 1988; Pelletier et al. 1978; Simmons et al. 1990). All other species of vertebrate animals possess only one GH gene, which is expressed predominantly in the pituitary. The pituitary-specific transcription factor, variously known as GHF-1/Pit-1 was shown to be the sole important factor for the pituitary- and somatotroph cell-specific expression of the GH-N gene (Ingraham et al. 1988; Lefevre et al. 1987). However, the GH-N related CS-L, CS-A, GH-V and CS-B genes, which are characteristic only to the primates, are exclusively transcribed in the syncytiotrophoblastic villus epithelial cell layer of the placenta during pregnancy (McWilliams and Boime, 1980, Parks, 1989). Initially, it was thought that the placental CS/GH-V genes were also regulated at the transcriptional level in placenta, in a similar way as that of the pituitary GH-N gene owing to the striking sequence similarity. However, it was believed that placenta does not express the GHF-1/Pit-1 transcription factor (Bodner and Karin, 1987) and therefore, it was assumed that the regulatory mechanisms involved in the expression of the placental CS/GH-V genes may be under the control of different transcription factors which could be related to the GHF-1/Pit-1 proteins. Although it was reported that the CS-L, CS-A and CS-B genes possess an enhancer region in their 3'-flanking sequences (Rogers et al. 1986; Walker et al. 1990; Jacquemin et al. 1994a), the regulatory mechanisms involved in the placenta-specific expression of the CS/GH-V genes are still not clear, and this will be discussed in detail in later sections.

The transcriptional regulation of a particular gene is the primary controlling mechanism in gene expression. It is an interesting situation, with regard to the GH/CS

genes, wherein highly homologous genes are expressed in two different tissues. Thus, a study of the regulatory mechanisms of these genes might determine how this tissue-specificity was achieved. Generally, a careful analysis of the genomic sequences located immediately upstream of these genes will reveal the important attributes involved in this specific transcriptional regulation. One of the advantages with the GH/CS gene cluster is that all five genes are tandemly arranged as a single locus on chromosome 17, and the complete genomic DNA sequence (66,495 bp) is known (Chen et al. 1989). These five genes share more than 92% sequence similarity in their primary transcripts and in the immediate 5'-flanking proximal promoter (-1 to -500 bp) DNA (Chen et al. 1989). The latter raises the possibility that these promoters can bind the same transcription factors and can be active in the pituitary somatotrophs, at least *in vitro*. Indeed, the ability of GHF-1/Pit-1 to bind to the GH/CS promoter regions was demonstrated by DNase I protection assays (Lemaigre et al. 1989; Nickel et al. 1991).

An extensive polymorphism in the GH-N promoter was reported (Giordano et al. 1997). The Chi-like sequences (GCTGGTC), which are known to be involved in DNA rearrangements and gene conversions, are present in the GH-N promoter (at nucleotide position -4/-10; Giordano et al. 1997 and references within). In these studies, it was revealed that whole stretches of the GH-N gene promoter sequences were replaced by homologous DNA stretches from the CS/GH-V genes by repeated gene conversion-like events without affecting GH-N gene transcription. These observations suggest that proximal promoter sequences of GH/CS genes are equally competent in the human pituitary, but still the CS/GH-V genes are not expressed *in vivo*.

The major drawback in studying transcriptional regulation of these primate genes, has been the unavailability of cell lines of primate pituitary origin. Initial characterization of the rat GH, prolactin (PRL) and human GH-N promoter sequences led to the identification of the pituitary-specific transcription factor, GHF-1/Pit-1 (Cattini et al. 1986; Lefevre et al. 1987; Nelson et al. 1986). Comparative analysis of the proximal promoter sequences of the human and rat GH-N gene revealed homology as well as conservation of the presence of

the two GHF-1/Pit-1 sites (Bodner and Karin, 1987; Cattini et al. 1986; Lefevre et al. 1987; Nelson et al. 1986; West et al. 1987). Furthermore, it was noticed that the proximal promoter sequences of the rat GH is sufficient for the efficient pituitary-specific expression both in cell culture (Nelson et al. 1986) and in the transgenic mouse (Behringer et al. 1988; Lira et al. 1988, 1993). Subsequently, it was shown by Lew and Elsholtz (1991) that the cDNA coding for human GHF-1/Pit-1 has a high degree of similarity (>96%) with rat GHF-1/Pit-1 and very high degree of homology was also revealed at the protein level. Because of these observations, one would expect a similar mechanism for transcriptional regulation of the human GH/CS and PRL genes in the pituitary. With the availability of the rat pituitary somatotroph (GC), lactotroph (235-1) and somatolactotroph (GH3 and GH4C1) tumor cell lines, many investigators used these cells successfully to study the transcriptional regulation of the primate GH/CS as well as PRL genes. Indeed, the functional characterization of the proximal promoter sequences (+1 to -496) of the GH-N and CS-A genes, revealed that both promoters are equally active in rat pituitary somatotroph GC cells after gene transfer (Cattini and Eberhardt, 1987). Furthermore, it was shown that CS-A promoter (CS-Ap) activity was dependent on the binding of the GHF-1/Pit-1 factor, since activity was abolished once the GHF-1/Pit-1 site(s) were deleted (Nachtigal et al. 1989). These observations as well as the high level of sequence similarity between the human and rat GHF-1/Pit-1 factors suggest that human GHF-1/Pit-1 should be able to activate both GH-N and CS genes in the pituitary, although CS and GH-V genes are not expressed in that tissue. Thus, it does not seem that the ability of the human CS-A promoter to function in rat GC cells can be easily explained by the minor structural differences between human and rat GHF-1/Pit-1. In this context it is noteworthy, that many transcription factors with similar or even slightly less sequence similarity are interchangeable and substitutable for similar functions among species ranging from yeast to humans. For example, the human transcriptional enhancer factor-1 (TEF-1) and *Drosophila* scalloped (sd) factors belong to a family of proteins that have a evolutionarily conserved TEA-DNA binding domain (TEA-DBD) (Burglin, 1991; Davidson et al. 1988). Both these factors share ~98% homology in their TEA-DBD and only ~50% similarity in their activation domain. It was observed that human TEF-1 can substitute for sd function in

Drosophila (Deshpande et al. 1997). Similarly, the human transcriptional modulator, TRIP-1/SUG-1 could substitute for a homologous gene in yeast (Lee et al. 1995b). Thus, it is possible that the activation of the placental CS genes in rat pituitary GC cells, as was observed by Cattini and Eberhardt (1987) and Nachtigal et al. (1989), was not an artificial phenomenon. Hence, this raises the question- ***what are the possible mechanisms for the differential regulation and restriction of the expression of the CS/GH-V genes in the pituitary?***

There are several possible mechanisms that could account for tissue-specific regulation of GH/CS genes. These include: (1) differential methylation of gene sequences, (2) chromatin structure and configuration, (3) presence of locus control regions, (4) presence of either pituitary-specific or placenta-specific repressor or negative regulators, and (5) other mechanisms such as differential responsiveness of GH/CS genes to environmental stimuli.

The available sequence information for the hGH/CS gene cluster reveals the absence of HTF/CpG island sequences in the immediate upstream or downstream flanking sequences of the each member of the GH/CS gene family (Bird, 1986; Chen et al. 1989). The cytosine residues of the HTF/CpG sequences are methylated and this methylation process was shown to be essential for differential expression patterns of many genes (Bird, 1986 and references therein). Furthermore, analysis of the relative levels of cytosine methylation in GH/CS gene locus in both GH/CS gene expressing and non-expressing tissues revealed that the extent of methylation of Hpa II and Hha I sites in the GH and CS genes was leukocyte>>pituitary>placenta=hydatidiform mole (Hjelle et al. 1982). At that time it was thought that the leukocytes do not express the GH gene, but later studies revealed some low level GH gene expression in peripheral blood leukocytes and lymphocytes (Lytras et al. 1993; Melen et al. 1997; Weigent et al. 1988). Hydatidiform mole, a form of trophoblastic neoplasm expresses little or no CS mRNA (Hjelle et al. 1982; Lytras et al. 1993; Mochizuki et al. 1976). Hence, these studies did not reveal a correlation between gene expression and the observed methylation pattern (Hjelle et al. 1982), thus a

role for differential (hypo or hyper) methylation of the GH/CS genes in the pituitary and the placenta in the control of GH/CS gene expression and, more specifically, restriction of CS/GH-V gene expression in the pituitary is not supported by available data. However, hypomethylation of the GH-N promoter region was shown to be partially responsible for the overexpression of the GH-N gene in human somatotrophinomas (Huttner et al. 1994).

It is possible that the differences in the chromatin structure of the GH/CS gene locus in the pituitary and the placenta might be responsible for their expression in two different tissues. In this context, it is interesting to observe that the expression of the GH and PRL genes in pituitary, although controlled by the GHF-1/Pit-1, is restricted to two different cell types in the pituitary. The GH and PRL genes are expressed in the somatotrophs and the lactotrophs (Pelletier et al. 1978), respectively. Moreover, the majority of the lactotrophs were shown to be derived from the somatotrophs (reviewed by Andersen and Rosenfeld, 1994). However, the PRL gene expression is also regulated by estrogen (Maurer, 1985; Shull and Gorski, 1985; Simmons et al. 1990). Recently it was shown that possible differences in chromatin configuration, and as reflected in changes in DNase I sensitivity are responsible for this cell-specific expression of these two genes (Aizawa et al. 1996). However, DNase I sensitivity assays on the nuclei isolated from human pituitary revealed that the chromatin of the CS/GH-V genes is as sensitive as that of the GH-N gene, suggesting the open configuration of the GH/CS genes in the pituitary (Nickel and Cattini, 1996). These findings suggest that this decondensed chromatin structure at the GH/CS gene locus would be accessible to DNA-binding factors including GHF-1/Pit-1. In this scenario, the open chromatin structure of the CS/GH-V genes would also be accessible to some repressor factor(s) involved in the pituitary-specific restriction of these genes. Therefore, our laboratory investigated this possibility and searched for the presence of a repressor mechanism.

3.1.3.2. Identification of PSF sequences

As stated earlier, the complete sequence information of the GH/CS gene cluster is

known (Chen et al. 1989). Dr. Seeburg's group had identified several interesting and characteristic sequence regions in the flanking sequences of the CS/GH-V genes. In the 5'-upstream flanking regions, a highly conserved element of a 1.0 kb repeat sequence located 1.7-2.1 kb upstream of the placental genes was identified (Chen et al. 1989). This sequence is absent for the GH-N gene. As this region is characteristic of placental CS/GH genes, it was designated as P-sequence (Chen et al. 1989). It was thought that insertion of P-sequence might have occurred during the duplication process of the GH-N ancestral gene in the human genome. It was further suggested that the P-region may harbor the transcriptional regulatory elements which could influence the expression of the placental CS/GH genes in either the placenta or pituitary (Chen et al. 1989).

Our laboratory investigated the P-sequence region and the initial functional characterization of a 2.4 kb fragment comprising the P-sequence (2.4P) repressed both the GH-N and CS-A promoter activities in pituitary GC cells after gene transfer (Nachtigal et al. 1993). This was the first study that revealed the presence of a pituitary specific repressor mechanism associated with P-sequences (Nachtigal et al. 1993). Furthermore, detailed analysis of this sequence led to the localization of repressor activity to a 263 bp fragment (263P) (Nachtigal et al. 1993). Subsequently, determination of sequence-specific protein binding activity on the 263P fragment using deoxyribonuclease I (DNase I) protection assays identified two footprint regions, P-Sequene Factor/Footprint (PSF)-A and PSF-B. This 263P fragment possessed the pituitary-specific repression activity in the forward but not reverse orientation, and this was similar to the repression seen with the 2.4P fragment (Nachtigal et al. 1993). These binding elements were reported to be "novel" elements as the PSF-A and PSF-B sequences had not been reported previously (Nachtigal et al. 1993). Hence, it was proposed that the factors (PSFs) recognizing the PSF-A and PSF-B elements are responsible for the pituitary-specific repression of the placental CS/GH genes (Nachtigal et al. 1993). Furthermore, it was also observed that the PSFs are ubiquitous with the exception of the human placenta and are evolutionarily conserved (Nachtigal et al. 1993). At this juncture, the role of these ubiquitous factors in other cells and tissues and of course in other species of animals is not known. This necessitates one to

identify and purify the PSF factors and subsequently isolate the genes encoding these factors to characterize and answer the functionality of these evolutionarily highly conserved factors.

Purification of the PSF (A and B) protein(s) was carried out by the two widely used methods. The first approach was purifying the protein (s) from nuclear extracts by a series of ion-exchange and DNA-affinity chromatography, to near homogeneity, and subsequently attempting to microsequence the protein to get N-terminal amino acid sequence. This information can be used to synthesize the oligonucleotides to screen cDNA libraries. The second method attempted was screening the expression cDNA libraries with radioactively labeled concatemeric PSF recognition elements.

3.2. PURIFICATION OF PSF-A AND PSF-B FACTORS

3.2.1. Source (cell or tissue) of the PSF-A and PSF-B proteins for purification

The factors recognizing the PSF-A and PSF-B elements of the P sequence were shown to be expressed in a wide variety of cells and tissues of different species origin (Nachtigal et al. 1993). The initial identification and the role of the PSF factors were studied in the rat anterior pituitary tumor GC cells (Nachtigal et al. 1993). In addition to this, GC cells were also the source of GHF-1/Pit-1 purification (Castrillo et al. 1989). GHF-1/Pit-1 was the first tissue-specific transcription factor to be isolated and more importantly it is the transcription factor involved in pituitary-specific expression of the GH-N gene. Hence, GC cells were used as the source to purify the PSF proteins. These cells are usually grown as monolayers. Although it was reported that the GC cells can grow in suspension (Bodner and Karin, 1987), our initial trials to repeat this were not successful.

Thus, we chose to purify PSF-A and -B factors from GC cells grown as monolayers in 150 mm culture dishes. The cells were harvested at 70% of confluency (12-

15 x 10⁶ cells/plate). Each time, cells from a batch of 20 culture dishes were harvested and stored at -70 °C. Nuclear protein extracts were prepared according to established protocol (Dignam et al. 1983). After processing the GC cells with hypotonic buffer-A and subsequent Dounce homogenization, the cytoplasmic fraction was discarded after a low speed centrifugation, and the nuclear pellet was extracted with a buffer-C containing 420 mM NaCl and dialyzed against buffer-D (Dignam et al. 1983). Approximately, 220 mg of crude nuclear protein was obtained from 300 x 150 mm culture dishes. The protein content was estimated by Bradford's (Bio-Rad) assay (Bradford, 1976). Every batch of nuclear extract was assayed for the presence of PSF-A and -B binding activity by the DNase I protection (footprints) assay on the 263P fragment of the P-sequence containing the PSF-A and -B elements (Nachtigal et al. 1993).

Many investigators used an ammonium sulfate (40% to 53%) precipitation step (Briggs et al. 1986, Karsenty et al. 1991) to concentrate and differentially precipitate the crude nuclear proteins before separation by sequential column chromatography. Although we did not use the ammonium precipitate step, attempts were made to purify the PSFs using heparin agarose, gel-filtration, phosphocellulose, hydroxylapatite and finally DNA-affinity columns.

3.2.2. Heparin-Agarose Chromatography

Initially, on a pilot scale, fractionation of the GC nuclear protein extract was performed with 12.5 mg/5 ml of protein. The extract was incubated with 1.5 ml bed volume of the heparin agarose (HA) resin for 1 h at 4 °C to allow nuclear proteins to bind to the resin. Fractionation of the nuclear proteins was done by serially treating the bound resin with 1.5 ml each of 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M KCl-buffer-D as was described by Dynan and Tjian (1983) and as detailed in Materials and Methods section. The eluted samples were dialyzed against buffer-D and were tested for the presence of PSF (A and B) factors. At least 5-10 µg of each of the dialyzed fractions was incubated with radiolabelled 263P fragment containing PSF-A and -B elements. Subsequently the probe

was digested with DNase I as detailed in the Materials and Methods section. The majority of the PSF (A and B) recognizing factors were eluted at 0.4 M and 0.6 M KCl (Fig. 3.1).

After confirming the elution profile of the GC nuclear proteins from the HA resin, a large scale purification of the GC nuclear proteins was performed. Approximately 125 mg of GC nuclear protein/50 mL was mixed with 15 ml bed volume of HA resin and the nuclear proteins were fractionated as indicated above. Twenty milliliters of each KCl concentrations were used for elutions. From the protein estimations, it was determined that from the starting material of approximately 125 mg of GC nuclear protein, an amount of 40 mg of protein was seen in the flow-through and approximately 6-10 mg was eluted at 0.2 M KCl. Significant amounts of protein, 28 and 30 mg were observed at 0.4 and 0.6 M KCl, respectively. Finally, 12 mg of protein was eluted at 1.0 KCl concentration (Table. 3.3.). After dialysis and bringing the KCl concentration to 0.1 M in all eluted samples, the samples (5-10 µg) were tested for the presence of PSF proteins. As observed with the small scale purification, the PSF (A and B) factors were present in the 0.4 M and 0.6 M KCl fractions (Fig. 3.2).

Table. 3.3. Amounts of protein recovered from the Heparin-Agarose chromatography

<u>Sample</u>	<u>Protein in mg</u>	<u>% of Recovery</u>
Load	125	100.0
Flow-through	40	32.0
0.2 M KCl	10	8.0
0.4 M KCl	28	22.4
0.6 M KCl	30	24.0
1.0 M KCl	12	9.6

Fig. 3.1. Heparin agarose fractionation of GC cell nuclear extract. Rat pituitary tumor GC cell nuclear proteins were fractionated using heparin agarose resin as described in Materials and Methods section. The eluted fractions were tested for the presence of PSF-A and PSF-B factors using nuclease protection assay on the radiolabelled 263P fragment. Lanes represent the DNase I pattern generated from the 263P fragment in the absence of GC cell nuclear protein (a, i and j) or in the presence of GC cell nuclear protein used as load on to the resin (b), flow-through from the resin (c), 0.2 M (d), 0.4 M (e), 0.6 M (f), 0.8 M (g) and 1.0 M (h) KCl eluted GC cell nuclear protein fractions from the resin. Two regions, PSF-A and PSF-B, protected by the nuclear proteins are indicated.

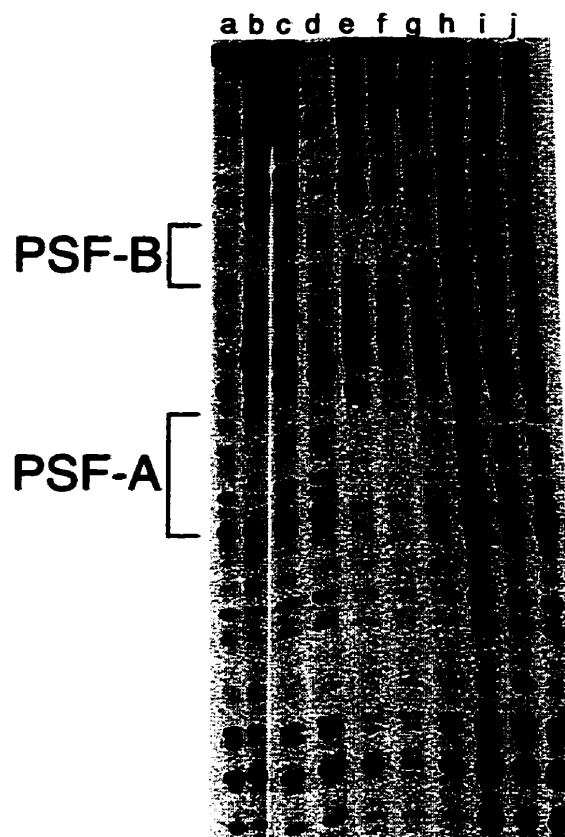
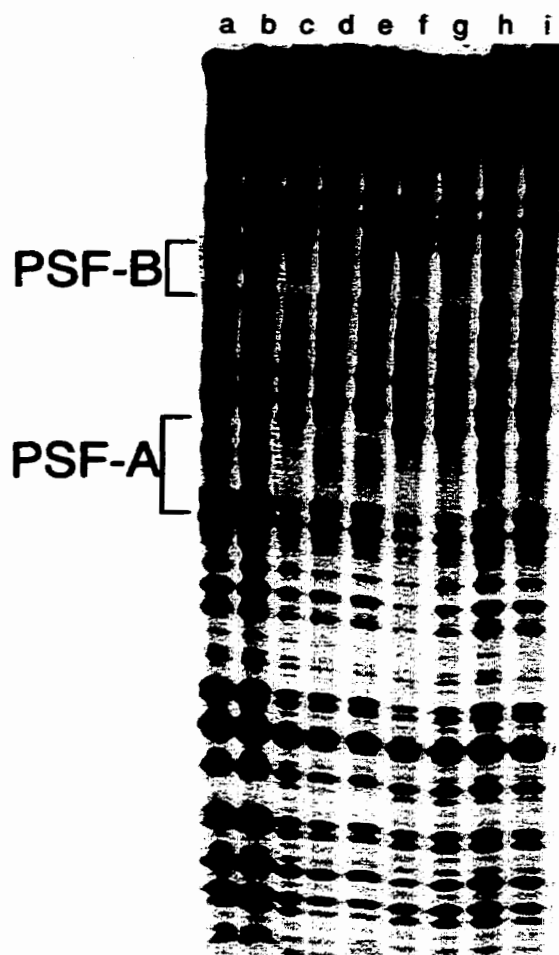


Fig. 3.2. Large scale heparin agarose fractionation of GC cell nuclear extract. Rat pituitary tumor GC cell nuclear proteins were fractionated using heparin agarose resin as described in Materials and Methods section. The eluted fractions were tested for the presence of PSF-A and PSF-B factors using nuclease protection assay on the radiolabelled 263P fragment. Lanes represent the DNase I pattern generated from the 263P fragment in the absence of GC cell nuclear protein (a, b and i), or in the presence of GC cell nuclear protein used as load on to the resin (c), flow-through from the resin (d), 0.2 M (e), 0.4 M (f), 0.6 M (g), 1.0 M (h) KCl eluted GC cell nuclear protein fractions from the resin. Two regions, PSF-A and PSF-B, protected by the nuclear proteins are indicated.



At a later time, a second batch of GC cell (150 mg) and several small scale purifications of the HeLa cell nuclear extracts were also performed using HA chromatography. Similar yields and fractionation profiles were observed with each batch of purification.

Other chromatography techniques were also employed to fractionate the GC nuclear protein, such as phosphocellulose or hydroxylapatite (Prywes and Roeder 1987) resins (described in the Materials and Methods section). An amount of 10 mg of nuclear extract was incubated with 1 ml of settled bed resin of either phosphocellulose or hydroxylapatite resins and the proteins were eluted with increasing salt concentrations. Similar to the HA resin fractionated GC nuclear proteins, the protein fractions from phosphocellulose or hydroxylapatite resins were tested for the PSF binding activity using 263P DNA fragment in nuclease protection assays (Figs. 3.3; 3.4). It was observed that the phosphocellulose resin has very low binding capacity as was evidenced by the presence of the PSF proteins even in the flowthrough fraction (Fig. 3.3; lane: c). In contrast, the PSF factors bound very strongly to the hydroxylapatite resin and could not be eluted by increasing salt concentrations as was revealed by nuclease protection assay (Fig. 3.4). Gel filtration chromatography was also employed to determine the approximate molecular size of the PSF factors by applying 100 mg of GC nuclear extract on to Sephacryl S-300 High Resolution grade resin (Pharmacia). However, glycerol (20%) associated clogging of the column was encountered. Hence, these chromatography techniques were not employed in subsequent purification steps. Only heparin agarose resin was used to fractionate the GC nuclear proteins, as heparin agarose had a very high binding capacity and gave a very clear fractionation.

Figs. 3.3 & 3.4. Nuclease sensitivity assay of 263P fragment with GC cell nuclear proteins fractionated on Phosphocellulose and Hydroxylapatite resins. a) and i) represent the DNase I pattern generated from the 263P fragment in the absence of GC cell nuclear protein. b) GC cell nuclear protein used as load on to the resins, c) flow-through from the resins, d) 0.2 M, e) 0.4 M, f) 0.6 M, g) 0.8 M and h) 1.0 M KCl eluted GC cell nuclear proteins from the resins. Two regions, PSF-A and PSF-B, protected by the nuclear proteins are indicated.

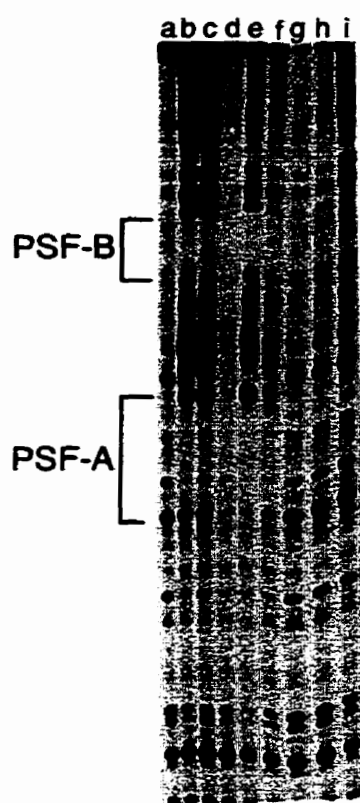


Fig. 3.3

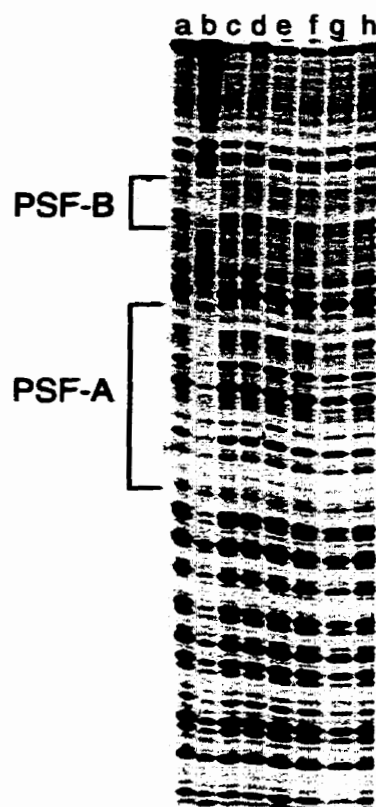


Fig. 3.4

3.2.3. DNA-affinity chromatography

In order to purify the PSF-A and PSF-B proteins separately, we prepared DNA-affinity columns using their corresponding binding sites (Table. 3.4), as were identified by Nachtigal et al. (1993).

Table. 3.4 Sequences of oligonucleotides (from -5' to -3') used in the preparation of DNA-affinity columns.

PSF-A(sense)	gatcTTTTCAATGTTGGTTGCCAACACCACTGCCAACCACCTTCTAG
PSF-A (antisense)	gatcCTAGAAGTGGTTGGCAGTGGTGTGGCAACCAACATTGAAAA
PSF-B(sense)	gatcAAGTGGGGAGATGGCAGGGCCCCAGCATTCACATCCTAGGCC
PSF-B(antisense)	gatcGGCCTAGGATGTGAATGCTGGGGCCCTGCCATCTCCCCACTT
D8 (sense)	gatcCGATCCAAGAATGTTGCAACACCTTTAGATCTG
D8 (antisense)	gatcCAGATCTAAAGGTGTTGCAACATTCTTGGATCG
PSF-Bmt(sense)	gatcAAGTGGCAGAAGTATTCGTAGCTGCACATCCTAGGCCACAGG
PSF-Bmt(antisense)	gatcCCTGTGGCCTAGGATGTGCAGCTACGAATACTTCTGCCACTT

The affinity columns were prepared using Sepharose CL-2B resin as was described by Kadonaga (1991) and detailed in the Materials and Methods section. The PSF-A and -B (both sense and antisense) oligodeoxynucleotides (ODNs) were synthesized with a 5'-GATC overhang, so that annealing of ODNs would lead to concatemerization. Subsequent ligation of these concatemers resulted in a ladder as visualized in agarose gel electrophoresis. The quality of an affinity column mainly depends on the ability of the majority of the population of the ODNs forming concatemers representing multiple binding sites. It was suggested that when 21 or 42 bp are used to prepare affinity columns, the ODNs tend to bend or kink and subsequently circularize on ligation (Kadonaga, 1991), thus affecting the quality of the column. Therefore, the length of each sense and antisense ODN of both PSF-A and PSF-B was selected to 42 bases and added a GATC overhang to

the 5'-end of each ODN. A similar strategy was also used to prepare affinity columns with the D8 (33 bases + GATC) and mutant PSF-B (42 bases + GATC) ODNs. The D8 binding site was previously identified in the distal sequences of the human PRL gene (Peers et al. 1990) and was characterized as a repressor element (Leite et al. 1996; Peers et al. 1990) and bears a high level of sequence similarity with the PSF-A element (Fig. 3.5, Nachtigal et al. 1993).

Fig. 3.5. Sequence similarity between PSF-A and D8 elements. Sequence of the sense strand is shown. The palindromic sequence is underlined.

PSF-A (sense)	TTTTCAATGTTGGTTGCCAACACCACTGCCAACCACTTCTAG
D8 (sense)	CGATCCAAGAATGTTGCAACACCTTTAGATCTG

TGTTGG ttg CCAACA (PSF-A)

TGTTG CAACA (D8)

Approximately 250 µg of both sense and antisense ODNs were used for preparing 5 ml (settled bed resin volume) of affinity resin. The extent of DNA bound to the column was monitored by the level of radioactivity associated with the resin.

Protection of the PSF-B site on the 263P fragment was consistently stronger, therefore, it was assumed that the factor recognizing the PSF-B had a higher affinity for the DNA element than with that of PSF-A. Thus, initial attempts were made to identify the factor recognizing the PSF-B site. DNA-affinity columns were prepared with PSF-B elements.

3.2.3.1. Purification on a PSF-B affinity column

Initially, a small scale purification of the 0.4 M and 0.6 M HA fractions of the GC

nuclear extract was performed on PSF-B affinity resin as detailed in the Materials and Methods section. Approximately, 3 mg/2 ml from each HA fractions was incubated with 36 μ g of poly [dIdC] for 10 minutes on ice and the nonspecific and insoluble complexes were pelleted. The supernatant was mixed with 200 μ l of PSF-B affinity resin and proteins were eluted with 200 μ l of 0.2 M, 0.3 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M KCl-buffer-Z. These fractions were dialyzed against buffer-Z on a 0.0025 μ M Millipore filters floating in a petridish with 100 ml of buffer-Z. From these dialyzed samples, 12.5 μ l of protein fraction was used for footprint analysis to identify the PSF-B and/or PSF-A binding activities in the fractions. Interestingly, both the PSF-B and PSF-A binding activities were observed in the same salt eluted fractions with both 0.4 and 0.6 M heparin agarose samples (Fig. 3.6). From the footprinting analysis of the PSF-B affinity purified GC nuclear extract, it was observed that the 0.4 M KCl eluted fraction of the 0.4 M HA material (Fig. 3.6, lane: e) and both the 0.3 and 0.4 M KCl eluted fractions of the 0.6 M HA material (Fig. 3.6, lanes: f and g) were positive for both PSF-A and PSF-B binding activities. More interestingly, the PSF-A region was better footprinted with 0.3 M eluted sample (Fig. 3.6, lane: f). These results indicate the possibility of co-purification of the PSF-A and PSF-B factors or their close association and/or also raise the possibility that the same factor may be recognizing both elements. Similarly, 12.5 μ l of above fractions were also analyzed on a SDS-PAGE and the proteins were silver stained. From these gels (Fig. 3.7) it was noticed that the proteins in the molecular mass range from 45 to 70 kDa were enriched by affinity purification. To further characterize the proteins interacting with PSF-A and PSF-B, the partially fractionated GC nuclear material was further purified on both PSF-A and PSF-B and as well as the PSF-A related D8 affinity columns. The HA fractionated, dialyzed, 28 mg/18 ml of 0.4 M KCl heparin-agarose fraction was preincubated with 365 μ g of poly [dIdC] for 10 minutes at 4° C and centrifuged at 12,000 x g to pellet down the non specific, insoluble complexes. The resulting supernatant from the GC nuclear extract was divided into three aliquots of 6.0 ml and was further purified by DNA-affinity chromatography on PSF-A, PSF-B and D8 specific affinity resins. The nuclear extract (~9 mg/6.0 ml) was mixed with 2 ml each of either PSF-A, PSF-B or D8 affinity resin and purified as was described in the Materials and Methods. The proteins were eluted with 2 ml each of 0.2 M,

0.3 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M KCl-buffer Z. The eluants were brought to 0.1 M KCl by dialysis against buffer Z, and 12.5 μ l of each fraction was used to test binding activity of PSF factors by footprint analysis as described earlier. The PSF-A and PSF-B binding activities were present in the same fractions eluted from both the PSF-A and PSF-B affinity columns. The majority of the PSF binding activity was observed in the 0.4 M fraction of PSF-A (lane: f) and the 0.3 M and 0.4 M KCl fractions of PSF-B (lanes: n and o) affinity columns (Fig. 3.8).

However, attempts to purify PSFs on the D8 affinity resin did not give encouraging results, as no PSF-A and PSF-B binding activity was observed in any of the fractions. This elution profile of PSFs from the D8 affinity column suggests that the factor binding to D8 and the PSFs may be different. Similarly, the PSFs bound very poorly to the PSF-Bmt affinity resin and the PSF binding activity was observed both in flow-through and 0.1 M-buffer Z wash.

Approximately, 12.5 μ l of the above purified fractions from either the PSF-A or the PSF-B resins were also analyzed on a SDS-10% PAGE. The silver stained protein gels revealed similar pattern as was shown in Fig. 3.7, in that enrichment of proteins in the range of 45 kDa and above was seen.

The PSF-B affinity column was used for further purification of the HA fractionated GC nuclear extract largely because of the more readily observed footprint with GC nuclear extract. The material 30 mg/20 ml fractionated with 0.6 M HA was concentrated to approximately 6 ml using a Centricon concentrator with molecular weight cutoff of 3 kDa by centrifuging for 1 h at 5,000 x g. This material was preincubated with 400 μ g of poly [dIdC] to remove insoluble complexes and was purified on a PSF-B affinity column, essentially as was done above. From this purification step, most of the PSF-A and PSF-B proteins were eluted in the 0.4 M KCl fraction.

Fig. 3.6. Small scale purification of heparin agarose fractionated (0.4 M and 0.6 M) GC cell nuclear extract on a PSF-B affinity resin. Heparin agarose (HA) fractionated PSF-A and PSF-B positive (0.4 M and 0.6 M) rat pituitary tumor GC cell nuclear protein fractions are purified separately on a PSF-B DNA affinity resin as described in Materials and Methods section. The eluted fractions were tested for the presence of PSF-A and PSF-B factors using nuclease protection assay on the radiolabelled 263P fragment. Lanes: a) represent the DNase I pattern generated from the 263P fragment in the absence of GC cell nuclear protein, b) GC cell nuclear protein (0.4 M of HA), c) flow-through of the 0.4 M HA fractions from the PSF-B affinity resin, d) 0.3 M, e) 0.4 M fractions from PSF-B affinity resin using 0.4 M HA material as load, f) 0.3 M, g) 0.4 M fractions from PSF-B affinity resin using 0.6 M HA material as load, h) and i) 0.6 M HA material tested for PSF binding activity after concentrating using Centricon (MW 3,000 cutoff) concentrator. Two regions, PSF-A and PSF-B, protected by the nuclear proteins are indicated. The extended PSF-A footprint in 0.3 M fraction (f) was indicated by a line with arrow heads.

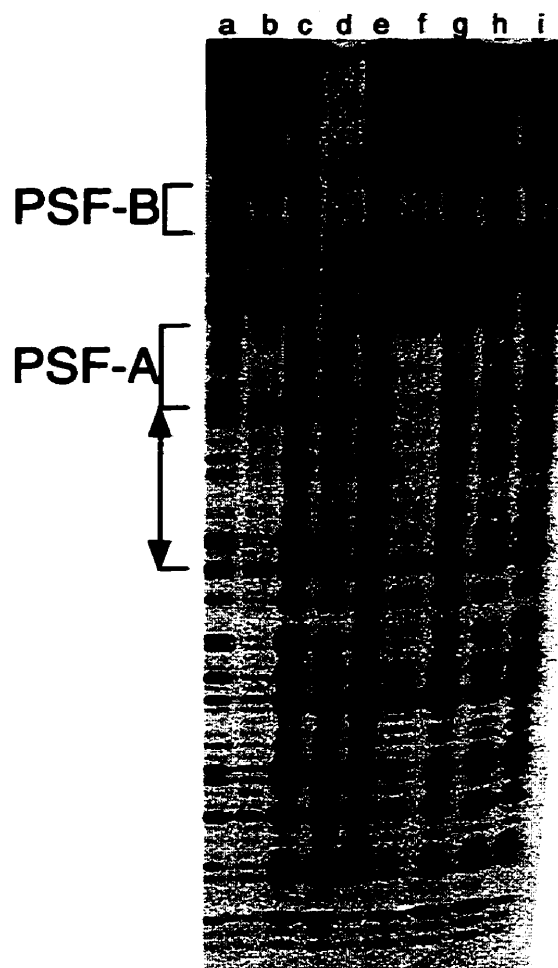


Fig. 3.7. Silver stained protein gel of the PSF-B affinity purified fractions reveals that proteins between 50 and 70 kDa recognize the PSF-A and PSF-B binding sites. Approximately 12.5 μ l of 0.3 M (a), 0.4 M (b) fractions from PSF-B affinity resin using 0.4 HA GC nuclear material and 0.3 M (c), 0.4 M (d) fractions from PSF-B affinity resin using 0.6 HA GC nuclear material were fractionated on a SDS-10% PAGE and silver stained. Protein-molecular weight marker sizes are indicated.

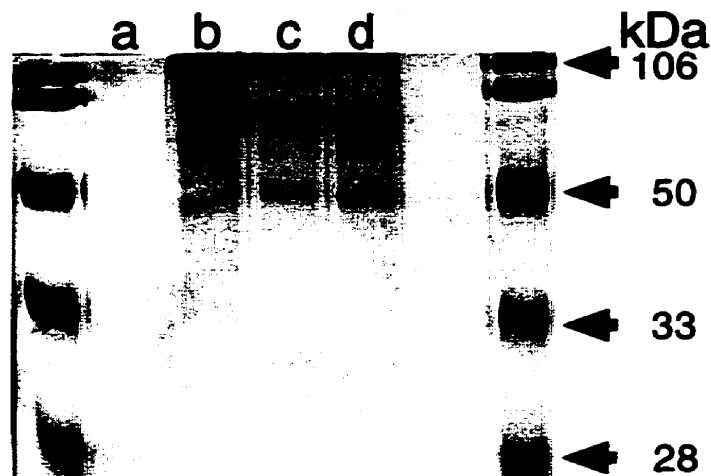
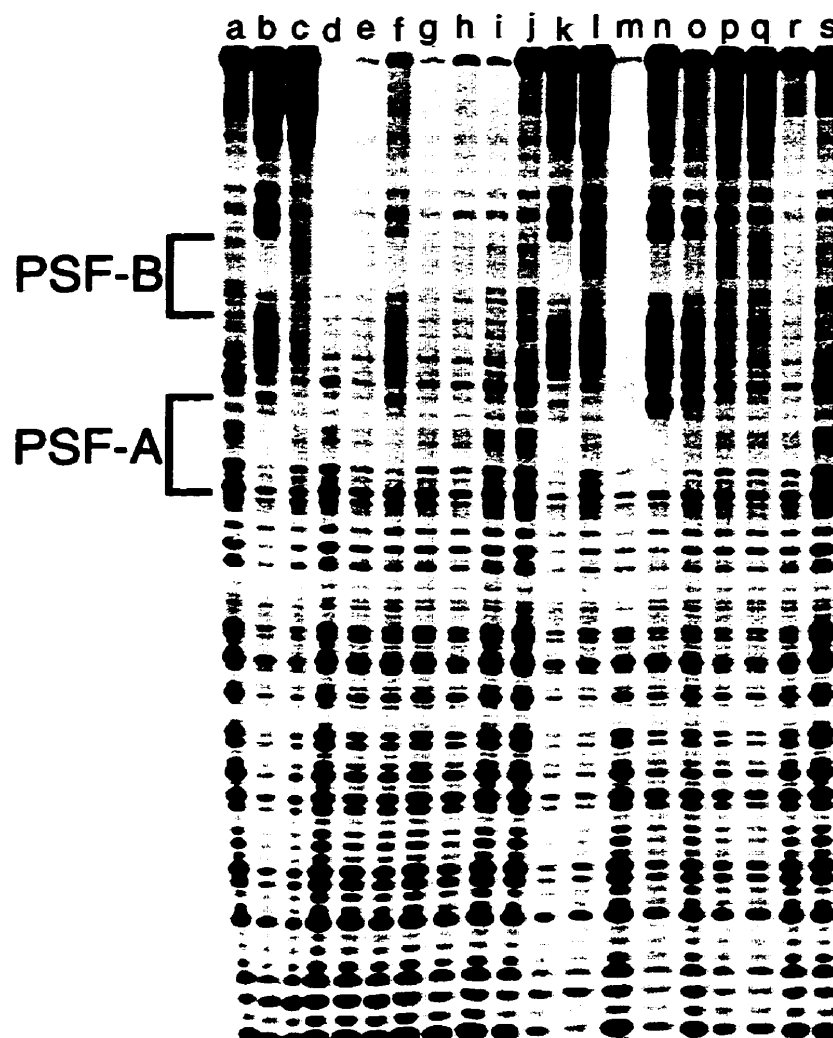


Fig. 3.8. Purification of heparin agarose fractionated (0.4 M) GC cell nuclear extract on PSF-B and PSF-A affinity resins. Heparin agarose (HA) fractionated PSF-A and PSF-B positive (0.4 M) rat pituitary tumor GC cell nuclear protein fraction is purified separately on PSF-B and PSF-A DNA affinity resins as described in Materials and Methods section. The eluted fractions were tested for the presence of PSF-A and PSF-B factors using nuclease protection assay on the radiolabelled 263P fragment. Lanes: a), j) and s) represent the DNase I pattern generated from the 263P fragment in the absence of GC cell nuclear protein, b) and k) GC cell nuclear protein (0.4 M of HA). Lanes c) to i) correspond to the fractions eluted from PSF-A affinity column and lanes l) to r) represent the fractions eluted from PSF-B affinity resin. c) and l) flow-through from the affinity resins, d) and m) 0.2 M, e) and n) 0.3 M, f) and o) 0.4 M, g) and p) 0.6 M, h) and q) 0.8 M and i) and r) 1.0 M salt eluted fractions from affinity resins. Two regions, PSF-A and PSF-B, protected by the nuclear proteins are indicated.



All the fractions (a total of 6 ml) from the above purification steps (from both PSF-A and PSF-B affinity purified) were pooled and subjected to a second round of affinity purification on the regenerated PSF-B resin. The nuclear protein (3 ml each time) was preincubated with 50 µg of poly [dIdC], bound to the regenerated PSF-B resin (1 ml bed resin volume) and eluted with 1 ml each of 0.2 M, 0.4 M and 0.6 M KCl-buffer Z. Subsequently after dialysis of the fractions against buffer Z, footprint and SDS-PAGE analysis of protein fractions followed by silver-staining were also performed. At this stage only the 0.4 M fraction was positive for PSF binding activity (Fig. 3.9, lane: c). The positive fractions, a total of 2 ml, from both 0.4 M elutions were pooled and subjected to another round of purification on the PSF-B affinity column. The nuclear material was preincubated with 50 µg of poly [dIdC] and further purifications steps were followed as mentioned earlier. This third round (1 ml fractions) of purification resulted in “very clean” footprints of the PSF-A and PSF-B sites (Fig. 3.9, lane: g). However, silver stained gels revealed negligible amounts of proteins. Finally, the 1 ml fraction was concentrated to approximately 200 µl using 200 µl of PSF-B affinity resin by eluting with 200 µl of 0.4 M KCl-buffer Z. Each round of purification resulted in the detection of four to five bands suggesting low amounts of proteins as was observed in the silver stained SDS-PAGE gels and gave very clear footprints of PSF-A and PSF-B (Fig. 3.9). By the fourth round of purification, we were **unable** to observe any binding activity except the **generation of a hypersensitive site that is characteristic of PSF-A region** (Fig. 3.9, lane: k).

However, the nuclear protein fraction that was used for footprinting showed at least four major protein bands in the range of 45-70 kDa (Fig. 3.10). Thus, it is possible that the PSF proteins might be present at very low amounts or degraded in this fraction. A further batch of GC cell nuclear extract was prepared to generate more material for the protein microsequencing.

Fig. 3.9. Detection of PSF binding activity in the GC cell nuclear extract after four rounds purification on the PSF-B affinity column. Partially purified GC cell nuclear extract was purified on PSF-B affinity resin as described in the Materials and Methods. The eluted fractions were tested for the presence of PSF-A and PSF-B factors using nuclease protection assay on the radiolabelled 263P fragment. Lanes: a), b), l), m) and n) represent the DNase I pattern generated from the 263P fragment in the absence of GC cell nuclear protein, c) correspond to PSF positive fraction from second round PSF-B affinity purified material used as load for third round purification. Lanes d) to g) and h) to k) represent the fractions eluted from PSF-B affinity column after third and fourth rounds of purification, respectively. Lanes d) and h) flow-through from the affinity resin, e) and i) 0.1 M wash, f) and j) 0.2 M and g) and k) 0.4 M salt eluted fractions from PSF-B affinity resin. Two regions, PSF-A and PSF-B, protected by the nuclear proteins are indicated. The hypersensitive site characteristic of PSF-A is indicated by an arrow head.

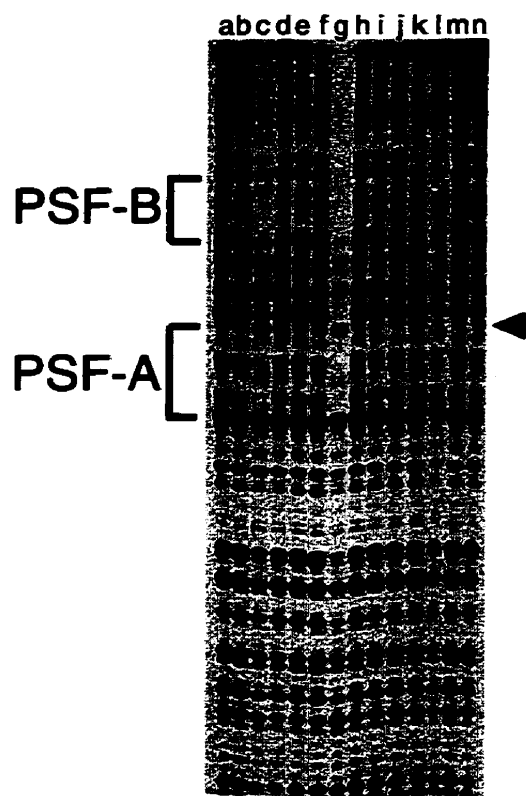
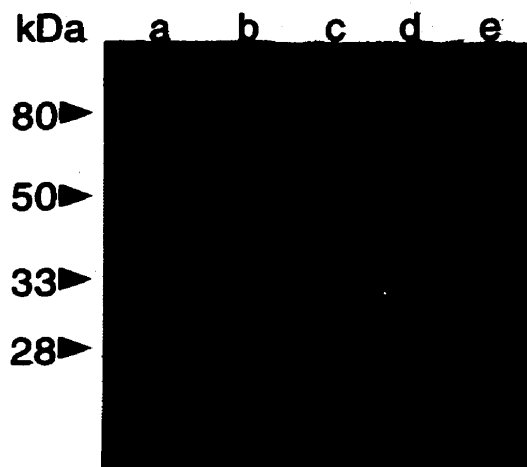


Fig. 3.10. PSF-A and PSF-B binding activity is associated with proteins in the molecular range of 50-70 kDa as revealed by the presence of four bands in 4 times purified GC cell nuclear extract on a PSF-B affinity column. Protein fractions from the fourth round purified GC nuclear extract from a PSF-B affinity resin were fractionated on a SDS-10%PAGE gel and silver stained. Lanes a) and b) are protein-molecular weight markers. Lane c) 0.2 M, d) 0.4 M, and e) 0.6 M KCl eluted GC nuclear protein fractions.

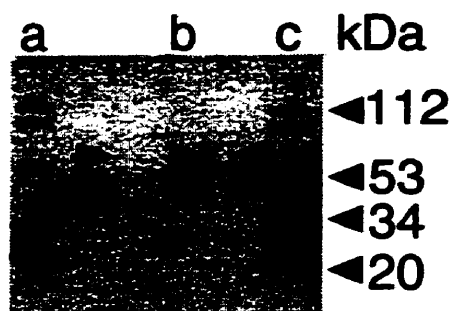


A second batch of GC nuclear extract was subjected to HA fractionation and PSF-B affinity purification. Instead of processing the 0.4 M and 0.6 M heparin agarose samples separately, we pooled and purified the fractions on the PSF-B affinity column. The nuclear protein was subjected to only three rounds of purification on PSF-B affinity column. This time the PSF binding activity was observed in both 0.4 and 0.6 M KCl-buffer Z fractions (Fig. 3.11; please see page # 102). By the third round of PSF-B affinity purification, the GC nuclear material was approximately 1 ml. Using this material in the DNase I protection assay revealed prominent PSF-A and PSF-B footprint regions on the 263P fragment indicating purification of the PSF factors (Fig. 3.11)

The affinity purified materials from the first (200 μ l and four times purified) and second batch (1 ml and three times purified) of purifications were pooled and concentrated to $\sim$ 70 μ l using a Centricon concentrator with 3 kDa molecular cutoff by centrifuging at 5,000 x g. This sample was processed for microsequencing. The sample was treated with 25 μ l of 4 X protein sample preparation buffer and loaded onto a SDS-10% PAGE gel. After electrophoresis, the proteins were transferred to a Bio-Rad protein sequencing grade,

PVDF membrane. Coomassie-blue staining and subsequent destaining of the membrane revealed two prominent bands with other three or four minor bands in the region of 50-70 kDa range (Fig. 3.12). Based on the intensity of staining, it was estimated the amount of protein in each band on the membrane could be in the range of 0.5 to 1 μ g. After destaining the membrane, the membrane was submitted for microsequencing. Though the microsequencing revealed some amino acid composition, we were informed that the amount of protein was not sufficient for sequencing, requiring 4-5 μ g protein per band.

Fig. 3.12. PSF-B DNA affinity purified GC cell nuclear protein fraction analyzed for protein microsequencing. GC nuclear extract purified on a PSF-B affinity column was fractionated on a SDS-7.5% PAGE, transferred to a sequencing grade PVDF membrane and stained with Coomassie-blue stain. Lanes a) and c) are prestained-protein molecular weight markers. Lane b) PSF-B purified GC cell nuclear protein. Two prominent protein bands are indicated by arrow heads.



It was found from thorough search of literature that the amounts of nuclear material that were used as the starting material to purify the PSFs was far less than what was used to purify many transcription factors (Table. 3.5). The year 1988 can be considered as the year of transcription factor as many ubiquitous DNA binding factors were purified in that year. Many investigators had started with as much as 500 mg of nuclear extract and after a series of chromatography procedures including DNA affinity columns, the yield of the protein of interest was only 1-5 μ g. Many proteins in the above cited examples (Table. 3.5) were purified from the cells that grow as suspensions and large amounts of nuclear material can be prepared from these cells. GC cells grow as monolayers.

Fig. 3.11. Detection of PSF binding activity in the GC cell nuclear extract after three rounds purification on the PSF-B affinity column. Partially purified GC cell nuclear extract was purified with the PSF-B affinity resin as described in the Materials and Methods. The eluted fractions were tested for the presence of PSF-A and PSF-B factors using nuclease protection assay on the radiolabelled 263P fragment. Lanes: a), l), and n) represent the DNase I pattern generated from the 263P fragment in the absence of GC cell nuclear protein. Lanes b) and g) correspond to PSF positive fraction from first and second rounds PSF-B affinity purified material used as load for second and third rounds purification, respectively. Lanes c) to f) and h) to k) represent the fractions eluted from PSF-B affinity column after second and third rounds of purification, respectively. Lanes c) and h) flow-through from the affinity resin, d) and i) 0.2 M, e) and j) 0.4 M and f) and k) 0.6 M salt eluted fractions from PSF-B affinity resin. Lane m) HeLa crude nuclear extract. Two regions, PSF-A and PSF-B, protected by the nuclear proteins are indicated.

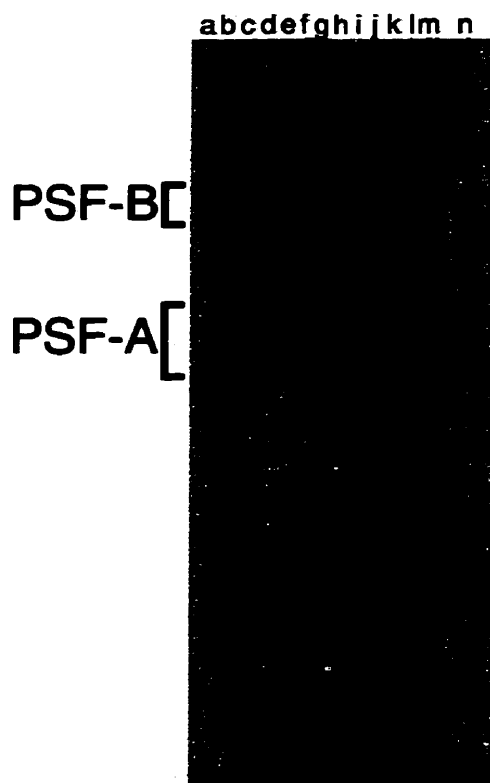


Table. 3.5. Amounts of near homogeneous DNA-binding proteins obtained from the nuclear protein extracts. Several DNA-binding proteins were purified from the nuclear protein extracts prepared from different cells/tissues. Amounts of starting material as nuclear extract (NE) in milligrams (mg) and the final product in micrograms (μ g) are indicated. For some DNA-binding proteins either cells or tissue in grams (g) were used.

Factor	NE in mg	Final product in μ g	References
Sp-1	600	10	Briggs et al.1986
NF-1	1010	100	Jones et al. 1987
AP-1	100 g of cells	20	Lee et al. 1987
AP-2	1000	30	Mitchel et al. 1987
AP-2	25 g of cells	2-5	Imagawa et al. 1987
NF-kB	900	2	Kawakami et al. 1988
NF-kB	3600	5	Lenardo et al. 1988
GHF-1	800	210 **	Castrillo et al. 1989
f-EBP	1830	21	Prywes and Roeder, 1987
μ EBP-E	60 g tumor	3	Peterson et al. 1988
UBF-1	60 g of cells	5	Bell et al. 1988
GRF-1	1400 WCE	20	Buchman et al. 1988
TEF-1	100 g of cells		Davidson et al. 1988
GAGA	790	70	Biggin and Tjian, 1988
Zeste	800	15	Biggin et al. 1988

** denotes a mixture of proteins

WCE-whole cell extract

3.3. DETERMINATION OF THE MOLECULAR WEIGHT(S) OF THE FACTORS RECOGNIZING THE PSF ELEMENTS

Several attempts with different approaches were made to determine the approximate molecular weight of the factors binding to the PSF-A and PSF-B elements. In spite of 3-4 rounds of purification on a PSF-B oligo affinity column, the PSF-A as well as the PSF-B was protected in a footprint analysis of the 263P fragment. Thus the data supported the possibility of a single factor or factors which copurify recognizing both the elements (Figs. 3.6, 3.8, 3.9, 3.11). The following methods were used to identify the PSFs molecular sizes.

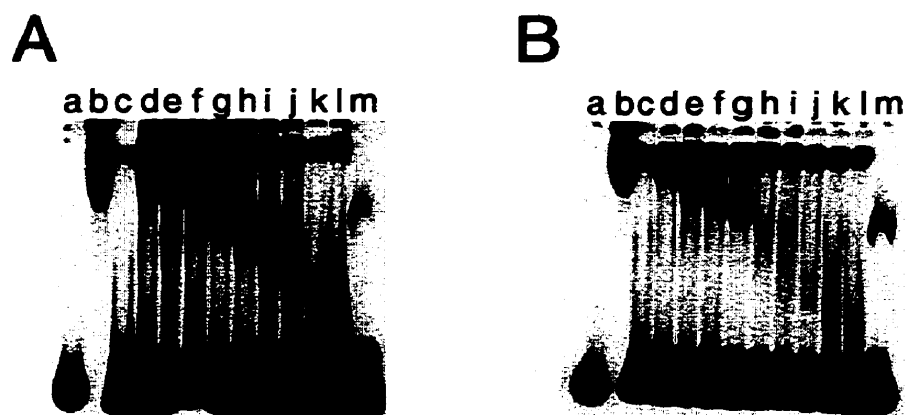
3.3.1. Attempts to identify the PSF proteins from SDS-PAGE fractionated, eluted and renatured protein samples

The partially purified (HA fractionated) GC cell nuclear material, 2 mg/2 ml, was first concentrated to 100 μ l using a Centricon concentrator with 3 kDa cut off by centrifuging at 5,000 x g. This material was separated on a SDS-10% PAGE gel. The gel lane with the nuclear material was cut into 8 equal size pieces and the proteins were eluted and renatured as described in the Materials and Methods section. The renatured proteins (15/75 μ l) were used to detect the PSFs binding activity by footprint analysis, without the addition of the non-specific competitor poly [dIdC]. No binding activity of the PSFs in any of the renatured proteins fractions was observed in footprint reactions (data not shown). However, the incomplete elution or renaturation of the proteins from the gel pieces could not be ruled out. Subsequently, it was found that the amount of sample used for the footprint analysis did not show the presence of proteins when separated on a SDS-PAGE minigel (data not shown).

In a repetition of above experiment using a minigel for SDS-PAGE gel electrophoresis. The gel lane with GC nuclear extract was cut into 11 small pieces and the elution and renaturation steps were repeated. Approximately 15 μ l of the extracted fraction

was used in gel shift binding reactions in the presence of 2 μ g of poly [dIdC]. Although shifted complexes were observed in mobility shift assays using both the PSF-A and PSF-B elements as probes (Figs. 3.13A, 3.13B), the PSF specific complexes could not be detected as all fractions resulted in complex formation with varying intensities. However, the 5th fraction which corresponds to a gel piece between 50-70 kDa resulted in a prominent complex both with PSF-A and PSF-B probes suggesting that PSFs may be in this size range. Moreover, prominent complexes were also observed with protein fractions eluted from gel pieces 3 and 4 corresponding to and above 106 kDa. The molecular size of the PSFs was also determined by UV crosslinking experiment.

Fig. 3. 13. Size-fractionated GC Cell nuclear proteins eluted from a SDS-PAGE gel form complexes with radiolabelled PSF-A (A) and PSF-B (B) elements in gel mobility shift assays. GC nuclear extract was fractionated on a SDS-7.5% PAGE and the gel was cut into 11 equal size pieces and proteins from each gel piece were eluted and renatured as described in the Materials and Methods. Gel mobility shift assays were performed with renatured protein fractions using radiolabelled PSF-A and PSF-B elements. Lane a) represent radiolabelled PSF-A or PSF-B probes without the addition of proteins. Lanes b) to l) are representative of gel pieces 1 to 11 respectively. Lane m) represent the GC nuclear protein complexes formed either PSF-A or PSF-B probes. Gel pieces 1, 2 and 3 contain proteins of above 106 kDa, piece 4 is representative of 106-80 kDa, pieces 5 and 6 correspond to sizes between 50 and 80 kDa and gel pieces 7 to 11 contain protein of below 50 kDa.



3.3.2. Crosslinking of PSF proteins to the PSF-B3 DNA element

The persistent problem encountered with the popular native polyacrylamide gel pieces containing the shifted complexes, was loading the gel piece into the wells of a SDS-PAGE gel. To circumvent this difficulty, the vertical 4% native polyacrylamide gel was replaced with a horizontal 0.5% low melting point (LMP) agarose gel and the cross-linked complexes were separated at 4 °C. Approximately 40 µl of GC cell nuclear extract (0.3 M fraction PSF-B affinity purified, positive for PSFs binding activity) was incubated with the PSF-B3 element (Fig. 3.14; which is shorter in length than the PSF-B and comprises the sequences of the footprint region as was mapped by Nachtigal et al. 1993) as a probe (1 x 10⁵ cpm, 0.1-1.0 ng) and the binding reactions were done as described in the mobility shift assay (Materials and Methods).

Fig. 3.14. Sequences of PSF-B and B-3. The nuclease protected regions in PSF-B sequence was shown as upper case letters. The sequence of PSF-B3 in PSF-B is underlined.

PSF-B 5'-aaagtgGGGAGATGGCAGGGCCCCAGCATTCACatcctaggccacagggg-3'
PSF-B3 5'-GATGGCAGGGCCCCAGCA-3'

Incubations were done for 15 minutes in a 96-well polystyrene plate with U-shaped wells. The plate was placed on ice and was exposed to ultra-violet rays by inverting UV transilluminator (302 nm, Spectorline transilluminator, Model TR-302) for 30 minutes. The distance between the top of the well to the transilluminator was about 5 cm. The cross-linked complexes were loaded onto the 0.5% LMP agarose gel and separated at 4 °C. The wet gel was exposed to an X-ray film overnight. The autoradiogram (Fig. 3.15) was used as a template to mark the complexes and free probe locations on the gel. The PSF-B3 probe revealed only one complex (Fig. 3.15). The pieces carrying these complexes and the free

probe were put into separate tubes and protein sample preparation buffer was added before heating to 65 °C for 3 minutes and loading onto a SDS-10% PAGE gel. Subsequent exposure of the dried gel and autoradiography revealed a complex size larger than 142 kDa (Fig. 3.16). Hence, results from above two experiments suggest that PSFs form large size protein complex (Figs. 3.13, 3.16). It is not known whether this very large protein complex represents PSF multimer or an association between PSFs and other factors including, possibly GHF-1/Pit-1 (Nachtigal et al. 1993). To assess these possibilities, it is essential to isolate and purify the PSFs.

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Figs. 3.15 and 3.16. Determination of molecular size of the protein recognizing the PSF-B3 element using UV-crosslinking and subsequent size fractionation of the crosslinked complex.

Fig. 3. 15. PSF-B affinity purified GC nuclear extract positive for PSF binding activity was UV crosslinked with a radiolabelled PSF-B3 element and the complexes were separated in a 0.5% low melting agarose gel. The wet gel was exposed to X-ray film. Using autoradiogram as template, the gel pieces containing the DNA-protein complex (lane, a; boxed) as well as probe (lane, b; boxed) were isolated, treated with protein sample preparation buffer and heated for 5 min at 95 °C.

Fig. 3.16. The UV crosslinked PSF-B3 DNA and GC protein complexes were fractionated on a SDS-7.5% PAGE, gel was dried and exposed to X-ray film. Lanes a) represent the PSF-B3/GC nuclear protein complex and lane b) is radiolabelled PSF-B3 DNA without protein

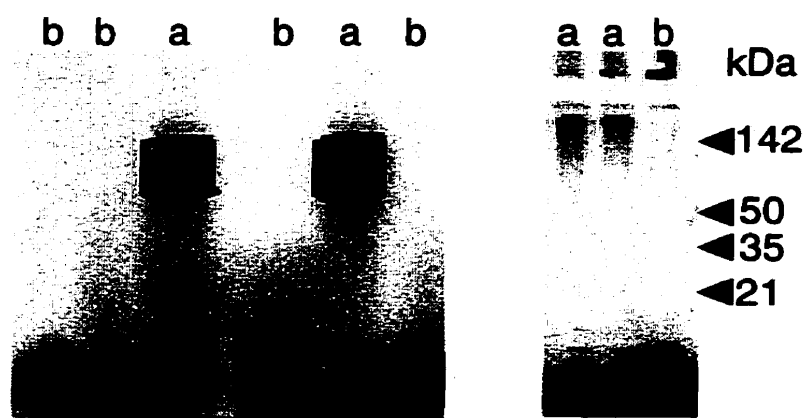


Fig. 3.15

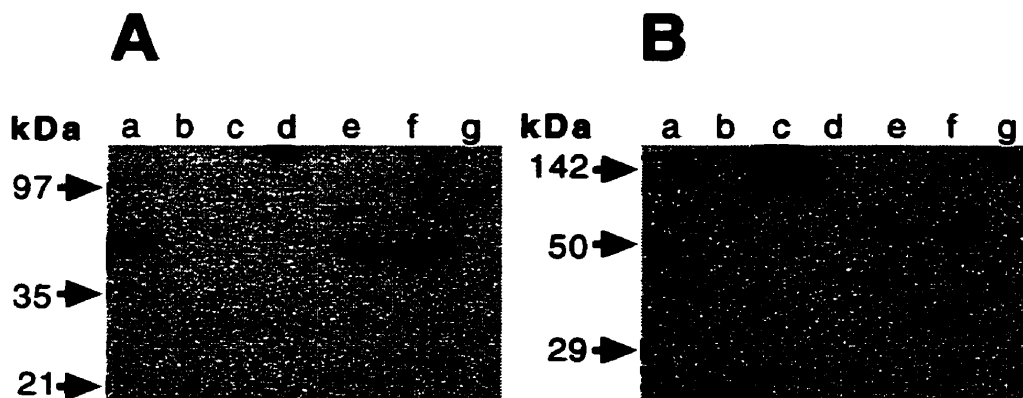
Fig. 3.16

3.3.3. Southwestern blotting

As described in earlier sections, it was not possible to correctly estimate the size of the PSFs using the SDS-PAGE elution and renaturation and UV-cross linking assays. Since the prospects for successful isolation of a clone for a given sequence specific DNA binding protein is greatly enhanced if the native protein can be detected by southwestern blotting using a DNA probe (Singh, 1993), a series of southwestern blotting assays were attempted. Many trials gave non-specific recognition of several proteins by the PSF probes (data not shown, Nachtigal, 1993). However, when the nitrocellulose membrane carrying the heparin-agarose fractionated GC nuclear material (50 µg of each fraction) was subjected to a series of denaturation and renaturation steps in the presence of guanidium hydrochloride (as detailed in Materials and Methods section) and then probed with a radiolabelled 90 bp fragment containing both PSF-A and PSF-B elements (or a similar probe but carrying a PSF-A mutation), a protein of ~54 kDa was detected (Fig. 3.17A, 3.17B). This signal was observed only in GC NE (load onto HA), 0.4 M and 0.6 M HA fractions which were positive for the PSF binding activity as determined by the footprint analysis. This result is particularly interesting as in a previous experiment a prominent complex was formed with proteins eluted from a gel piece corresponding to sizes 50-70 kDa (Fig. 3.13). A protein of 55 kDa was observed previously using the 263P fragment as a probe (Nachtigal, 1993). The molecular sizes of other proteins recognized by the 263P probe were 29, 27, 19 and 17 kDa and these proteins could be seen with both the GC and HeLa crude nuclear extracts (Nachtigal, 1993). Several weaker signals (about 66, 40, 35 kDa) were also observed with GC nuclear extracts (Nachtigal, 1993). The detection of several proteins interacting with 263P could be of non-specific nature because of the large piece of the DNA used as a probe. Interestingly, in footprint assays these non-specific interactions were not observed even in the presence of very low amounts of poly [dIdC] (1 µg/ml). It is known that different DNA binding proteins can recognize the same element (an example of such a DNA element is CCAAT which is recognized by c/EBP, NF-1/CTF, Y box factors, CDP, NF-Y or HSP-CBF (Mantovani, 1998) and several protein bands observed might represent some of these proteins. In the present study, HA fractionated

nuclear material was used as opposed to the crude nuclear extract as used by Nachtigal (1993). This fractionation may eliminate most of the non-specific interactions. Secondly, the probe was a only a fragment (90 bp) of 263P which would be expected to reduce the number of non-specific recognitions. Finally, the proteins bound to nitrocellulose were subjected to denaturation and renaturation steps and it is possible that during this process the protein of interest was brought to a near native conformation and this allowed the identification of the protein by southwestern blotting. Regardless, given the detection of a 54 kDa protein (Fig. 3.17) a cDNA library was screened to isolate a PSF clone.

Fig. 3.17. Determination of Molecular size of the factors recognizing PSF-A/-B elements. South western blotting was performed using a radiolabelled 90 bp DNA fragment containing PSF-A and PSF-B (A) or containing PSF-A mutant and PSF-B (B) binding sites. GC cell nuclear extract was fractionated on heparin agarose (HA) resin, 50 µg of each salt eluted fraction was subjected to size separation in a SDS-7.5% PAGE and transferred to a nitrocellulose membrane. Southwestern blotting was done using denaturation and renaturation procedure as described in the Materials Methods section. Lane: a) GC cell nuclear protein used as load to fractionate on HA, b) flow-through, c) 0.1 M wash, d) 0.2 M, e) 0.4 M, f) 0.6 M and g) 1.0 M salt eluted HA fractions. The location of the prestained-protein-molecular weights were indicated.



3.4. SCREENING OF THE cDNA EXPRESSION LIBRARIES

During the process of purification many attempts were made to identify the PSF protein by screening cDNA expression libraries. As the PSF proteins were widely expressed in different cells and tissues of various species origin (Nachtigal et al. 1993), we chose at least two different cDNA libraries generated from rat pituitary (in Lambda ZAP II vector, Stratagene; the GC tumor cell line was treated with estrogen to induce *in vivo* tumor formation, then adapted to tissue culture) and the mouse fibroblast 3T3 cell line. The screening of libraries was done according to the method detailed by Singh et al (1988). A minimum of half-a-million plaques were screened.

Two types of probes comprising PSF-B binding elements were used to screen libraries. The PSF-B binding element was subcloned as a 3 mer (a concatemer with 3 tandem repeats of the PSF-B recognition site) in the pUC 19 vector, and used as a probe. For probe preparation, this fragment (~50-100 ng) was isolated as *Eco*RI and *Hind*III fragment and the ends were Klenow filled using [α - 32 P]dATP. The second probe was the concatemerized and then ligated PSF-B oligonucleotide with multiple repeats of PSF-B binding sites used for the preparation of the affinity columns. The mixture of these concatemerized ODNs were radiolabelled by a double-strand DNA nick translation method (Vinson et al. 1988).

Though the southwestern blotting revealed the PSFs may be ~54 kDa proteins, the denaturation and renaturation method with guanidium hydrochloride was not used to screen the library. Another reason for not identifying a cDNA clone could also be due to lack of proper conformation or post-translational modifications of the DNA-binding protein. It is still not known whether the 54 kDa protein band detected in the southwestern blotting is PSF. However, during the process of PSFs purification using affinity columns, enrichment of proteins in the range of 45-70 kDa was always observed. It is possible that one of these bands correspond to the 54 kDa protein. The presence of other proteins in the affinity purified samples suggests that these proteins may also be involved in PSF DNA

binding and the PSFs may be a heteromer of these proteins. This is also supported by the appearance of the large DNA-protein complex (> 142 kDa) in the cross linking studies (Fig. 3.16).

3.5. ATTEMPTS TO GENERATE MODEL SYSTEMS TO STUDY THE FUNCTIONALITY OF THE INTERACTION BETWEEN GHF-1/PIT-1 AND THE PSF PROTEINS

As stated earlier, Nachtigal et al. (1993) suggested that PSFs and GHF-1/Pit-1 proteins participate in a common interaction. In the competition mobility shift assay, the GHF-1/Pit-1 element competed the pituitary nuclear complexes formed on the PSF-A or PSF-B elements, whereas in a competition DNase I protection assay on the 263P fragment, the PSF-A and PSF-B footprint regions were competed out by a GHF-1/Pit-1 element in a dose dependent manner (Nachtigal et al. 1993). Furthermore, the antibodies raised against GHF-1/Pit-1 protein recognized the pituitary (GC cell) nuclear complexes formed on the PSF elements in a "far-supershift" assay (unpublished data). These observations led to the suggestion that PSFs and GHF-1/Pit-1 interact, and this leads to inactivation of GHF-1/Pit-1 and the pituitary-specific restriction of CS/GH-V gene expression. This observation was the basis for the repression observed in GC cells with the CS-A promoter when the PSF sequences (263P) were placed upstream of the CS-A promoter in the hybrid CAT gene construct, CS-Ap.CAT.Enh (Nachtigal et al. 1993). Hence, it was hypothesized, in a novel way, that introduction of an excess number of PSF-A and PSF-B elements into GC cells could "squelch" GHF-1/Pit-1 activation of a promoter and, thus, be monitored by repression of GHF-1/Pit-1 dependent target gene activation.

3.5.1. Objective

It was proposed that in a pituitary somatotroph, the interaction between the PSFs and GHF-1/Pit-1 forms an inactive complex, thereby restricting the pituitary expression of CS/GH-V genes (Nachtigal et al. 1993). As stated earlier, as no human pituitary cell lines

are available, rat pituitary GC cells were used as a pituitary model system to study transcriptional regulation of hGH/CS genes. In this context, the question asked was if the PSF and GHF-1/Pit-1 form an inactive complex, **can GHF-1/Pit-1 mediated CS-A promoter activity be repressed in the presence of excess amounts of PSF elements in GC cells?**

3.5.2. Experimental design

Hybrid CAT genes (-163/+1) or (-133/+1) CS-Ap.CAT as well as the GC cell line with stably integrated CS-Ap.CAT (-133/+1 of CS-A promoter) were described by Nachtigal et al (1989). The stable cells were selected with G-418 (100 µg/ml) upon seeding and were maintained in growth medium. The double stranded oligodeoxynucleotides (ODNs) representing the GHF-1/Pit-1, PSF-A, PSF-B and TEF-1 binding elements or the pUC 19 constructs carrying these elements as inserts were transiently transfected into the GC cells by calcium phosphate method. Approximately 0.3×10^6 cells were plated onto a 60 mm culture dishes and, after 16 hours, 30 µg (10 µg/ml of medium) of the double-strand oligonucleotides or 15 µg of each pUC 19 construct were transfected into these cells. The cells were harvested 48 hours after the nucleic acids transfer and CAT assay was performed.

3.5.3. Results and Discussion

3.5.3.1. CS promoter in its chromatin configuration is highly and pituitary-specifically active

Transient transfections with (-163/+1) or (-133/+1) CS-Ap.CAT constructs revealed that these proximal promoter sequences of the CS-A gene poorly activate transcription in GC cells *in vitro* (not shown, n=3). These results were similar to those observed with rat GH-N as well as hGH-N gene proximal promoter sequences (Cattini et al. 1986; Nelson et al. 1986).

Initial studies to unravel the mechanism of the pituitary-specific expression of the growth hormone and prolactin genes led to the identification of the pituitary-specific *cis*-elements in the proximal promoter sequences of both rat and human genes (Cattini et al. 1986; Nelson et al. 1986). Both the rat and human GH-N genes appear to depend on their proximal -235 bp of 5'-flanking DNA for pituitary-specific activity, while a deletion to -181 bp almost abolished this activity (Cattini et al. 1986; Nelson et al. 1986). Further analysis revealed that both the rat and human GH-N genes possessed two GHF-1/Pit-1 binding sites (proximal and distal) within the region -140/+1 of the promoter sequences, and that GHF-1/Pit-1 was responsible for the pituitary-specific expression of these genes (Lefevre et al. 1987; Nelson et al. 1988). However, although these GHF-1/Pit-1 sites are necessary, they are not sufficient to support pituitary-specific activity of the rat or human GH-N promoters (Cattini et al. 1986; Nelson et al. 1986).

Comparison of the proximal promoter sequences from -140/+1 bp of GH-N, GH-V and CS-A genes reveals that the GH-V gene includes only the distal GHF-1/Pit-1 site and the CS-A gene contains only the proximal GHF-1/Pit-1 binding site (Fig. 3.18). This physical binding of GHF-1/Pit-1 protein to the two binding sites in GH-N, to the distal site in GH-V and to the proximal site of the CS-A genes was demonstrated by footprint analysis with GC nuclear extracts (Lemaigre et al. 1989; Nickel et al. 1991). Furthermore, the functionality of the CS-A promoter as well as the necessity of the GHF-1/Pit-1 binding elements was established by stably integrating different deletion constructs of CS-Ap.CAT into the chromatin of the rat pituitary GC cells (Nachtigal et al. 1989). Transient transfections performed with -163/+1 or -133/+1 CS-Ap.CAT constructs did not result in efficient CAT gene expression. This raises the possibility that upstream sequences are important. It was suggested that, unlike the GH-N gene sequences (Cattini et al. 1986), the fragment from -486/-134 bp of the CS-A gene may contain putative repressor elements as the -133/+1 CS-Ap.CAT was expressed more efficiently after stable integration (Nachtigal et al. 1989), suggesting a positive influence through the resulting chromatin configuration.

brought about by the chromatin structure (Kornberg and Lorch, 1995). These results indicate that the CS-Ap constructs could overcome the effect of the site of integration and suggests that at least one GHF-1/Pit-1 site could be sufficient for pituitary-specific activity of the GH/CS promoters. Similarly, the rat GH proximal promoter sequences containing only the two GHF-1/Pit-1 sites (-145/+1) supported pituitary-specific reporter gene expression in transgenic mice (Lira et al. 1988; 1993). However, further enhanced expression of the reporter gene was observed by addition of upstream sequences (up to -1.7 kb) to the transgene (Lira et al. 1988; 1993). Hence, these results suggest GHF-1/Pit-1 alone and in conjunction with basal transcription factors can support the pituitary-specific expression of GH gene *in vivo*.

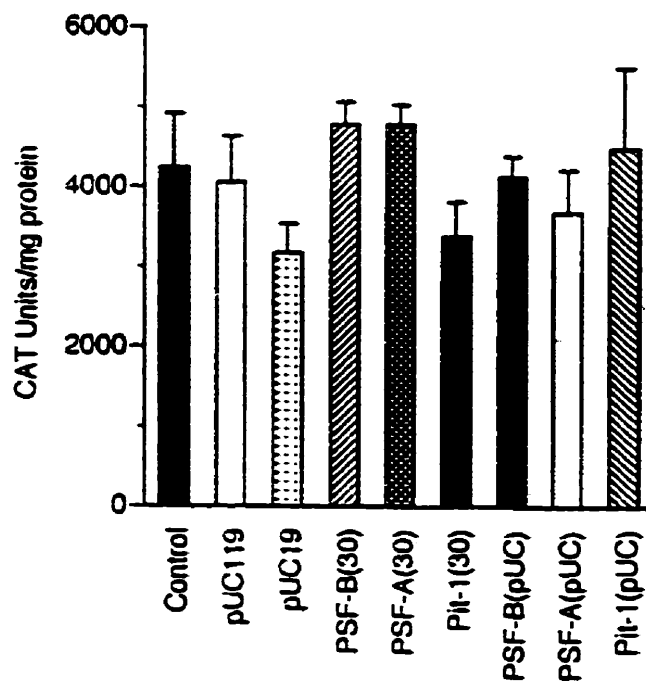
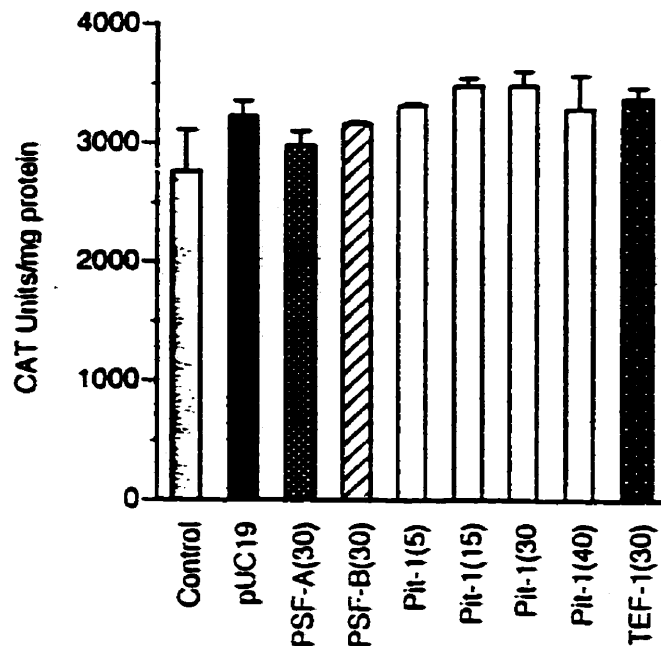
3.5.3.2. Provision of PSF binding elements did not influence the activity of the stably integrated CS promoter

In a novel approach and analogous to the antisense technology and competition assays performed to titrate the transcription factors (Colantuoni et al. 1987; Guerin et al. 1992; Scholer and Gruss, 1984; Seguin et al. 1984), attempts were made to titrate the GHF-1/Pit-1 factor. In this experiment, the GC cells with stably integrated -133/+1 CS-Ap.CAT were used as model system to unravel the functionality of the interaction between GHF-1/Pit-1 and PSFs. As stated, the objective was to test if the interaction between GHF-1/Pit-1 and PSFs can be used to squelch GHF-1/Pit-1 and whether this can be monitored by the level of expression of a GHF-1/Pit-1 dependent CAT gene.

Approximately 48 hours after introduction of either the GHF-1/Pit-1, PSF-A, and PSF-B binding sites or GHF-1/Pit-1, PSF-A, PSF-B and TEF-1 elements in a pUC19 vector, the GC cells were harvested and the cell extract was used to measure the CAT activity. Interestingly, the endogenous transcriptional activity of the -133/+1 CS-Ap.CAT was unaltered in the presence of either the PSF-A and PSF-B binding sites (ODNs) or their corresponding pUC 19 constructs (Figs. 3.19, 3.20). Furthermore, no influence on the activity of -133/+1 CS-Ap was observed even in the presence of the GHF-1/Pit-1 binding

elements which were thought to titrate the GHF-1/Pit-1 and thereby influence the endogenous GHF-1/Pit-1 dependent promoter activity (Figs. 3.19, 3.20).

Figs. 3.19 and 3.20. A stably integrated CS-A promoter (-133/+1) activity in GC was not influenced by the provision of excess amounts of PSF-A, PSF-B and Pit-1 DNA elements. Approximately 0.3×10^6 GC cells, with stably integrated (-133 -1)CS-Ap.CAT, were plated onto a 60 mm culture dish and were transfected with either double stranded oligodeoxynucleotides (ODNs) corresponding to PSF-A, PSF-B, GHF-1-Pit-1, TEF-1 or pUC19 constructs bearing these elements by the calcium phosphate method. For each ODN or plasmid construct, at least three culture dishes were used. Amounts of ODNs/culture dish used were indicated in parenthesis and 15 μ g/culture dish of each of the pUC19 construct or pUC19 alone were used in transfections. Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. CAT units are expressed per milligram of protein. Error bars indicate standard error of the mean.



Similar kinds of all or none phenomena were also observed in several antisense experiments while trying to influence expression of a particular gene (Wagner, 1994). The doses of binding elements (10 µg/ml) used in this study were comparable with the doses that were used in antisense studies (Goodchild et al. 1988; Matsukura et al. 1989), therefore, it was assumed that 'no influence' of the ODNs was not due to the use of too low doses, but could be due to degradation or not being translocated to nucleus in this study. To overcome the degradation and nuclear translocation problems, the plasmid constructs containing the binding elements were used for further studies. However, this was also unsuccessful and did not reveal any influence on the activity of the CS-A promoter (Fig. 3.20). At present, it is not clear why the pUC19 constructs with the binding elements did not show any effect.

It was shown by others that SV40 enhancer binding factors, which are present in limiting concentrations, were competed out by transfecting the enhancer elements (as plasmid constructs) into cells by effectively working as 'competitors' and thereby influencing SV40 enhancer activity (Scholer and Gruss, 1984). Similarly, other investigators were able to compete out regulatory factors using their corresponding elements as a plasmid construct, and thereby influencing target genes such as, metallothionein gene (Seguin et al. 1984), retinol-binding protein gene (Colantuoni et al. 1987) and rat growth hormone gene (Guerin et al. 1992). This squelching effect appeared to be specific since provision of either SV40 promoter or TATA elements did not affect the enhancer activity (Scholer and Gruss, 1984) indicating that the factors recognizing these elements are not present in limiting amounts. This might explain why GHF-1/Pit-1 protein was not competed out with GHF-1/Pit-1 elements and no influence on the CS promoter activity could be observed. Indeed, it was suggested that in rat pituitary tumor cell lines, GHF-1/Pit-1 might not be the limiting factor (Fox et al. 1990; Shepard et al. 1994) and furthermore, that GHF-1/Pit-1 elements should be considered as promoter but not as enhancer elements (Sittler and Reudelhuber, 1990). In the above studies, the dose of plasmid constructs containing the regulatory elements used as competitors was 10-50 µg/100 mm culture dish, and was comparable to the doses used in the present study (5

µg/ml of culture medium or 15 µg/60 mm culture dish).

The reason these studies did not establish a functional interaction between GHF-1/Pit-1 and PSFs could be due to the high levels of GHF-1/Pit-1 in these tumor cell lines (Fox et al. 1990; Shepard et al. 1994) as well as enhanced transcriptional mechanisms operating on a gene at the chromatin level (Kornberg and Lorch, 1995). In the above examples cited (Colantuoni et al. 1987; Guerin et al 1992; Scholer and Gruss, 1984; Seguin et al. 1984), the competition assays were performed using transiently introduced, naked reporter constructs. As stated earlier, regulation of a gene may be different when considered at the level of chromatin. Although initial transient transfection experiments were performed with (-163/+1) or (-133/+1) CS-Ap.CAT constructs in a similar way as was done in the cited examples, these constructs were not active in GC cells (data not shown), hence the stables were used as a model system. However, it was reported recently that genes integrated into cellular chromatin are resistant to the activator mediated-squelching effect (Natesan et al. 1997) and failure to see any modulation of CS-Ap activity in stable cells could also be attributed to the resistance of the CS-Ap.CAT gene.

Regardless, different model systems were developed in an attempt to study the primary objective of determining the functionality of any GHF-1/Pit-1 and PSFs interaction.

3.6. DETECTION OF THE FACTORS RECOGNIZING PSF-A/-B ELEMENTS IN HUMAN PLACENTAL (JEG-3) CELLS

3.6.1. Introduction

In spite of their described function in pituitary cells, PSF-A and PSF-B factors are expressed in pituitary as well as various cells and tissues of different species origin (Nachtigal et al. 1993). The protection of PSF-A and PSF-B regions were identical with nuclear extracts prepared from human cervical (HeLa), breast (MCF-7, MDA-MB231),

prostate (Du145, PC-3) cell lines, rat lateral prostate tissue and green monkey kidney (COS-1) cells (Nachtigal et al. 1993). However, it was noted that these ubiquitously expressed and evolutionarily conserved PSF-DNA binding factors were not detectable or were absent in nuclear extracts from human placenta or a cell line (JEG-3) derived from human placenta (Nachtigal et al. 1993). Even in the presence of three times the amount of the placenta cell nuclear protein material to that of rat pituitary GC cell or human pituitary tissue nuclear extract, the placental nuclear proteins could not protect the PSF-A and PSF-B binding sites in a DNase I protection assay (Nachtigal et al. 1993). It was suggested that the placenta cells may express the PSF factors at very low levels or that these factors might be present, but in a modified form or that the accessory co-factor necessary for the PSF factors to bind to their recognition elements may be deficient in placenta (Nachtigal et al. 1993). It was suggested that with the possible exception of the placenta, PSFs may belong to a family of ubiquitous factors, conserved among various species, but all sharing a common DNA binding domain (Nachtigal et al. 1993). In this context, it is interesting to note that there are a number of transcription factors such as the TEFs, that all contain the TEA-DNA binding domain, which would represent one such family of transcription factors, which recognize similar sequences and show some tissue-specificity in distribution (Jacquemin et al. 1996a; 1997; 1998).

3.6.2. Results and Discussion

3.6.2.1. PSFs are expressed in the placental JEG-3 cells

During the course of purification and characterization of the nuclear protein extracts prepared from various cells, an interesting observation was made. The nuclear extracts prepared from the human placenta (JEG-3) cells protected the PSF-A and PSF-B regions in a similar manner to that of nuclear material from GC and HeLa cells (Fig. 3.21; please refer to Fig. 3.30). These results were further confirmed by gel-mobility shift assays performed with placental (JEG-3 and JAR) and non-placental (GC and HeLa) nuclear extracts (please refer to Fig. 3.29). Thus, this result extends and reconfirms the earlier observation that

PSFs are ubiquitous in nature (Nachtigal et al. 1993).

Fig. 3.21. Detection of PSF-A and PSF-B binding activity in human choriocarcinoma JEG-3 cells. DNase I protection assay of the radiolabelled 263P fragment. Lanes: a) GC cell nuclear protein (10 μ g), b) JEG-3 cell nuclear protein (10 μ g) and c) represent the DNase I pattern generated from the 263P fragment in the absence of nuclear protein. Two regions, PSF-A and PSF-B, protected by the nuclear proteins are indicated.



The presumption of an ubiquitous nature for PSFs could be due to the recognition of PSF-A and PSF-B binding sites by the same factor or multiple distinct factors present in cells of different tissue and species origin. This is one of the major themes in the study of eukaryotic transcriptional control mechanisms. There could be a positive or negative effect on the transcription of a particular gene depending on the factor recognizing the the same

motif (Diamond et al. 1990). Examples of such motifs being recognized by different factors include CCAAT element which can be recognized by either c/EBP (CCAAT/enhancer binding protein, Landshultz et al. 1988; Umek et al. 1991), CTF/NF-1 (CCAAT transcription factor, Jones et al. 1985), Y box factors (Didier et al. 1988), CDP (CCAAT displacement protein, Barberis et al. 1987; Superti-Furga et al. 1988), HSP-CBF (Lum et al. 1990), or NF-Y (also known as CBF, alpha-CP1, and CP-1, Dorn et al. 1987). Similarly, the ubiquitous NF-A1, now known as Oct-1 (Singh et al. 1986) and the lymphoid-specific NF-A2, also known as Oct-2 (Staudt et al. 1986) bind the octamer motif, ATGCAAAT. It was also shown that the symmetrical sequence TGGGGATTCCCCA in an MHC class I gene enhancer (Baldwin et al. 1990) is recognized by distinct transcription factors including H2TF1 (Baldwin and Sharp. 1987), NF-kB (Baldwin and Sharp. 1988), KBF1 (Israel et al. 1987) and EBP-1 (Clark et al. 1988). GATA elements (Ko and Engel, 1993), ets motifs (Sharrocks et al. 1997) and multiple sites in the SV40 enhancer region (Davidson et al. 1986) are recognized by different tissue-specific factors. Similarly a 25 bp fragment located upstream of the proliferin gene can be recognized selectively by both the glucocorticoid receptor and by AP-1 depending on the physiological context. Moreover, these factors exert opposite effects on proliferin gene expression (Diamond et al. 1990). It is also known that the nature of a transcription factor, for example phosphorylation, can bring a positive or a negative effect on the regulation of gene expression (Lamph et al. 1990). Thus, PSFs could be the same or different proteins present in GC, JEG-3 and HeLa cells recognizing the same DNA sequence.

Interestingly, based on their sequence similarity with PSF-A or the region adjacent to PSF-B, two additional regulatory elements RF-1 and DF-1 were identified in our laboratory (Lytras and Cattini, 1994) within the 1-80 bp of the 240 bp sequence of the placental lactogen transcriptional enhancer (Walker et al. 1990). These PSF-related sequences are present in a reverse orientation within this 1-80 bp region (Fig. 3.22; Lytras and Cattini, 1994).

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Fig. 3.22. Sequence comparison of PSF-A and PSF-B with sequences (RF-1 and DF-1) in the 1-80 bp fragment. Sense strand of PSF-A and PSF-B elements, whereas antisense strand of RF-1 and DF-1 elements are compared for sequence similarity.

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PSF-B      5'-AAGTGGGGAGATGGCAGGGCCCCAGCATTCACATCCTAGGCC-3'
DF-1                               5'-AGAAACATCCTAGGCATCTC-3'

PSF-A      5'-TTCAATGTTGGTTGCCAACACCACTGCCAACCACTTCT-3'
RF-1                               5'-GCCGTCC:ACACCAAGTTGATGAG-3'

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It was also suggested that these elements are recognized by factors, RF-1, a repressor and DF-1, a derepressor (Lytras and Cattini, 1994). Subsequently, by gene transfer experiments, mutations in the RF-1 element revealed that the repressor activity could be abolished both in the human placenta JEG-3 and cervical HeLa cells, suggesting a generalized function for the RF-1 element (Lytras and Cattini, 1994). Furthermore, RF-1 and DF-1 regions were protected with nuclear extracts from both the placental (tissue, BeWo and JEG-3) and non-placental (HeLa and rat pituitary, GC) cells (Jacquemin et al. 1994a, Jiang and Eberhardt, 1994) in footprint analysis. However, the boundaries of the footprints were different in both studies. Hence, it is possible that similar ubiquitous factors are present in these cells types as well as in human and rat tissues recognizing the elements in both the PSF and CS-B enhancer. Thus, the identification of PSFs in the placenta strongly emphasizes that they may play a role in hGH/CS gene transcriptional regulation, possibly in a tissue-specific manner or in tissues where the hGH/CS genes are expressed. As was suggested earlier by Nachtigal et al. (1993), the PSFs and GHF-1/Pit-1 may interact to elicit the pituitary-repression of the placental CS/GH-V genes.

3.7. ROLE OF FACTORS RECOGNIZING PSF ELEMENTS AND EFFECT OF OVER-EXPRESSION OF THE PITUITARY SPECIFIC, GHF-1/PIT-1 TRANSCRIPTION FACTOR IN PLACENTAL CELLS

3.7.1. Introduction

From the above footprint analysis of the 263P fragment and gel shift analysis with placental nuclear extracts, it was established that the PSFs are also expressed in human placental JEG-3 cells (Figs. 3.21, 3.30). For a long time it was believed that the pituitary-specific transcription factor GHF-1/Pit-1 was not expressed in the placenta (Bodner and Karin, 1987). As was discussed earlier, a possible interaction between the PSFs and GHF-1/Pit-1 could result in the pituitary-repression of the placental CS/GH-V genes (Nachtigal et al. 1993). DNase I sensitivity assays revealed that the CS genes are as equally open as the pituitary GH-N gene in the pituitary (Nickel and Cattini, 1996). Thus, the possibility exists that the chromatin structure of the CS/GH-V genes are same in these tissues (Nickel and Cattini, 1996). This structure would increase the chances of the PSFs to recognize the PSF elements in the pituitary. Based on these assumptions, all components for repression except GHF-1/Pit-1 would be present in a placental cell.

3.7.2. Objective

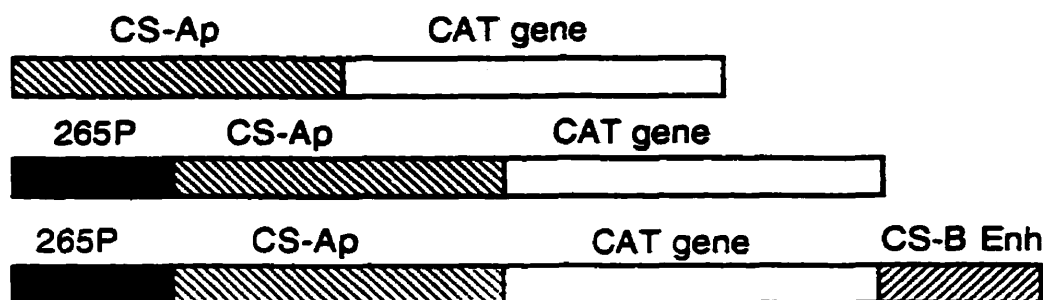
On the basis that PSF factors are expressed and GHF-1/Pit-1 is absent in placental cells, experiments were designed to test whether overexpression of GHF-1/Pit-1 in placental JEG-3 cells will stimulate the CS promoter activity as well as whether the combination of transfected GHF-1/Pit-1 and endogenous PSF, (together with corresponding DNA elements) can repress the CS promoter activity as seen in pituitary GC cells (Nachtigal et al. 1993).

3.7.3. Experimental design

The design and construction of reporter vectors CS-Ap.CAT, CS-Ap.CAT.Enh and PSF.CS-Ap.CAT.Enh (Fig. 3.23) were detailed by Nachtigal et al. (1993). The expression vector CMVp-Pit-1 was a gift from Dr Maria Vrontakis (Winnipeg, MB). The growth and culturing conditions for human placenta JEG-3 cells, the calcium phosphate precipitation

method of gene transfer and detection of CAT gene expression (CAT assay) are described in the Materials and Methods section.

Fig. 3.23. Hybrid CAT gene constructs. CAT gene was fused to the CS-A gene proximal promoter sequences (-496/+1) generating CS-Ap.CAT. The CS-B enhancer (240 bp) fragment was ligated into downstream sequences of CS-Ap.CAT resulting in the CS-Ap.CAT.Enh. The PSF.CS-Ap.CAT construct was generated by inserting 263P fragment upstream DNA of the CS-Ap.CAT.Enh construct.



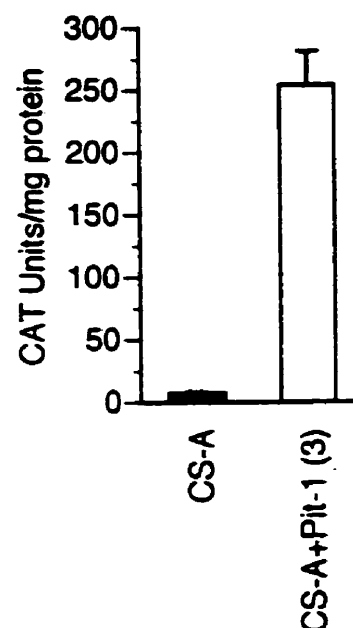
3.7.4. Results and Discussion

3.7.4.1. Activation of the CS promoter by GHF-1/Pit-1

Transient gene transfer of the CS-A promoter (-496/+1) into the human placental (JEG-3) cells revealed that the CS-A promoter is usually not very active (Fig. 3.23A). In rat pituitary GC cells, the CS-A promoter is more active than the GH-N promoter (Nachtigal et al. 1993), although the CS-A promoter has only the proximal GHF-1/Pit-1 site of the two present in equivalent sequences of the GH-N gene (Lemaigre et al. 1989, Nachtigal et al. 1989, Nickel et al. 1991). The low level of CS-A promoter activity in placental JEG-3 cells is consistent with the possibility that GHF-1/Pit-1 may be the limiting factor and thereby responsible for the weak activity associated with the GH/CS promoters. When 3 μ g of the expression vector CMV.Pit-1 was co-transfected with 15 μ g of CS-

Ap.CAT construct into JEG-3 (1×10^6) cells in 100 mm culture dish, an ~30 fold increase in CS-A promoter activity compared to control (no GHF-1/Pit-1) was observed ($n=5$, $p<0.0001$) (Fig. 3.23A). Similarly GHF-1/Pit-1-dependent transactivation of the GH-N, PRL and CS-A promoters was observed in non-GHF-1/Pit-1-expressing HeLa and green monkey kidney CV-1 cells (Fox et al. 1990; Ingraham et al. 1990; Mangalam et al. 1989; please refer to Figs. 3.27 and 3.28). These observations strongly suggested that GHF-1/Pit-1 is the sole important transcription factor required for pituitary-specific expression of GH-N and PRL genes and can activate GHF-1/Pit-1-dependent GH-N and PRL promoters in non-GHF-1/Pit-1 expressing cells.

Fig. 3.23A. Overexpression of GHF-1/Pit-1 activates CS-A promoter activity in human placental choriocarcinoma JEG-3 cells. Approximately 1×10^6 JEG-3 cells per 100 mm culture dish were transfected with 15 μ g of CS-Ap.CAT (CS-A) along with or without 3 μ g of a Pit-1 overexpressing CMVp.Pit-1 (Pit-1) construct. Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. The CS-A promoter was stimulated over ~30 fold ($p<0.0001$) in the presence of GHF-1/Pit-1. The mean value in CAT units per milligram of protein is shown and was derived from at least five determinations. Error bars indicate standard error of the mean. (A DNA fragment containing nucleotides from -496 to +1 of the CS-A gene was used as CS-Ap).



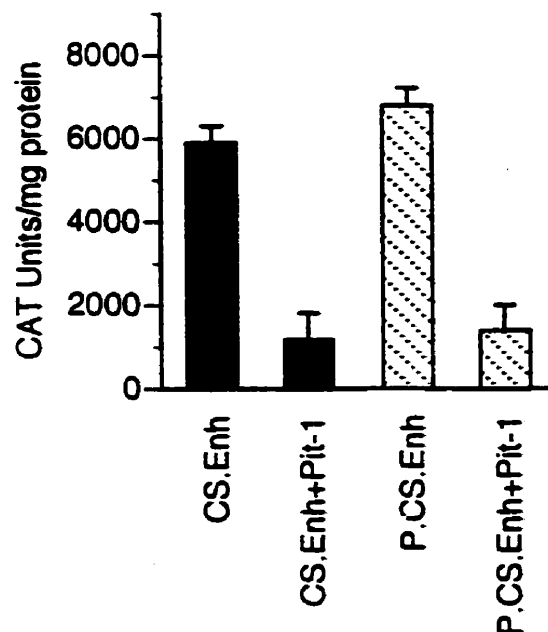
3.7.4.2. Effect of GHF-1/Pit-1 overexpression on CS-B enhancer activity

In order to unravel the importance and the functionality of the suggested physical interaction between the GHF-1/Pit-1 and PSFs (Nachtigal et al. 1993), similar co-

transfection experiments were done in JEG-3 cells with 15 μg of either CS-Ap.CAT.Enh or PSF.CS-Ap.CAT.Enh genes and 3 μg of CMVp.Pit-1. As was reported earlier, the CS-B enhancer-dependent CS-A promoter activity was not affected by the presence or the absence of PSF sequences in placental JEG-3 cells (Fig. 3.23B, Nachtigal et al. 1993). Surprisingly, however, the overexpression of GHF-1/Pit-1 dramatically repressed the CS-B enhancer associated stimulatory activity (Fig. 3.23B; $n=6$ $p<0.0001$). Interestingly, this repressor activity was independent of the presence of the PSF sequences (Fig. 3.23B; $n=6$ $p<0.0001$). The CAT gene expression was measured in terms of CAT units/mg of protein.

Although, an expression vector CMVp.luciferase was used to normalize the transfection efficiency, this CMV promoter activity was also repressed by over-expression of the GHF-1/Pit-1 (data not shown). Analysis of the CMV immediate early gene promoter sequences (GenBank Accession Number: X03922) revealed the presence of a consensus GHF-1 Pit-1 binding site. At present it is not known whether GHF-1/Pit-1 binds to these sequences. Regardless the CMV promoter appears to be influenced by GHF-1/Pit-1 levels. Hence, the CAT activity was standardized to protein.

Fig. 3.23B. Downregulation of stimulatory activity associated with CS-B enhancer by overexpression of GHF-1/Pit-1 in human placental choriocarcinoma JEG-3 cells. Approximately 1×10^6 JEG-3 cells per 100 mm culture dish were transfected with 15 μg of CS-Ap.CAT.Enh (CS.Enh) or PSF.CS-Ap.CAT.Enh (P.CS.Enh), with or without 3 μg of a Pit-1 overexpressing CMVp.Pit-1 (Pit-1) construct. Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. The CS-B enhancer-associated stimulatory activity was downregulated in a very significant manner ($p<0.0001$) in the presence of GHF-1/Pit-1. There was no significant difference ($p>0.1$) in the CAT gene expression in the presence or absence of PSF sequences. The mean value in CAT units per milligram of protein is shown and was derived from at least six determinations. Error bars indicate standard error of the mean. (A DNA fragment containing nucleotides from -496 to +1 of the CS-A gene was used as CS-Ap and a 240 bp fragment was used as an CS-B enhancer).



These results indicate that GHF-1/Pit-1 influenced repression of the CS promoter is independent of PSF sequences. Although this present study did not establish an interaction between PSFs and GHF-1/Pit-1, the results suggest a novel interaction between GHF-1/Pit-1 and the factors recognizing the placental CS enhancer elements. It is possible, as observed with the squelching experiments in a placenta cell environment, it may not be possible to study the functionality of the interaction between the GHF-1/Pit-1 and the PSFs. However, it is interesting to note at this juncture that the CS-B enhancer sequences possess RF-1 and DF-1 elements which resemble sequences containing PSF-A or associated with PSF-B binding sites (Lytras and Cattini, 1994). Thus, it is possible that these elements/factors may be interacting with GHF-1/Pit-1 and result in the repression. Interestingly, it was reported that a nuclease protected region comprising part of the RF-1 sequences was non-functional in placental cells (Jacquemin et al. 1994b). However, the effect could require a combinatorial mechanism and the PSFs may play some role in a pituitary environment. Thus, the above observations may not be reflective of non-participation of the PSFs in the repression, as was seen in the pituitary GC cells by Nachtigal et al. (1993).

However, a major limitation in transient gene transfer systems is the degree of transcriptional regulation of the naked DNA, which could be typically very low unless enhancers are used, whereas the extent of activation *in vivo* may be a thousandfold or more (Kornberg and Lorch, 1995). Thus, the chromatin structure enables additional levels of regulation and may be required for faithful reproduction of the natural process (Kornberg and Lorch, 1995). Moreover, it is difficult to monitor transcriptional regulation of the transfected genes in transient transfections assays (Nakayama et al. 1998). Hence, the GHF-1/Pit-1 cDNA was stably integrated into placental JEG-3 and JAR cells, to examine the effect of GHF-1/Pit-1 on endogenous hGH/CS gene transcriptional regulation. As stated in the earlier section, at the time of undertaking these experiments, it was assumed that the GHF-1/Pit-1 was not expressed in placenta. Furthermore, the hGH/CS genes excluding the GH-N gene are in an open-chromatin configuration in the placenta as these genes are expressed in this tissue (Nickel and Cattini, 1996).

It is noteworthy that many transcription factors originally described as activators have subsequently been shown to repress under certain conditions. The activator can inhibit its own activity (self-interference) or that of heterologous activators in transient transfection experiments (Gill and Ptashne, 1988; Berger et al. 1990). It was suggested that this 'squenching effect' was a result of titration of one or more general transcription factors (GTFs; Hwang et al. 1993; Orphanides et al. 1996). Furthermore, it was noticed that some transcription factors can repress promoter activity independent of their binding site in the promoter sequences, i.e., repression of heterologous activators (Gill and Ptashne, 1988). It was suggested recently that the reason for the transcriptional squenching is not only due to the limited supply of GTFs, but is also restricted to episomal target genes, because genes integrated into cellular chromatin are immune to the squenching effect (Natesan et al. 1997).

3.7.5. Generation of JEG-3 and JAR cells stably expressing GHF-1/Pit-1

Placental JEG-3 and JAR cells ($1 \times 10^6/10$ cm culture dish) were transfected with a mixture of 10 μ g of CMVp.Pit-1 and 1 μ g of pDX.neo expression plasmids using the calcium phosphate-DNA precipitation method. Maintenance and selection of stable cell lines were detailed in the Materials and Methods section. After G-418 selection with different doses for 15 days, individual clones with JAR and JEG-3 cells were identified, grown separately into 24 well culture dishes. Once a sufficient number of cells from each clone was grown, the cells were harvested and maintained on larger sized dishes and finally each stable cell line was established and frozen as stocks in 90% FBS-10% DMSO medium at -70 °C.

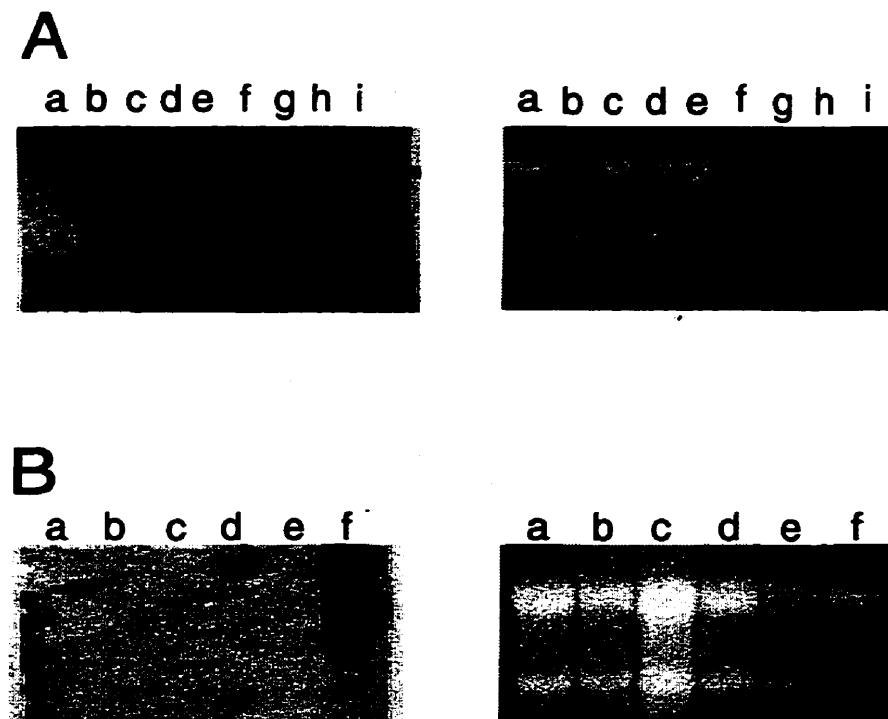
3.7.5.1. Detection of GHF-1/Pit-1 mRNA in the JEG-3 and JAR cells stably expressing GHF-1/Pit-1

The GHF-1/Pit-1 stable JEG-3 and JAR clones were grown to ~70 % confluency on 100 mm culture dishes. Total RNA was prepared from at least two 100 mm culture

dishes for each clone according to established protocols (Chomczynski and Sacchi, 1987) and 100 µg of total RNA of each sample was fractionated on a formaldehyde-1.5% agarose gel as detailed in Materials and Methods. After transfer of the RNA onto a nitrocellulose membrane, the membrane was probed with rat Pit-1 cDNA fragment (*Xba*I and *Kpn*I) from the CMVp.Pit-1 expression vector. From two clones of JAR.GHF-1/Pit-1 stable lines, only one (#2) showed a GHF-1/Pit-1 specific transcript (Fig. 3.24A), whereas one (#7) out of ten clones from JEG-3.GHF-1/Pit-1 was positive for GHF-1/Pit-1 (Fig. 3.24B). Expression of GHF-1/Pit-1 was not observed in control JEG-3 and JAR cells (Fig. 3.24).

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Fig. 3.24A and 3.24B. Expression of Pit-1 in human placenta choriocarcinoma JAR and JEG-3 cells stably expressing Pit-1. Clones of cell lines (human cervical HeLa, human placental JAR, JEG-3 and rat pituitary GC) were generated and total RNA was prepared from these clones as described in the Materials and Methods section. Approximately 100 µg of total RNA was fractionated in a 1.5% agarose-formaldehyde gels and transferred to a nitrocellulose membranes. Membranes were probed with the radiolabelled Pit-1 cDNA and after washings exposed to X-ray film. Both the autoradiogram and RNA gel pictures were shown. **Fig. 3.24A.** Lanes represent RNA from: a) HeLa, b) GC, c) JAR, d) JEG-3 e)HeLa.Pit-1, f) GC.Pit-1, g) JAR.Pit-1 clone # 1, h) JAR.Pit-1 clone #2, and i) JEG-3.Pit-1 clone # 1. **Fig. 3.24B.** Lanes represent RNA from: a) HeLa, b) HeLa.Pit-1, c) JEG-3, d) JEG-3.pit-1 clone # 5 e)JEG-3.pit-1 clone # 6, and f) JEG-3.pit-1 clone # 7.



Nuclear extracts were also prepared from these cell lines according to a rapid method described by Andrews and Faller (1991) and the presence of GHF-1/Pit-1 binding activity was determined by gel mobility shift assays (Figs. 3.25, 3.26). However, no differences were observed in the binding activities and complexes formed on a radiolabelled GHF-1/Pit-1 binding element (sense strand 5'-CGTCAGTGGCCCCATGCATAAATGTACACAGAAACAGGTG-3', Lefevre et al. 1987) with nuclear extracts prepared from the control JEG-3 and JAR or their corresponding GHF-1/Pit-1 stables. Furthermore, these complexes were specifically competed by excess amounts of unlabelled GHF-1/Pit-1 binding element (Fig. 3.26). Initially, it was assumed that the binding activity observed with nuclear extracts from both the control and GHF-1/Pit-1 stables was due to the binding of Oct-1, which was known to recognize the GHF-1/Pit-1 elements (Aurora and Herr, 1992). Oct-1 is also a homeodomain protein and was known to form heterodimers with GHF-1/Pit-1 (Voss et al. 1991; Verrijzer et al. 1992). However, the reason for no differences in binding activity has become evident, since GHF-1/Pit-1 was shown to be expressed in the placenta (Bamberger et al. 1995; Schanke et al. 1997).

Fig. 3.25. Detection of GHF-1/Pit-1 DNA binding activity in human placental JAR and JEG-3 cell nuclear extracts. Nuclear extracts were prepared from GC, JAR, JAR.Pit-1, JEG-3 and JEG-3.Pit-1 cells by rapid method and were used in gel mobility shift assays with a radiolabelled GHF-1/Pit-1 binding site as a probe (100 pg/10,000 cpm). The complexes formed on GHF-1/Pit-1 element were separated in a 4%PAGE gel. The gel was dried and exposed to X-ray film. Lanes: a) GHF-1/Pit-1 probe without nuclear extract, b) GC (2 µg), c) GC (5 µg), d) JAR (10 µg), e) JAR.Pit-1 # 2 (10 µg), f) JEG-3 (10 µg), and g) JEG-3.Pit-1 # 7 (10 µg).

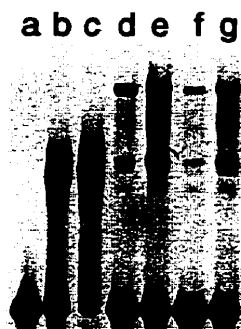


Fig. 3.26. Detection of GHF-1/Pit-1-specific complexes in nuclear extracts prepared from human placental JAR and JEG-3 cells stably expressing Pit-1. Nuclear extracts were prepared from GC, JAR, JAR.Pit-1, JEG-3 and JEG-3.Pit-1 cells by rapid method and were used in gel mobility shift assays with a radiolabelled GHF-1/Pit-1 binding site as a probe (100 pg/10,000 cpm). The complexes formed on GHF-1/Pit-1 element were competed in the presence of either 50 or 100 ng of GHF-1/Pit-1 element. DNA-protein complexes were separated in a 4%PAGE gel. The gel was dried and exposed to X-ray film. Lanes: a) GC (5 μ g), b) GC (5 μ g) + GHF-1/Pit-1 element, (50 ng), c) GC (5 μ g) + GHF-1/Pit-1 element (100 ng), d) JAR.Pit-1 # 2 (10 μ g), e) JAR.Pit-1 # 2 (10 μ g) + GHF-1/Pit-1 element (50 ng), f) JAR.Pit-1 # 2 (10 μ g) + GHF-1/Pit-1 element (100 ng), g) JEG-3.Pit-1 # 7 (10 μ g), h) JEG-3.Pit-1 # 7 (10 μ g) + GHF-1/Pit-1 element (50 ng), i) JEG-3.Pit-1 # 7 (10 μ g) + GHF-1/Pit-1 element (100 ng) and j) GHF-1/Pit-1 probe without nuclear extract.



3.7.5.2. Influence of GHF-1/Pit-1 on endogenous GH/CS gene expression in the stable JEG-3 and JAR cells overexpressing GHF-1/Pit-1

The effect of GHF-1/Pit-1 (over)expression on endogenous GH/CS gene expression in JEG-3 and JAR cells was assessed. Approximately 100 μ g of total RNA from these stable (=7 JEG-3.GHF-1/Pit-1 and #2 JAR.GHF-1/Pit-1) and control (JEG-3 and JAR) cells was subjected to RNA (northern) blotting after fractionation on a formaldehyde gel, and the membrane was probed with the hGH cDNA. Surprisingly, no GH/CS positive transcripts were detected in any of the samples including the GHF-1/Pit-1 stables (data not shown). Initially it was assumed that the expression of GHF-1/Pit-1 in these cells led to the general repression of the placental growth hormone genes, as the

placental cells express the PSFs and the factors recognizing the CS enhancer elements. Previous experiments strongly suggested the possibility of an interaction between the GHF-1/Pit-1 and factors binding to the CS enhancer elements, which could result in repression of CS promoter activity (Fig. 3.23B). However, control cells without transfected GHF-1/Pit-1 were also negative for GH/CS transcripts. Although the JEG-3 and JAR cells were initially isolated as placental lactogen (CS) expressing cells (Kohler and Bridson, 1971; Pattillo et al. 1971), subsequent reports indicate that these cells produce very low to undetectable levels GH/CS transcripts (Jimenez et al. 1993; Nickel and Cattini, 1991; Quinn et al. 1996; Yamashita et al. 1987).

Consistent with this idea of a change in the pattern of GH/CS gene expression, it was reported that the phenotype of placental cells undergo a rapid drift and this will result in variations in the transcriptional activity in these cells (Jacquemin et al. 1994b). Indeed, this was the case with rat choriocarcinoma, Rcho cells, which change their placental lactogen (these proteins are more like PRL) gene expression pattern with continuous differentiation resulting from serial passaging of cells (Soares et al. 1991). Interestingly, it was suggested that JEG-3 cells may possess similar regulatory transacting properties to human pituitary cells (Kohler and Bridson, 1971). In support of this notion, in one study, after gene transfer, the transfected hGH-N gene (2.6 kb EcoRI fragment) was expressed in JEG-3 cells and this expression was responsive to treatment with 8-Bromo-cAMP and hydrocortisone (Yamashita et al. 1987). Furthermore, GH-N expression was inhibited by IGF-1, which is a typical feedback inhibition of GH gene expression (Yamashita et al. 1987). This suppression of GH-N gene transcription is analogous to the reduction of human GH-N mRNA seen with IGF-1 treatment human pituitary tumor cells (Yamashita et al. 1986). However, non transfected JEG-3 cells, with 10 µg of poly (A⁺) RNA did not exhibit hybridization of mRNA to the hGH cDNA probe (Yamashita et al. 1987) suggesting the absence of CS gene expression in these cells. Expression of the transfected GH-N gene was also detected in JEG-3 cells (Rogers et al. 1986a.. as referred in Yamashita et al. 1987). Surprisingly, no induction of endogenous placental CS/GH-V could be detected in these cells even after treatment with 8-Bromo-cAMP and

hydrocortisone (Yamashita et al. 1987).

Although JEG-3 cells were first isolated as CS producing cells (Kohler and Bridson, 1971) and as these cells were of tumor origin, one can expect possible changes in terms of sequence deletions and gene rearrangements in the GH/CS locus in these cells, that could account for deficiency in CS gene expression. The GH/CS gene locus is comprised of at least 48 regions of Alu sequence repeats which are known to be involved in gene duplications and rearrangements (Chen et al. 1989). To assess the possibility of gross rearrangements, genomic DNA was isolated from these cells and digested with *Bam*HI, *Bgl*II, *Eco*RI, *Xba*I and *Xho*I, the fragments were separated on a 1% agarose gel and transferred to a nitrocellulose membrane. Subsequent hybridization with hGH-N cDNA (Martial et al. 1979) revealed the expected restriction sizes specific to the GH/CS genes (please refer to Fig. 5.9) and agreed with the sequence information available (Chen et al. 1989) as well as corroborated published results from other investigators (Lytras et al. 1993; Nickel and Cattini, 1991; Vnencak-Jones et al. 1988). Similarly, the insertion of the GHF-1/Pit-1 gene in these stable cells did not alter the arrangement of the GH/CS genes as was confirmed by similar Southern hybridizations of the genomic DNA from these stable cell lines.

The lack of response seen with JEG-3 cells was also noticed with other gene systems unrelated to the GH/CS gene family. In many cell lines, fibronectin (FN) gene expression is serum responsive and this was reflected in the serum induced binding of proteins to the cAMP responsive element (CRE) as was detected in gel retardation assays. However, in JEG-3 cells, FN gene expression was not serum responsive and concomitantly, there was no serum induced proteins binding to the CRE in these cells (Dean et al. 1990). However, the essential components required for a cAMP response are available in a JEG-3 cell, as 8-Bromo-cAMP stimulates the α and β subunit mRNAs for human chorionic gonadotropin (Johnson et al. 1997), Bcl-2 (Sakuragi et al. 1994) and GHF-1/Pit-1 (Schanke et al. 1997) in these cells. It is also known that cAMP plays a critical role in syncytial trophoblast formation (Wice et al. 1990; Strauss et al. 1992).

A combination of more sensitive RT-PCR of the RNA from the JEG-3, JAR and BeWo cells, with subsequent restriction digestion analysis of the PCR products, revealed that these cells also express GH-V, CS-A and CS-B genes, but at different ratios (Lytras et al. 1994). In this study, it was also shown that hydatidiform moles express the CS genes (Lytras et al. 1994), but initially it was suggested by other investigators that in patients with hydatidiform molar pregnancy, the abnormal trophoblasts do not express the CS genes (Mochizuki et al. 1976; Hjelle et al. 1982). However, it is yet to be confirmed whether the CS/GH-V RNA levels in these cells (Lytras et al. 1994) correlate with corresponding protein levels.

As indicated earlier, GHF-1/Pit-1 mRNA was detected in placental cells (Bamberger et al. 1995; Lee et al. 1996; Schanke et al. 1997) by RT-PCR. In addition, the same investigators identified GHF-1/Pit-1 protein in these cells by immunohistochemistry and western blotting (Bamberger et al. 1995; Schanke et al. 1997). Surprisingly, as stated earlier, no GHF-1/Pit-1 transcripts were detected in these cells by northern blotting (Fig. 3.24A, B). Low levels of GHF-1/Pit-1 expression were detected in both the early and the term placenta relative to those observed in human pituitary. The levels in JEG-3 cells were even lower than seen in the placenta (Bamberger et al. 1995). Interestingly, the GHF-1/Pit-1 transcript and protein levels were increased upon treatment with 8-Bromo-cAMP in JEG-3 cells (Schanke et al. 1997) which is similar to the GHF-1/Pit-1 gene upregulation observed in the pituitary (McCormick et al 1990). The renin promoter contains a GHF-1/Pit-1 binding site which is essential for the promoter activity (Catanzaro et al. 1994). Further studies showed that the renin promoter is active in pituitary GC, human placental as well as kidney juxtaglomerular cells. Initially it was thought that renin promoter activity in non-pituitary cells was due to recognition of the GHF-1/Pit-1 element by either GHF-1/Pit-1 related or Hox proteins (Catanzaro et al. 1994). However, at least in placental cells the renin promoter activity could be due to binding of placental GHF-1/Pit-1.

3.8. ROLE OF FACTORS RECOGNIZING PSF-A AND PSF-B ELEMENTS AND THE EFFECT OF PITUITARY SPECIFIC GHF-1/PIT-1 TRANSCRIPTION FACTOR OVEREXPRESSION IN NON-PITUITARY AND NON-PLACENTA, HeLa CELLS

3.8.1. Introduction

The functionality of any physical interaction between the GHF-1/Pit-1 and PSFs could not be established using both pituitary and placental cells. This may be due to the complex cell (pituitary and placenta) environments. Furthermore, these cells originate from tissues where members of the GH/CS gene family are expressed. It is possible that a better understanding of these control mechanisms can be achieved with cells of other tissue origins in which the GH/CS genes are not expressed. The factors recognizing the PSF-A and PSF-B elements are present whereas GHF-1/Pit-1 is absent in human cervical carcinoma HeLa cells. Hence, these cells were used for further studies.

3.8.2. Objective

As PSF factors are expressed in HeLa cells, it was hypothesized that overexpression of GHF-1/Pit-1 in HeLa cells will stimulate CS promoter activity but down-regulate CS promoter activity in the presence of PSF elements in a similar way to that observed in pituitary GC cells (Nachtigal et al. 1993).

3.8.3. Results and Discussion

3.8.3.1. Examination of the interaction between GHF-1/Pit-1 and PSFs in a cellular environment

This experiment was performed to detect the influence of a possible physical interaction between GHF-1/Pit-1 and the PSFs (Nachtigal et al. 1993). Although the PSFs

are ubiquitous, it was suggested that their role as pituitary-specific repressors was due to the interaction of these factors with the pituitary-specific factor, GHF-1/Pit-1 (Nachtigal et al. 1993). HeLa cells are deficient in the GHF-1/Pit-1 factor and it was already demonstrated by other investigators that the provision of GHF-1/Pit-1 alone is sufficient to activate the GH promoter in HeLa cells (Fox et al. 1990; Ingraham et al. 1990). Thus, in order to simulate the pituitary environment in the HeLa cells, the expression vector, CMVp.Pit-1 was introduced into these cells. Hence, in this environment, the activity of CS-Ap.CAT.Enh should be repressed if a 263P fragment harboring the PSF-A and PSF-B elements was present upstream of the CS-Ap driven reporter gene. Approximately 5×10^5 HeLa cells in a 100 mm culture dish were transiently transfected with 15 μ g of CS-Ap.CAT.Enh or PSF.CS-Ap.CAT.Enh. Co-transfections were also carried out with 5 μ g of CMVp.Pit-1 expression vector. Extracts prepared from the HeLa cells transfected with either the CS-Ap.CAT.Enh or the PSF.CS-Ap.CAT.Enh alone did not show any significant CAT enzyme activity (Fig. 3.27; n=6). This indicates that the CS-A promoter is not active in these cells. There was a GHF-1/Pit-1 dependent activation of the CS promoter upon overexpression of GHF-1/Pit-1 in HeLa cells (Fig. 3.27; n=6, $p < 0.0001$). However, no repression of CS-A promoter activity was observed in the presence of PSF (263P) sequences in HeLa cells upon co-transfection with GHF-1/Pit-1 (Fig. 3.27; n=6).

Although the PSFs did not repress CS-A promoter activity in the presence of GHF-1/Pit-1 in HeLa cells which were imitating a pituitary environment (as the CS-A promoter could be activated), it was not known whether the amount of GHF-1/Pit-1 being expressed in HeLa cells in this experiment was appropriate (too low or too high) to show the repression. It was observed in several studies that subtle differences in the amount of transcription factors (either the amount could be low or high) can bring differential effects both *in vivo* and *in vitro* (Grosveld et al. 1998; Hwang et al. 1993). If this is the case, the type of interaction between the PSFs and GHF-1/Pit-1 that was suggested in pituitary GC cells may be difficult to achieve. To address this problem, different amounts of CMVp.Pit-1 expression vector ranging between 0.1, 0.3, 1.0, 3.0 and 10 μ g per culture dish were used along with 15 μ g of the reporter constructs as stated earlier, in a further round of

transient transfection experiments. Although, a GHF-1/Pit-1- and dose-dependent activation of the CS-A promoter was observed in HeLa cells with very significant activation at 3 μ g of CMVp.Pit-1 (Fig. 3.28; n=3, p<0.05), the CS-A promoter alone was activated better than that of CS-A promoter accompanied by enhancer sequences. However, as seen in the previous experiment (Fig. 3.27), there was no significant difference between the activities of CS-A.CAT.Enh and PSF.CS-A.CAT Enh, suggesting the presence of PSF sequences did not bring any significant repression (Fig. 3.28; n=3). A similar reduction in the GH promoter activity at higher doses (10 μ g) was also observed in experiments done by Fischberg et al (1994). These results indicate that this approach may also not be appropriate to study the functional importance of the interaction between the GHF-1/Pit-1 and the PSFs. It is possible that the complete reconstitution of a pituitary environment was not achieved in this study except for the activation of CS promoter by GHF-1/Pit-1. In this context it is interesting to observe that in the presence of GHF-1/Pit-1, the CS-Ap.CAT.Enh was repressed both in pituitary GC and placental JEG-3 cells (results from this study; Jiang and Eberhardt, 1997), suggesting an interaction between GHF-1/Pit-1 and the factors (possibly TEF-like factors) recognizing the CS-B enhancer elements. In contrast, overexpression of GHF-1/Pit-1 in HeLa cells did not result in similar repression of the CS-Ap.CAT.Enh construct, instead there was stimulation (Fig. 3.27). However, the magnitude of CS promoter activation in the presence of CS-B enhancer sequences was less than that of the CS promoter alone (Fig. 3.28). This observation does raise the possibility that tissue-specific mediators exist and interact with the GHF-1/Pit-1/enhancer factors thereby eliciting either a positive or negative effect. It was reported recently that GHF-1/Pit-1 forms complexes with positive or negative regulators of transcription (Xu et al. 1998).

Furthermore, the results from the above experiments, which were done to establish the functional importance of any interaction between GHF-1/Pit-1 and PSFs, supports the hypothesis that transcription factors act cooperatively and combinatorially. These results also suggest that it may be difficult sometimes to recreate an *in vitro* phenomenon, such as interaction between GHF-1/Pit-1 and PSFs, *in vivo*. These assumptions were also supported by many trials of targeted disruption or alterations in the levels of the

transcription factors in mice, which often resulted in only moderate reductions in target gene expression (Duncan et al. 1998).

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Fig. 3.27. GHF-1/Pit-1 dependent CS-A promoter activity in HeLa cells is not influenced in the presence of PSF sequences. Approximately 0.5×10^6 HeLa cells per 100 mm culture dish were transfected with 15 μ g of CS-Ap.CAT.Enh (CSp.E) or PSF.CS-Ap.CAT.Enh (P.CSp.E) and with or without 5 μ g of a Pit-1 overexpressing CMVp.Pit-1 (Pit-1) construct. Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. The CS-A promoter was stimulated very significantly in the presence or absence of PSF sequences ($p < 0.0001$) when GHF-1/Pit-1 was overexpressed. The mean value in CAT units per milligram of protein is shown and was derived from at least six determinations. Error bars indicate standard error of the mean. (A DNA fragment containing nucleotides from -496 to +1 of the CS-A gene was used as CS-Ap and the number in parenthesis indicates the amount of Pit-1 expression vector used).

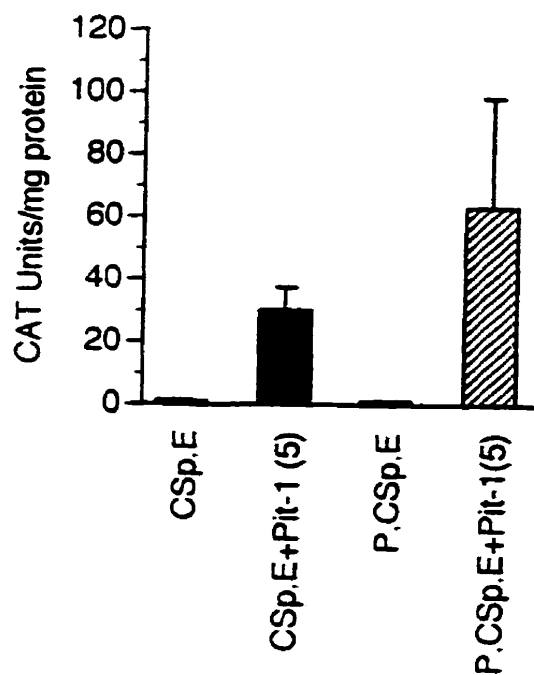
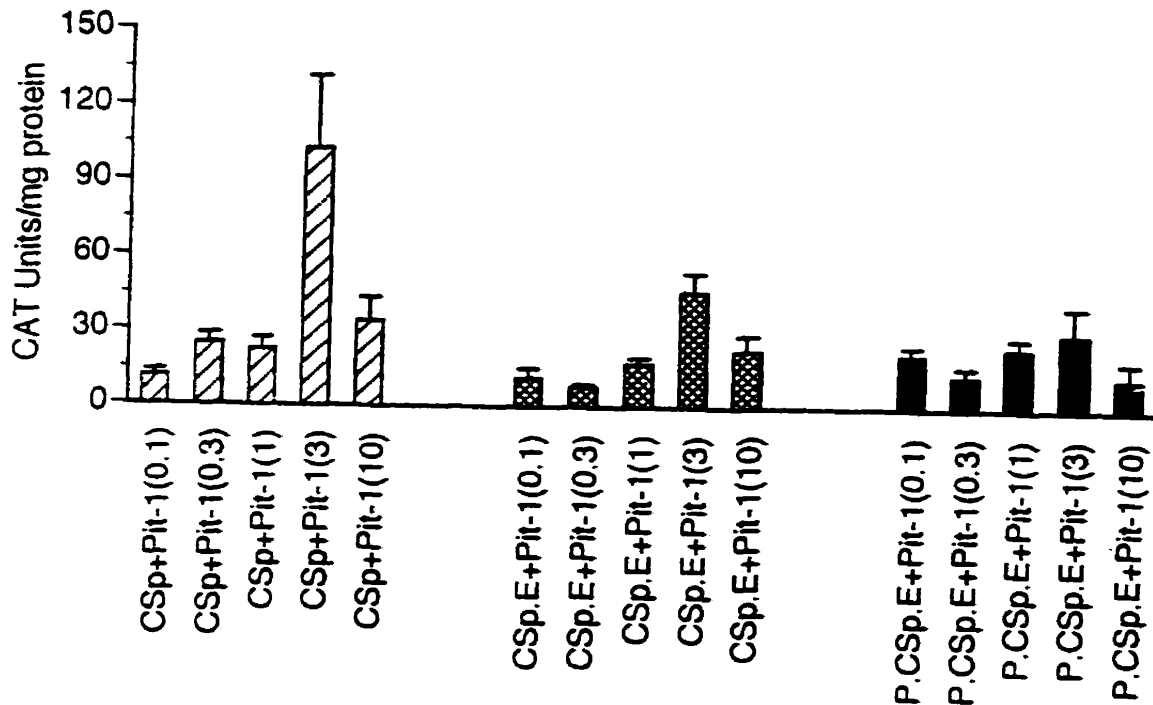


Fig. 3.28. Modulation of GHF-1/Pit-1 concentration in HeLa cells still activates the CS-A promoter in a GHF-1/Pit-1- and dose-dependent manner, but this activation of CS-A promoter is not influenced in the presence of PSF sequences. Approximately 0.5×10^6 HeLa cells per 100 mm culture dish were transfected with 15 μ g of each CS-Ap.CAT (CSp), CS-Ap.CAT.Enh (CSp.E) or PSF.CS-Ap.CAT.Enh (P.CSp.E), with or without 0.1, 0.3, 1.0, 3.0 and 10 μ g of a Pit-1 overexpressing CMVp.Pit-1 (Pit-1) construct. Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. There was a significant stimulation ($p < 0.05$) of CS-A promoter when 3 μ g of CMVp.Pit-1 was cotransfected, however there was no significant difference ($p > 0.05$) among the groups with other concentrations of CMVp.Pit-1. The mean value in CAT units per milligram of protein is shown and was derived from at least three determinations. Error bars indicate standard error of the mean. (A DNA fragment containing nucleotides from -496 to +1 of the CS-A gene was used as CS-Ap and the numbers in parenthesis indicate the amounts of CMVp.Pit-1 used in transfections).



3.9. PHYSICAL AND FUNCTIONAL CHARACTERIZATION OF PSF-A AND PSF-B ELEMENTS AND FACTORS

Previous studies from our laboratory revealed that the complexes formed on either PSF-A or PSF-B can compete for each other (Nachtigal, 1993; Nachtigal et al. 1993). In nuclease protection as well as in gel mobility shift assays, it was observed that both PSF-A and -B regions or complexes were competed in the presence of either binding site (Nachtigal, 1993; Nachtigal et al. 1993). These observations led to the suggestion that PSF-A and -B are recognized by the same protein or by a complex which is a heteromer. Furthermore, the PSF-A and -B elements individually possessed repressor activity (Nachtigal et al. 1993) suggesting their modular nature. Interestingly, the repressor activity observed with the 263P fragment was orientation dependent and was associated only with its "endogenous" orientation in the genome (Nachtigal et al. 1993). However, individual binding sites could repress the CS-A promoter activity in pituitary cells in an orientation independent manner (Nachtigal et al. 1993). This orientation independence effect was not explained. However, it is possible that in the 263P fragment, both binding elements form an integral part to exert the repression. To address these possibilities and to understand the nature of the individual elements, fragments were generated containing (i) both PSF-A and -B regions or (ii) either PSF-A or PSF-B mutated (iii) both PSF-A and PSF-B regions mutated. Structural characterization of these regions was carried out to unravel the nature of PSF protein binding to these altered regions. Previously, the mutant PSF regions (either PSF-A or PSF-B mutants, individually) were shown to be completely devoid of repressor activity (Nachtigal et al. 1993).

3.9.1. Generation of truncated fragments containing PSF sequences

Using the 263P fragment as the template and with different pair combinations of either PSF-As (sense, 5'-TTTCAATGTTGGTTGCCAACACCACAGCCAACCACTTC ATG-3'), PSF-Bas (antisense, 5'-CCCCTGTGGCCTAGGATGTGAATGCTGGGGCCC TGCCATCTCCCCACTTT-3'), PSF-Asmt (sense, mutant 5'-TTTCAATGTGAAGTAT

TCGTAGGTGCGCCAACCACTTCTAG-3') or PSF-Basmt (antisense, mutant 5'-CTGTGGCCTAGGATGTGCAGCTACGAATACTTCTGCCACTTT-3') oligonucleotides as primers, approximately 90 bp PSF fragments (PSFA/B, PSFA/Bmt, PSFamt/B and PSFamt/Bmt) were generated by PCR.

Using different pairs of primers (PSF-As/-Bas; PSF-As/-Basmt; PSF-Asmt/-Bas; PSF-Asmt/-Basmt), 100 µl PCR reactions were performed. After the initial step at 94 °C for 3 minutes for amplification (100 ng of plasmid with 263P insert in 10 mM of Tris-Cl pH 8.0, 1.5 mM MgCl₂, 50 mM KCl, 200 µg/ml gelatin, 200 µM dNTPs, 300-400 nM each primer and 2 U of Taq polymerase in a final volume of 100 µl), the reactions were performed for five cycles with each cycle consisting three 30 seconds steps of denaturation at 92 °C, annealing at 37 °C and extension at 72 °C. The reactions were further continued for another 30 cycles with similar three 30 seconds steps but the annealing temperature was raised to 55 °C. A final step of 10 minutes extension at 72 °C was included.

The PCR products (~90 bp) were isolated after gel electrophoresis. The ends of these products were blunted by the Klenow-end filling method. The fragments were subcloned into the *Sma*I site of the pUC19 plasmid. The fragments/mutations were confirmed by PCR sequencing. These 90 bp fragments were used to structurally characterize the PSFs by mobility shift and nuclease protection assays. To prepare the probes, the pUC19 constructs were first digested with *Eco*RI and this site was labeled with radioactive [α -³²P]dATP as described for labelling the 263P fragment. Finally, the radiolabelled 90 bp fragments were released by *Hind*III digestion and then isolated.

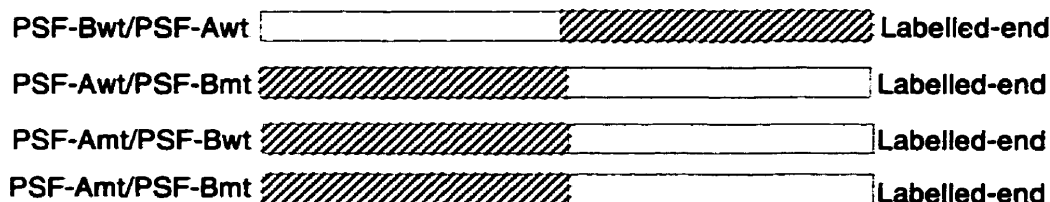
3.9.2. Gel-mobility shift assays with the 90 bp PSF fragments

Gel mobility shift assays were performed with 5-10 µg each of GC, HeLa, COS-1, JEG-3 and JAR nuclear extracts. Assays were done essentially as detailed for gel mobility shift assay in the Materials and Methods section. The nuclear proteins recognized by the 90 bp PSF DNA fragments were different in each cell as reflected by different

complex shift patterns (Fig. 3.29). The placental cell, JEG-3 and JAR, nuclear extracts resulted in similar complexes suggesting that the complexes were, at least, tissue-specific (Fig. 3.29). However, no detectable variations in the binding patterns among different probes comprising mutant sites were observed (Fig. 3.29). Although it is difficult to interpret these results, some of the complexes could be non-specific in nature due to binding of proteins to the DNA probe. It is surprising that these complexes were observed in the presence of 2 μ g/reaction of non-specific competitor, poly [dIdC]. Therefore, it was decided to perform footprint analysis on these probes. Sometimes shifts can be observed in a non-specific manner in mobility shift assays. In a nuclease protection assay, the DNase I can destabilize these non-specific interactions and thus result in specific-protected regions.

Fig. 3.29. Characterization of 90 bp PSF fragments by gel mobility shift assay. Gel mobility shift assays of 90 bp DNA fragments containing PSF-Awt/-Bwt (lanes: a-f in A), PSF-Awt/-Bmt (lanes: g-l in A), PSF-Amt/-Bwt (lanes: a-f in B) and PSF-Amt/-Bmt (lanes: g-l in B) were performed using 5-10 μ g each of GC (lanes : b and g), Cos-1 (lanes: c and h), HeLa (lanes: c and i), JEG-3 (lanes: d and j) and JAR (lanes: e and K). Lanes a and l represent represent the respective 90 bp PSF fragments in the absence of nuclear protein. The DNA probes used in the assay had a specific activity of 10,000 cpm/250 pg. Location of the PSF elements in the 90-mer DNA fragments and the the radiolabelled ends were shown.

Probes



A

a b c d e f g h i j k l



B

a b c d e f g h i j k l



3.9.3. DNase I protection assays on 90 bp PSF fragments

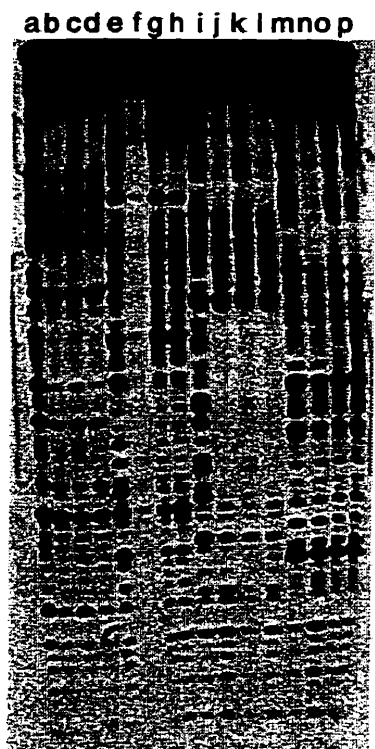
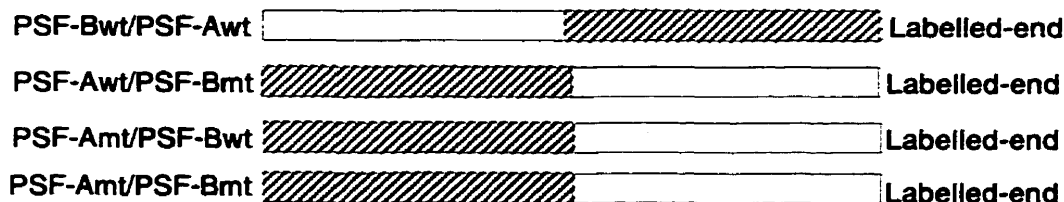
Nuclease protection reactions were performed as described in nuclease protection assay in the Materials and Methods section, using 10 µg each of GC, JEG-3 and HeLa nuclear extracts. The truncated fragment containing the wild type PSF-A and PSF-B regions essentially gave similar protected regions as was observed with 263P fragment including the formation of a hypersensitive site characteristic of the PSF-A region (Fig. 3.30). Nuclear extracts from all three cell types resulted in similar patterns of protection. However, the presence of either mutated PSF-A or the mutated PSF-B region did not affect the binding activity in the other region (Fig. 3.30), suggesting that the PSFs binding activities are independent of each other and this corroborates the repressor activities associated with the individual elements (Nachtigal et al. 1993). Furthermore, there was a complete loss of these footprints when both regions were mutated (Fig. 3.30).

3.9.4. PSFs possess weak modular repressor activity in GC and HeLa, but not in placental cells

Previous work by Nachtigal et al. (1993) had revealed that PSFs were repressors in pituitary but not placental cells, thereby showing some tissue-specificity. Furthermore, it was suggested that the PSFs could exert their function only on homologous promoters such as CS and GH as the PSFs need to interact with GHF-1/Pit-1 to exert the repressor activity (Nachtigal et al. 1993). The modular nature of individual PSF elements, either PSF-A or PSF-B, as repressors was also studied (Nachtigal et al. 1993). Although, these results suggested that PSFs could only act when associated with their homologous promoters, no data was available on the effect of PSF sequences on a heterologous promoter such as the viral promoter for Herpes Simplex thymidine kinase (TK).

Fig. 3. 30. Characterization of 90 bp PSF fragments by nuclease protection assays. Nuclease protection assays of 90 bp DNA fragments containing PSF-Awt/-Bwt (lanes: a-d), PSF-Awt/-Bmt (lanes: e-h), PSF-Amt/-Bwt (lanes: i-l) and PSF-Amt/-Bmt (lanes: m-p) were performed using 10 µg each of GC (lanes : b, f, j and n), JEG-3 (lanes: c, g, k and o) and HeLa (lanes: d, h, l and p). Lanes a, e, i and m represent the DNase I pattern generated from the respective 90 bp PSF fragments in the absence of nuclear protein. The DNA probes used in the assay had a specific activity of 10,000 cpm/250 pg. Location of the PSF elements in the 90-mer DNA fragments and the the radiolabelled ends were shown.

Probes



3.9.4.1. Objective and experimental design

In this study, attempts were made to determine the modular nature and effect of PSFs on TK promoter activity in GC, HeLa, JEG-3 and JAR cells after gene transfer. The 263P fragment was isolated as *EcoRI* and *HindIII* fragment and the ends were blunted. This fragment was subcloned into the *SmaI* site of the TK.CAT construct resulting in PSF.TK.CAT (this construct was generated by Nachtigal MW). Growth and maintenance of cells, gene transfer and CAT gene expression assays were described in the Materials and Methods section.

3.9.4.2. Results and Discussion

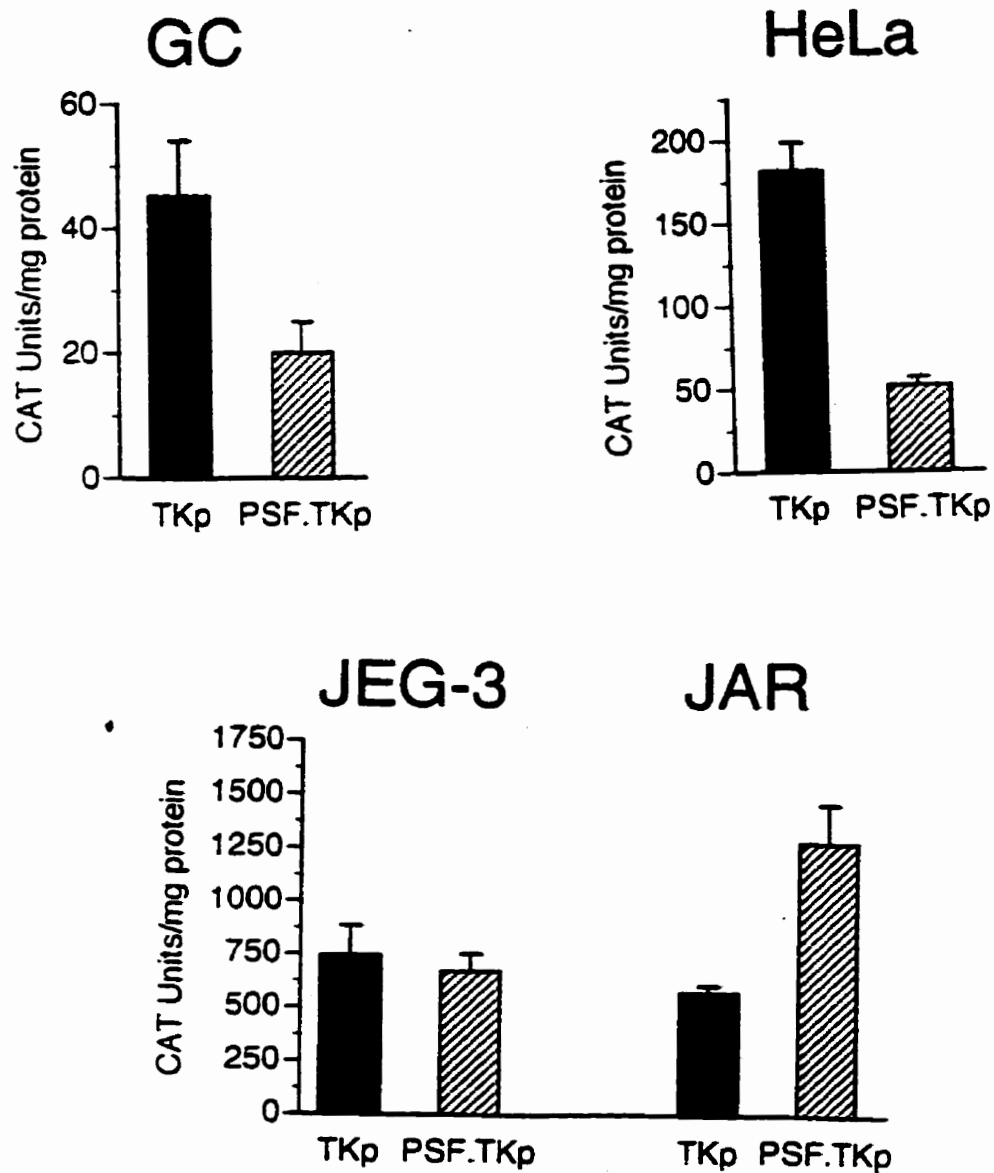
The hybrid CAT gene constructs TK.CAT and PSF.TK.CAT (10 µg/plate) were transiently transfected into GC, HeLa, JEG-3 and JAR cells. Expression analysis of the CAT gene revealed that in the presence of PSF sequences, the TK promoter activity was downregulated by at least 55% in GC, which was not quite significant ($n=3$, $p=0.06$), whereas in HeLa cells there was significant PSF associated repressor activity (~72%) ($n=3$, $p=0.0018$) on TK promoter (Fig. 3.31). However, no effect of PSF sequences was observed on TK promoter activity in placental JEG-3 and JAR cells (Fig. 3.31; $n=3$). In JAR cells, the PSF sequences stimulated TK promoter. These are the first results which show repressor activity outside of the pituitary cell environment. Furthermore, these results suggest that PSFs can also act independently of GHF-1/Pit-1.

The pattern of complexes formed on the PSF elements was different in placental versus non placental cells and suggests that related but different factors with similar binding specificities might be binding to PSF elements in these cell types (Fig. 3.29). For example, partial purification analysis of Sp1 from human placental nuclear extracts revealed that the active DNA binding species was a polypeptide of approximately 40 kDa rather than the 105 and 95 kDa proteins purified from HeLa cells (Briggs et al. 1986). Interestingly, this species of Sp1 from the placenta displayed the same affinity and specificity as that of the

Sp1 protein from HeLa cells for binding to SV40 Sp1 recognition elements, but had a lower potency with regard to transcriptional activity. This suggests that the lower molecular weight Sp1 might be a proteolytic product with an incomplete activation domain and thus, incapable of making complete interactions with other transcriptional apparatus (Briggs et al. 1986). It is possible that a similar situation occurs with PSFs in placenta explaining why the P-sequence or 263P fragment did not repress the CS promoter activity in placental cells after gene transfer (Nachtigal et al. 1993).

It is also possible that protein-protein and protein-DNA interactions, as well as post translational modifications of the DNA-binding factors may be different in the placental and non-placental cells. This would be reflected in the patterns seen in gel mobility shift assays and non-functional nature of PSFs in placental cells. However, it is yet to be determined, whether PSFs are recognized by the same factors in both GC, HeLa, COS-1, JEG-3 and JAR cells. This would best be accomplished by the identification and purification of the PSFs, thereby allowing the determination of whether the same factor(s) is recognizing the sequences *in vivo*.

Fig. 3.31. Heterologous promoter activity is repressed in the presence of PSF sequences in rat pituitary GC and human cervical carcinoma HeLa but not in human placental choriocarcinoma JEG-3 and JAR cells. Approximately 0.5×10^6 GC or HeLa, 1.0×10^6 JEG-3 or JAR cells per 100 mm culture dish were transfected with 15 μ g of each (-109/+53)TKp.CAT (TKp) or PSF.(-109/+53)TKp.CAT (PSF.TKp). Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. The presence of PSF sequences downregulated TK promoter activity very significantly in HeLa ($n=3$, $p=0.0018$), not that significantly in GC ($n=3$, $p=0.06$) but there was no effect in JEG-3 and JAR ($n=3$, $p>0.05$) cells. The mean value in CAT units per milligram of protein is shown and was derived from at least three determinations. Error bars indicate standard error of the mean.



3.10. POSSIBLE UBIQUITOUS FACTORS RECOGNIZING THE PSF ELEMENTS

Multiple approaches to characterize the factors binding to the PSF element were performed. Initial purification attempts to isolate near homogeneous PSFs from GC nuclear extracts were sufficiently successful, in that at least two protein bands corresponding to the sizes approximately 50 and 65 kDa were identified. However, the amounts purified were not sufficient to sequence the PSF proteins. Furthermore, the southwestern blotting revealed that the PSFs may include a 54 kDa protein. In addition, crosslinking experiments provided evidence that GC cells protein of >142 kDa interacts with the PSF-B element. Previous results from our laboratory also suggested that PSFs could be 30-40 kDa proteins (Nachtigal, 1993; Nachtigal et al. 1993). Structural characterization of the the PSF elements by gel mobility shift assays and footprint analysis, revealed that binding events on each of the PSF elements were independent of each other. These results also raised the possibility that PSFs are being recognized by a single factor as PSF-A and -B were copurified on one of the DNA affinity columns. Consistent with the latter, the PSF-A and -B were shown to compete for common complexes (Nachtigal, 1993). However, all these attempts could not help us to achieve the original objective of identifying the PSF proteins.

There have been recent developments in and availability of computational programs (such as MatInspector) to allow analysis of DNA sequences for the search of possible regulatory regions and the DNA-binding proteins recognizing these elements (Quandt et al. 1995). Using this approach, the ubiquitous factor, Nuclear factor-1 (NF-1)/CCAAT-binding transcription factor (CTF-1) was identified as a possible candidate for both PSF-A and -B. NF-1 was originally identified as adenovirus replication protein (Nagata et al. 1982). Purification and characterization of the CTF, the CCAAT-binding transcription factor revealed that NF-1 and CTF are the same proteins (Jones et al. 1987). Many eukaryotic promoters contain a CCAAT motif with variations in the recognition sequences, and this element and NF-1/CTF-1 were shown to be important for RNA polymerase II dependent transcription (Jones et al. 1987 and references therein). The consensus sequence

for NF-1/CTF was derived after analyzing 75 related sequences and is "NNH(A/C/T)TGGCNNNNNNCCNNN" (Jones et al. 1987; Quandt et al. 1995; Osada et al. 1996). Chicken NF-1 proteins were purified from liver nuclear extracts as TGGCA sequence recognizing protein (Rupp and Sippel, 1987). A half-site with just TTGGCA could also bind NF-1 with high affinity (Osada et al. 1996). However, variations in the binding sequences has led to the classification of NF-1 elements as high, medium and low affinity binding sites for NF-1/CTF (Jones et al. 1987). The high affinity binding site was TGGC(N)₆GCCAA whereas other sequences with only half sites were classified under medium and low affinity sites (Jones et al. 1987). The footprinted region in the PSF-B element, 5'-HTGGCAGGGCCCC-3' (PSF-B 5'-AAGTGGGGAGATTGGCAGGGCCCCAGCATTCACATCCTAGGCC-3'), has the core NF-1 consensus sequence, 5'-ATGGCNNNNNNCC-3'. Whereas, PSF-A has two TGGCA elements (PSF-A 5'-TTCAATGTTGGTTGCCAACACCACTTGCCAACCACTTCT-3').

The NF-1/CTF factors purified from HeLa cell nuclear extracts were shown to consist predominantly of a family of polypeptides with molecular masses between 52 and 62 kDa, although an unidentified 120 kDa protein was also co-purified (Jones et al. 1987). Diffley and Stillman (1986) have reported the identification of a 160 kDa polypeptide in highly purified preparations of NF-1. Generation of multiple forms of CTF/NF-1s were shown to be due, in part, to alternative splicing of RNA (Santaro et al. 1988; Mermod et al. 1989). However, in chicken, the NF-1s were shown to be encoded by at least four independent genes, NFI-A, NFI-B, NFI-C and NF1-X (Rupp et al. 1990; Kruse et al. 1991). CTF/NF-1 proteins can form homo or heterodimers, even in the absence of DNA (Apt et al. 1993; Kruse and Sippel, 1994; Mermod et al. 1989). NF-1 contains a proline-rich activation domain (Mermod et al. 1989) and this domain was shown to contain amino acid sequences similar to repeat units of the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (Kim and Roeder, 1994).

Although, NF-1 is an activator of transcription, there are a few examples indicating NF-1 might act as a repressor (Osada et al. 1997). Furthermore, the repressor activity was

localized to the C-terminal region of the NF-1A (Osada et al. 1997). However, the NF-1A repressor domains did not interact with transcription factor IIB, TATA-binding protein (TBP), or TBP-associated factors *in vitro* (Osada et al. 1997), as was shown for many transcription factors which act as repressors including TEF-1 (Jiang and Eberhardt, 1995).

3.10.1. 'Supershift' assays to determine PSFs are NF-1

In an attempt to examine whether the PSFs are being recognized by NF-1, commercially available NF-1 antibodies were obtained and used in a "supershift" assay with PSF elements and nuclear extracts from GC and HeLa cells. This was unsuccessful. However, during a subsequent effort to obtain an alternative source of NF-1 antibodies, we became aware that several investigators were unable to get the NF-1 antibodies from Santa Cruz to work in a "supershift" assay (personal communication of Dr. Naoke Tanese, NYU Medical Center, U.S.A. to Dr. Peter A. Cattini). A supershift of complexes formed on PSF-B sequences with a GC nuclear protein was observed when NF-1 antibodies, provided by Dr. Tanese, to the C-terminal half of NF-1 were used. The result of this experiment performed by Ms. Y. Jin is shown in Fig. 3.32.

Fig. 3.32. NF-1 specific antibodies supershift protein bound to PSF-B3 element. Radiolabelled PSF-B3 DNA probe (250 pg; 1×10^4 cpm) was incubated with 2 μ g of poly (dI-dC) in the presence (a) of pituitary GC cell nuclear extract, with (b) NF-1 specific antiserum, or (c) normal rabbit serum (NRS). "Supershifted" bands detected with NF-1 antibodies after gel electrophoresis and autoradiography are indicated with an arrowhead (This experiment was done by Ms Jin).



It will be interesting to see whether purified NF-1 proteins can shift or protect the PSF-A and PSF-B regions, which would indicate PSFs are NF-1/NF-1-like. Furthermore, a 54 kDa protein observed in southwestern (Fig. 3.17), enrichment of 50-70 kDa proteins in partial purification (Figs. 3.7, 3.10, 3.12) and formation of a prominent complex with proteins (50-70 kDa) eluted from a protein gel (Fig. 3.13A, B) are consistent with properties of NF-1/NF-1-like factors. Perhaps more important than these molecular sizes, the presence of a NF-1 binding consensus sequence in the PSF elements indicates that NF-1/NF-1-like factor is the most probable candidate for PSFs. Interestingly, results from gel shift analysis of proteins eluted from SDS-PAGE gel pieces (Figs. 3.13A, B) and the crosslinking experiments (Fig. 3.16) also support this suggestion as a large complex (>142.0 kDa) on the PSF-B DNA was seen with GC nuclear extracts and other investigators have reported the presence of high molecular weight proteins (120-160 kDa) in purified NF-1 fractions (Diffley and Stillman, 1986; Jones et al. 1987). In this context it is interesting to note a few cited examples, where NF-1 has been shown to act as a repressor.

3.10.2. Repressor activity associated with NF-1

3.10.2.1. In pituitary

Interestingly, the rat GH-N promoter (proximal -237 bp) is active not only pituitary cells, but also in fibroblasts and kidney cell lines (Larsen et al 1986). These proximal sequences contain GHF-1/Pit-1 sites. However, further analysis of upstream sequences led to the identification of non-pituitary cell-type repression elements between -237 and -554 bp of the rat GH-N gene (Larsen et al 1986). Furthermore, these sequences were shown to be responsible for one of the two mechanisms for the extinction of rat GH-N gene expression in hybrid cells derived from the fusion of rat pituitary GH₁ and mouse fibroblast L cells (Tripputi et al. 1988). It was suggested that a protein or proteins binding to these two similar sequences near nucleotide positions -300 and -500 were responsible for this mechanism (Tripputi et al. 1988). Characterization of these sequences led to a strong

indication that NF-1 like nuclear proteins may recognize these sequences as these sequences bear very high sequence homology to the NF-1 consensus (Roy et al. 1992; Guerin et al. 1992). However, crosslinking studies with one of the silencer regions, silencer-1 (-327/-290 bp) revealed that the protein was 45 kDa and, thus, not a size associated with NF-1 (Roy et al. 1992). Further analysis of their data reveals these authors mistakenly did not subtract the molecular size of the probe from the crosslinked protein complex. This could be important since it was also reported by some investigators that variants of NF-1 of molecular size 30.0 (rat liver, Paonessa et al. 1988), 36.0 (porcine, Meisterernst et al. 1989) and 36.8 to 29.8 kDa (Chicken liver, Rupp and Sippel, 1987) are also present.

Recently, it was reported that the PSF-A and PSF-B elements and rat GH silencer-1 (-327/-290 bp) bear similarities to NF-1-like binding sites (Leclerc et al. 1997). Furthermore, rat GH silencer-1 was shown to bind NF-1-like factor suggesting that PSF elements may also bind the same (or a very similar) combination of nuclear protein *in vitro* (Leclerc et al. 1997).

Although it was reported earlier that the repressor activity of the rat GH silencer-1 region was specific to non-pituitary cells (Larsen et al 1986; Tripputi et al. 1988), later studies with this silencer element alone revealed that it was also a repressor in pituitary cells (Roy et al. 1992). The same upstream region of the rat GH gene was also identified as a repressor element in pituitary cells by other investigators (Pan et al. 1990).

Previous studies in transgenic mice showed that 15 kb of the 5'-flanking DNA of the GHF-1/Pit-1 gene is necessary to target expression to the anterior pituitary gland (Lew et al. 1993; Rhodes et al. 1993). Analysis of the 15 kb upstream regions of the human GHF-1/Pit-1 promoter revealed two regulatory, proximal (-7.1/-2.3) and distal (-11.8/-10.9), enhancer regions (Rajas et al. 1998). Interestingly, the distal region was also found to possess silencer activity and two NF-1 binding sites in that region were shown to be responsible for this repressor activity in pituitary cells (Rajas et al. 1998). Both NF-1 sites

had variations from the NF-1 consensus sequences. One of the NF-1 sites was TTGGC(N₆)GtCAA and the other was TTtGt(N₆)GCCAt, where the lower case letter indicates variations (Rajas et al. 1998).

However, in contrast to the above observations of repressor activity associated with NF-1 in the pituitary, a NF-1 consensus sequence in the proximal promoter sequences of all hGH/CS genes does not appear to act as a repressor (Fig. 3.33, Courtois et al. 1990). NF-1 binding activity in this region was determined by gel mobility shift, footprinting and methylation interference assays with both GC and HeLa nuclear extracts. This NF-1 site could also support transcription in cell-free transcription assays (Courtois et al. 1990).

Fig.3.33. Location of the NF-1 core sequence upstream of the GH/CS genes. Precise location of the NF-1 element (underlined) in the GH/CS genes is indicated by the nucleotide positions upstream of the transcription start site for the individual gene. The numbers in parenthesis represent the nucleotide positions according to the GH/CS gene sequence published by Chen et al (1989).

GH-N	ACCATGGCCTGCGGCCAGAGGGC	-289/-267 (4874/4896)
CS-L	T.....	-290/-268 (12452/12474)
CS-A	.A.....A.....	-291/-269 (27118/27140)
GH-V	T.....	-289/-267 (41791/41813)
CS-B	T.....	-291/-269 (49942/49964)

Similarly, the GH-N gene promoter sequences from nucleotide position -496 to -180 containing the NF-1 element were shown to be necessary for GH-N promoter activity in pituitary cells in transient transfection (Cattini et al. 1986). However, a region -496/-164 containing the NF-1 sequences of the CS-A promoter was shown to possess repressor activity (Nachtigal et al. 1989). Thus, further experiments are needed to establish a differential role for NF-1 in the proximal promoter sequences of the hGH/CS genes.

3.10.2.2. Extra pituitary

Recently, a naturally occurring truncated isoform of human NF-1B, NF-1B3, was identified which forms heterodimers with other members of the NF-1 family of transcription factors, and reduces the affinity of these factors to bind DNA, thereby repressing transcription (Liu et al. 1997). The abundance of NF-1B transcripts varied significantly between different human cell lines and tissues, suggesting a potential involvement of these factors in the complex mechanisms that generate cell type specificity (Liu et al. 1997). Furthermore, it was reported that histone H1 isoforms can also bind non-specifically to the NF-1 recognition sequences and bring a generalized transcriptional repression (Gao et al. 1998). NF-1 was also suggested to be involved in the expression of the human alpha-globin gene. An examination of organization of the promoter region of the alpha-globin gene *in vivo* in two established cell lines, cervical carcinoma HeLa and erythroleukemic K562, which express alpha-globin differentially, revealed differences in NF-1 binding (Rein et al. 1995). Although both cell lines express NF-1, in HeLa cells, in which alpha-globin is repressed, the NF-1 site of the alpha-globin promoter appeared occupied, whereas, the same region was free of proteins in K562 cells (Rein et al. 1995). Therefore, it suggests that distinct forms of NF-1 may repress the alpha-globin transcription in HeLa cells (Rein et al. 1995). NF-1 also acts as a silencer of the cartilage matrix protein (CMP) gene promoter in mesenchymal cells (Szabo et al. 1995) and is a repressor of the HBV (hepatitis B virus) enhancer (Spandau and Lee, 1992). Reifel-Miller et al. (1991 and 1994) reported binding of a NF-1-like transcription factor, BK virus enhancer factor-1 (BEF-1), to an ubiquitous repressor binding site. This site has core similarity (5'-TGaNNNNNNAGCCA-3' on one strand and 5'-TGGNNNNNNNGCCCA-3' on the other strand) with the NF-1 consensus sequence, and the complexes formed on the BEF-1 site could be competed by an NF-1 binding site with very high affinity (Reifel-Miller et al. 1991).

In view of these cited examples, NF-1's role as a repressor seems to be real and a case to confirm a role for NF-1 in the regulation of expression of hGH/CS gene locus can

be made.

3.10.2.3. The PSFs could also be recognized by other factors

The above attributes of NF-1 and the preliminary physical characterization of PSFs and their reported repressor activity, strongly suggest that PSFs may belong to the NF-1 family of transcription factors. However, the possibility of PSFs being a different type of protein can not be ruled out. The sequence, TGGCAG in the PSF-B binding site is present in urokinase plasminogen activator (uPA) as TG(A/G)CAG (Fig. 3.34) and was shown to be recognized by a factor known as uPA enhancer factor 3/nuclear inhibitory protein (UEF3/NIP; Berthelsen et al. 1996). Attempt to purify this protein from HeLa cells (10^{11} cells = 4556 mg of nuclear extract) yielded 20 µg of protein with 5000 times purification and was found to a very low abundance factor (Berthelsen et al. 1996). This is similar to the yields we obtained with PSFs as well as what was obtained with NF-kB factors (Hansen et al. 1994). In our attempts to purify PSFs, similar folds of purification after affinity column chromatography were predicted. The molecular sizes of the UEF3/NIP were 64.0, 50.0 and 40.0 KDa (Berthelsen et al. 1996) and similar sizes were seen with PSFs purification. Tryptic digestion of the 64.0 kDa protein yielded a 17 amino acid sequence which was novel (Berthelsen et al. 1996). The NIP- element (TGGCAG) was also recognized by purified UEF3. The NIP element binds an unknown factor which acts as an AP-1 dependent repressor of a transcription factor in the IL-3 promoter (Mathey-Prevot et al. 1990). The UEF3 binding site has homology to the ICK-1 element (Fig. 3.34) of human LD-78 gene promoter (Nomiya et al. 1993) and its murine counter part, MIP-1A gene (Ritter et al. 1995).

Fig. 3.34. Comparison of sequences of NIP/UEF-3, ICK-1 and PSF-B binding sites.

NIP/UEF-3	AGCATG (G/A) CAGCA
ICK-1	AGCATG (A) CAGCA
PSF-B	gagATG (G) CAGgg

The identity of the factors that recognize the PSF elements can only be verified, once the PSFs are fully characterized.

CHAPTER 4

4.1. ROLE OF CHORIONIC SOMATOMAMMOTROPIN GENE ENHANCER SEQUENCES IN THE PLACENTA-SPECIFIC EXPRESSION OF GH/CS GENES

Placenta is an unique organ, by being the only tissue that normally develops and differentiates in the later part of an adult animal's life. More importantly, the placenta provides an environment for another life (fetus) and is a rich source of many growth factors and hormones. One of the major polypeptide hormones produced in the placenta syncytiotrophoblast is chorionic somatomammotropin (CS). While there has been a considerable advance in our knowledge of the molecular mechanisms involved in pituitary-specific expression of the GH-N gene, this is not the case for primate- and placenta-specific placental GH genes.

4.1.1. Ontogeny of CS in placenta

CS was first defined as placental lactogen which is present in human placenta extracts and the maternal circulation. More importantly it possessed immunocrossreactivity with pituitary GH-N (Josimovich and MacLaren, 1962). The presence of CS protein in the syncytiotrophoblast can be detected as early as 5-10 days after implantation (reviewed by Walker et al. 1991). The prerequisite for efficient expression of the CS gene in placenta is the formation of fully differentiated syncytiotrophoblast (Hoshina et al. 1982). Interestingly, it was observed that the amount of CS mRNA in a syncytiotrophoblastic cell remains constant during the entire period of pregnancy, and the increased amount of total CS mRNA during pregnancy could be attributed to the increase in the number of syncytiotrophoblasts (Braunstein et al. 1980; Hoshina et al. 1982; Pierce and Midgley, 1963 as in reviewed by Walker et al. 1991). During the third trimester, the CS protein levels reach 10-20% of total placental protein and as much as 1-3 g/day is synthesized. Thus, CS can be considered a polypeptide hormone produced in the highest amounts in the

body (Kaplan et al. 1968). Concomitantly, CS mRNA accounts for 5-10% of the total RNA in the placenta (Barrera-Saldana et al. 1982; Seeburg et al. 1977). The CS protein is rapidly cleared from the maternal circulation immediately after delivery (reviewed by Walker et al. 1991).

4.1.2. Variations in the expression of GH-V and CS genes in placenta

The above observations were drawn by collectively detecting the CS-A and CS-B proteins and CS/GH-V mRNAs. However, the placental GHs are expressed in a differential pattern. Although, the CS-A and CS-B mRNAs can be identified separately by their gene-specific oligonucleotide probes, they essentially encode the same CS protein in the syncytiotrophoblast of placenta (Barrera-Saldana et al. 1983; Hoshina et al. 1982). The CS-A/-B account for the major part of total GH-V/CS gene and protein expression in the placenta. The GH-V mRNA was suggested to be 0.001 to 0.06% (Chen et al. 1989; Cooke et al. 1988), whereas the CS-L mRNA was 0.01% of the total placental mRNA (Chen et al. 1989). Initially, the GH-V (Seeburg, 1982) and CS-L (Hirt et al. 1987) genes were considered as pseudogenes. However, several studies confirmed the presence of GH-V specific protein in the placenta and maternal circulation (Alsat et al 1997, 1998; Frankenne et al. 1987, 1990; Hennen et al. 1985; Jara et al. 1989; Liebhaber et al. 1989). Although CS-L specific transcripts were detected in the placenta as well as in a placental cDNA library, further analysis revealed these CS-L transcripts were incomplete due to defective splicing (Chen et al. 1989; Hirt et al. 1987; Misra-Press et al. 1994). It is still not clear whether CS-L produces a functional protein. A possible method to increase our understanding of the differential expression pattern is to unravel the transcriptional regulatory mechanisms associated with these genes.

4.1.3. Regulation of expression of placental growth hormones in the placenta

4.1.3.1. Possible role of an enhancer

- Much is known about the transcriptional regulation of pituitary GH-N gene. The striking similarity among the genes, CS, GH-V and GH-N, with 93-95% homology in their primary transcripts and proximal promoter sequences (Chen et al. 1989), suggests that very similar mechanisms should operate both in the pituitary and the placenta regulating the expression of these genes. However, until recently it was strongly believed that GHF-1/Pit-1 was not expressed in the placenta (Bodner and Karin, 1987). Furthermore, unlike in the pituitary cells, the proximal promoter region (-496/+1) of the GH/CS promoters are weakly active in placental tumor cells in transient transfection assays (Fitzpatrick et al. 1990; results from this thesis), suggesting the importance of other flanking sequences to achieve high levels of CS gene expression and mRNA levels in placental syncytiotrophoblasts.

Indeed, by analysing the flanking sequences of the hGH/CS genes, Rogers et al (1986) identified a transcriptional enhancer in a 3.9 kb 3' flanking region of the CS-B gene. Furthermore, deletion analysis localized the enhancer activity to a 1.0 kb fragment located approximately 2.0 kb downstream of the CS-B gene (Rogers et al. 1986). The functional studies suggested that the CS-B enhancer is not tissue specific, but is more active in human placental cells than pituitary 18-54, SF cells (Rogers et al. 1986). The CS-B enhancer requires the CS promoter region from -142 bp to -129 bp for its stimulatory activity (Fitzpatrick et al. 1990). Based on the sequence analysis, this CS promoter region was shown to contain a Sp1 site, suggesting an interaction between Sp1 and the proteins binding to the CS-B enhancer sequences to exert the activity (Fitzpatrick et al. 1990). Using transient transfections and progressively deleted regions of the 1.0 kb CS-B enhancer, the enhancer activity was further localized to a 138 bp region from nts 101 to 241 (Walker et al. 1990). This enhancer (1- 241 bp of the 1.0 kb fragment) typically like other enhancers, functions independently of orientation and position and is active with both homologous and heterologous promoters (Fitzpatrick et al. 1990; Jacquemin et al. 1994a; b; Jiang and Eberhardt, 1994; Lytras and Cattini, 1994; Rogers et al. 1986; Walker et al.

1990).

4.1.3.2. Transcription Enhancer Factor-1 (TEF-1) recognizes multiple elements in the CS-B enhancer

Walker et al (1990) localized the full CS-B enhancer activity to a 138 bp region, which maps to nts 101-241 of the 1.0 kb fragment (identified by Rogers et al. 1986). Furthermore, the same investigators identified a ubiquitous nuclease protected 22 bp region between nts 115-137 of the 1-241 fragment, which encompasses a TEF-1 motif (TGGAATGTG) located at positions 126-133 (Walker et al. 1990). However, recent reports indicate multiple footprint regions in the 1-241 fragment (Jacquemin et al. 1994a; b; Jiang and Eberhardt, 1994) and analysis of some of these protected sequences suggested interaction of TEF-1 with these sequences. Sequences in the 241 bp CS-B enhancer bear sequence similarity to SV40 enhancers (Jacquemin et al. 1994a, b; Jiang and Eberhardt, 1994). Thus, a TEF-1 like protein was shown to be important for the transcriptional activity of CS-B enhancer (Jacquemin et al. 1994a,b; Jiang and Eberhardt, 1994; Lytras and Cattini, 1994; Walker et al. 1990). TEF-1 is an ubiquitous factor with the exception that it is not present in lymphocytes (Xiao et al. 1987; Davidson et al. 1988). These observations raise the question of how the CS-B enhancer, with multiple TEF-1 sites, could achieve this high stimulatory activity in the placental cells. Recently, TEF-5, a TEF-1-like protein or gene was identified in human placenta or placental cell lines, implicating its possible role in CS enhancer activity (Jacquemin et al. 1997).

4.1.3.3. CS-B enhancer-like sequences also exist in the 3'-flanking sequences of the CS-A and CS-L genes

The five genes of human GH/CS locus are presumed to have arisen from a common ancestor by three duplications and a nonreciprocal cross-over event (Hirt et al. 1987). These events also resulted in duplication of intergenic sequences (Chen et al. 1989). Thus, the sequences responsible for CS-B enhancer activity might also be expected to reside in

similar regions of the other two CS genes (CS-L and CS-A). Indeed, examination of the GH/CS gene sequences revealed the presence of highly homologous sequences at the same relative location, 2.2 kb 3' to the CS-A and CS-L genes (Walker et al. 1990). More importantly, the 22 bp TEF-1 site, which is responsible for CS-B enhancer activity, was intact in both regions associated with the CS-A and CS-L genes, suggesting that these regions also serve as enhancers to their respective genes, CS-A and CS-B. However, these regions were shown to possess very weak to no activity (Rogers et al. 1986; Jacquemin et al. 1994a).

There is increasing evidence that the CS-A gene is expressed as much as 6 times higher than the CS-B gene in term placenta (Barrera-Saldana, 1998; Chen et al. 1989; MacLeod et al. 1992; Martinez-Rodriguez et al. 1997). The CS-L gene, although considered a pseudogene, is expressed at very low levels due to a mutation affecting transcript splicing and subsequent cytoplasmic translocation (Chen et al. 1989; Hirt et al. 1987; Misra-Press et al. 1994). Interestingly, initial studies by Rogers et al (1986) revealed that the highly homologous and CS-B enhancer-like region of CS-A gene possessed no enhancer activity. However, further studies with a 309 bp fragment taken from the 3'-flanking sequences of the CS-A and CS-L genes which bear very high sequence similarity to the CS-B enhancer, showed very weak activity in transfected JEG-3 cells (Jacquemin et al. 1994a). However, it was suggested that the DNA-protein interactions on these CS-A-/CS-L-specific regions were representative of those seen on the CS-B enhancer sequences with HeLa, JEG-3 and placental extracts (Jacquemin et al. 1994a). Thus, no clear explanation was given for the weaker activity of the CS-A and CS-L enhancer-like regions. Furthermore, this weak activity does not correlate with *in vivo* expression levels of CS-A and CS-B, where CS-A mRNA levels are more than CS-B mRNA levels (Barrera-Saldana, 1998; Chen et al. 1989; MacLeod et al. 1992; Martinez-Rodriguez et al. 1997).

The relative strengths of the enhancer-like regions of CS-A, CS-L and CS-B genes were deduced from functional studies by transient transfections using human placental cell lines including choriocarcinoma JEG-3, JAR and BeWo cells. However, these placental

cell lines may not provide a “good” physiological and transcriptional environment needed for GH-V/CS gene expression, as it was noted that these cell lines either express very low levels or no endogenous CS (Jimenez et al. 1993; Nickel and Cattini, 1991; Nickel et al. 1993; Quinn et al. 1996; Yamashita et al. 1987). However, these cell lines have retained all the necessary transcriptional machinery for CS-B enhancer activity as was revealed by the transient transfection studies from various laboratories (Fitzpatrick et al. 1990; Jacquemin et al. 1994a; b; 1996a; b; 1997; Jiang and Eberhardt, 1994; 1995; 1996; Jiang et al. 1995; 1997; Lytras and Cattini, 1994; Lytras et al. 1996; Rogers et al. 1986; Walker et al. 1990). One possible explanation for this discrepancy in the expression of these genes could be the stages of differentiation represented by each of these placental cells lines. As noted earlier, a fully differentiated syncytiotrophoblast is required for efficient CS gene expression (Hoshina et al. 1982). The factors associated with enhancer sequences (such as TEF-1) might not be dependent on the developmental status of the placental trophoblast. Recently it was reported that the human placenta and placental cell lines express TEF-1, but there was no correlation with the CS gene expression (Quinn et al. 1996). Thus, this does not explain why the CS enhancers possess differential activities.

To address this problem, Jacquemin et al (1996b) used primary cultures of trophoblasts generated from term placenta. These studies revealed the presence of enhancer activity associated with the CS-A and CS-L gene 3'-flanking sequences (309 bp). Also, the activity of CS enhancers was shown to be dependent on the differentiation of the cytotrophoblast to syncytiotrophoblast. However, the CS-B enhancer still possessed stronger activity than the CS-A and CS-L enhancer-like sequences (Jacquemin et al. 1996b). The contradictory nature between *in vivo* and *in vitro* observations might be due to 1) insufficient sequences used in the transient transfection studies, 2) an inability of transient transfection experiments to reflect the *in vivo* situation, or 3) the involvement of factors other than TEF-1 or TEF like DNA-binding proteins in CS enhancer activity. It is a well known phenomenon that the transcriptional regulation at the chromatin level *in vivo* is very different from that which occurs with naked DNA, as would be the case following transient transfections (Kornberg and Lorch, 1995). It was also reported that the

transcriptional activity of promoters and regulatory sequences fused to a reporter gene measured *in vitro* by transient transfection, may not reflect the activity of the promoter-regulatory sequences-reporter construct *in vivo* (Remmen et al. 1998). However, species-dependence and site of integration may also play a role in the regulation of a transgene. Similar observations were also made when a 15 kb genomic fragment of the human CS-A gene including 5.4 kb of 5'- and 7.2 of kb 3'-flanking sequences, was used for generating a transgenic mouse. This transgene contains both the PSF and the enhancer-like sequences present in the CS-A flanking DNA. This resulted in variable levels of expression of the CS-A gene in the mouse placenta (Jones et al. 1995). However, the anatomical differences between primate and murine placentas might play a role in this variable expression.

4.1.3.4. Upstream sequences of the 22 bp TEF-1 element are responsible for the differential activities associated with the CS enhancers

The above observations raise the possibility that flanking sequences around the 22 bp TEF-1 element may play a role in modulating the CS-B enhancer as well as CS-A and CS-L enhancer-like regions. Hence, the role of upstream flanking sequences of the TEF-1 site in the 241 bp fragment of the CS-B enhancer were examined. Initially, based on the sequence similarity to PSF sequences, Lytras and Cattini (1994) identified RF-1 and DF-1 elements. Subsequent studies revealed another region, AF-1 (Lytras et al. 1996), which was contained in a modulatory domain of about 81 bp located upstream of the TEF-1 site. Furthermore, a homology comparison of the 241 bp CS-B enhancer region with the equivalent region of the CS-A sequences revealed the existence of three nucleotide differences in the 81 bp regulatory region mentioned earlier. One nucleotide change was in the DF-1 region and the other two were in the AF-1 region (Lytras et al. 1996). Therefore, it was hypothesized that these sequence differences were responsible for the very minor enhancer activity of the CS-A 241 bp region in placental cells compared to the potent CS-B enhancer. Initially, the variant DF-1 region of the CS-A gene was mutated to the DF-1 sequence in CS-B (Lytras and Cattini, 1994). The "repaired" CS-A enhancer produced a 70 fold stimulation over the "wild" type CS-A enhancer (Lytras et al. 1996). However, this

enhancer activity was still less than the CS-B enhancer activity. Thus, the remaining two different nucleotides in the variant AF-1 region of the CS-A enhancer were repaired to resemble the AF-1 site on the CS-B enhancer. This resulted in a stimulation by the CS-A enhancer region which was equivalent to the CS-B enhancer in placental cells. These results suggested that the 81 bp modulatory domain with RF-1, AF-1 and DF-1 are important for the potent CS enhancer activity. Previously, the structural and functional activities of the RF-1 and DF-1 regions of the 81 bp regulatory domain of the CS-B enhancer were characterized in our laboratory (Lytras and Cattini, 1994), but the identity of the RF-1 and DF-1 factors is still not known. However, according to Jacquemin et al (1994a, b; 1997), the DF-1 element might be recognized by TEF-like factors. Although, the repair of either the AF-1 or the DF-1 element in the CS-A enhancer sequences imparted stimulatory activity to the CS-A enhancer, a full CS-A enhancer activity comparable to the CS-B enhancer activity was observed only when both the elements were repaired (Lytras et al. 1996). However, the modular nature of the AF-1 element as well as the factors recognizing the element are not known. Further work was done to understand the functionality of the AF-1 element.

4.2. Characterization of the AF-1 region of CS-B enhancer

4.2.1. Objective

Repair of the variant AF-1 region in the CS-A enhancer sequence revealed the importance of the AF-1 region with regard to CS-B enhancer activity. A five nucleotide mutation of the AF-1 region resulted in complete loss of stimulatory activity of the CS-B enhancer. However, characterization of this region was not done. The objective here was to determine whether the AF-1 element on its own has any modulatory function.

4.2.2. Experimental design

The hybrid CAT gene, SVp.CAT.TEF, directed by the SV40 promoter and

containing TEF-1 sequences was described previously (Lytras and Cattini, 1994). A 25 bp AF-1 (AF-1 sense 5'-CGGCAATTTCTGCAAATTTGAG-3' and AF-1 antisense 5'-CTCAAATTTGCAGAAATTGCC G-3') and a 21 bp DF-1 (DF-1 sense 5'-GAGATGCCTTTACGGTTTTCT-3' and DF-1 antisense 5'-AGAAAACCGTAAAGGCATCTC-3') element were generated by annealing these oligonucleotides. The AF-1 element was inserted into SVp.CAT.TEF at the blunted *Bam*HI site upstream of the TEF-1 element, to generate SVp.CAT.AF-1.TEF-1. Furthermore, a construct SVp.CAT.AF-1 was generated by inserting the AF-1 element into the blunted *Xba*I of SVp.CAT. The forward orientation of the AF-1 element in these constructs was confirmed by sequencing. A SVp.CAT.AF-1.DF-1 was generated by inserting the DF-1 element into a blunted *Sph*I site downstream of the AF-1 element in SVp.CAT.AF-1. Maintenance of JEG-3 cells, gene transfer assay and reporter gene analysis were described in the Materials and Methods section.

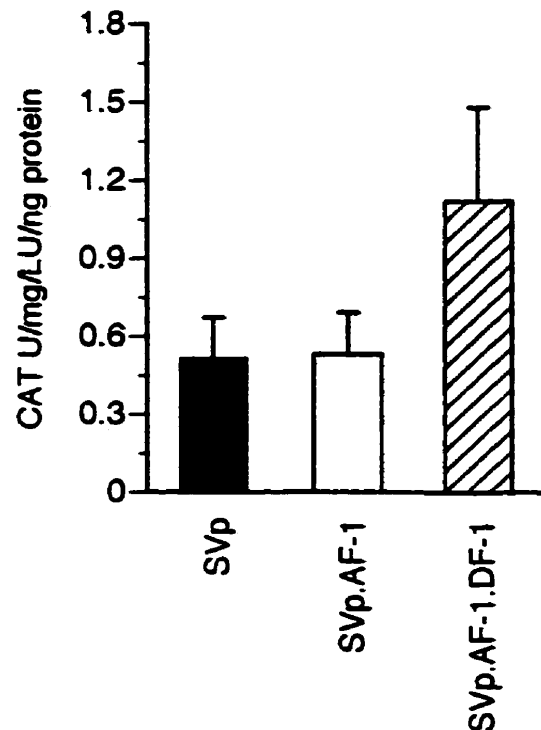
4.2.3. Results and Discussion

4.2.3.1. The AF-1 element alone does not possess any modulatory effect and AF-1/DF-1 together form a functional element

In order to examine the modular nature of the AF-1 region, the constructs SVp.CAT.AF-1 and SVp.CAT were used to transiently transfect JEG-3 cells. A comparison of CAT activity from these constructs revealed that the AF-1 could not stimulate transcription over basal levels seen with the SVp.CAT gene (Fig. 4.1; n=4; p=0.9). Interestingly, no activity was also observed when the DF-1 region was tested in the same manner (Lytras and Cattini, 1994). Furthermore, similarities were also observed between the DF-1 and AF-1 at the structural level. Although DF-1 has a consensus TEF-1 sequence, it was shown that it may be recognizing a different placenta protein as the DF-1/protein complex was not competed by a 35 bp double-strand oligonucleotide containing the 22 bp TEF-1 element (Lytras and Cattini, 1994). Similar observations were also made with the AF-1 element (Lytras et al. 1996). Interestingly, the AF-1/placenta protein complex could be competed by the DF-1 element, although with very low affinity (Lytras et

al. 1996). However, the TEF-1 element could compete for the major DNA protein complex formed on the 81 bp modulatory domain, that includes RF-1, AF-1 and DF-1 (Lytras and Cattini, 1994). This suggests that TEF-1 might bind to these sequences and the individual regions, RF-1, AF-1 and DF-1 might be lacking a complete nucleotide sequence required for TEF-1 binding (Jacquemin et al. 1997). However, the SV40 promoter was significantly activated by more than two fold, when both the AF-1 and DF-1 elements were placed downstream of the CAT gene as in the construct SVp.CAT.AF-1.DF-1 (Fig. 4.1; $n=4$, $p=0.022$). These results show that both AF-1 and DF-1 binding sites together form a functional element.

Fig. 4.1. AF-1 sequences alone do not possess any modulatory effect, but forms a functional element with DF-1 binding site. Approximately 1.0×10^6 JEG-3 cells per 100 mm culture dish were transfected with 15 μ g of either SV40p.CAT (SVp), SV40p.CAT.AF-1 (SVp.AF-1) or SV40p.CAT.AF-1.DF-1 (SVp.AF-1.DF-1) constructs. To determine the transfection efficiency, 250 ng of a luciferase expression vector CMVp.Luc construct was introduced along with each of the above constructs. Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. The presence of AF-1 element alone did not influence the SV40 promoter activity ($n=4$, $p=0.9$), whereas AF-1 and DF-1 together stimulated SV40 promoter ($n=4$, $p=0.022$). CAT units are expressed per milligram of protein and were corrected with luciferase units per ng of protein. Error bars indicate standard error of the mean.

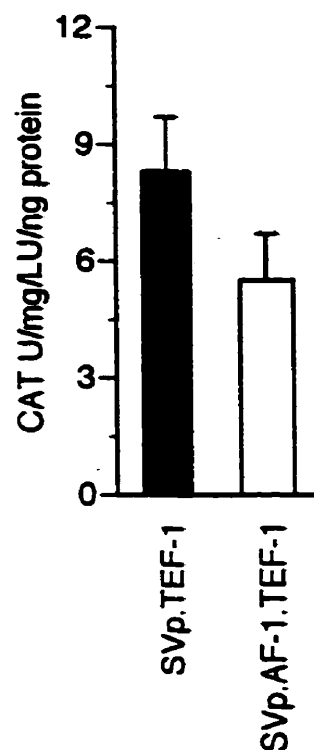


4.2.3.2. AF-1 is a weak repressor and represses the TEF-1 enhancer activity

The 'repair' of the variant AF-1 and the variant DF-1 elements in the 241 bp

sequences of the CS-A enhancer region resulted in a very strong stimulation of enhancer activity in JEG-3 cells (Lytras et al. 1996). Thus, the effect of the AF-1 element on the enhancer activity of the TEF-1 element was determined. The 22 bp TEF-1 alone possesses enhancer activity, although this stimulatory activity was less than the full length 241 bp CS-B enhancer (Lytras and Cattini, 1994). The activities of SVp.CAT.TEF-1 and SVp.CAT.AF-1.TEF-1 were measured after gene transfer into JEG-3 cells. Interestingly, AF-1 had a repressor effect on TEF-1 enhancer activity which was reduced significantly by 35% (Fig.5.2; $n=5$, $p=0.010$). These results suggest that AF-1 is a weak repressor. A similar, 50% repression effect of RF-1 on TEF-1 enhancer activity was also observed (Lytras and Cattini, 1994).

Fig. 4.2. AF-1 sequences downregulate the stimulatory activity associated with 22 bp TEF-1 element. Approximately 1.0×10^6 JEG-3 cells per 100 mm culture dish were transfected with 15 μ g of either SV40p.CAT.TEF-1 (SVp.TEF-1) or SV40p.CAT.AF-1.TEF-1 (SVp.AF-1.TEF-1) constructs. To determine the transfection efficiency, 250 ng of a luciferase expression vector CMVp.Luc construct was introduced along with above constructs. Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. AF-1 sequences downregulated (~35%) the stimulatory activity associated with 22bp TEF-1 element in a statistically significant manner ($n=5$, $p=0.01$). CAT units are expressed per milligram of protein and were corrected with luciferase units per ng of protein. Error bars indicate standard error of the mean.



Although, the influence of DF-1, which is a derepressor, on the TEF-1 enhancer activity was not tested (Lytras and Cattini, 1994), these results suggest that testing individually the effect of RF-1, AF-1 and DF-1 elements might not determine their actual role. However, a 134 bp fragment containing the 81 bp modulatory domain, and thus RF-

1, AF-1 and DF-1 elements retained only 2.4% of the stimulatory activity of the full length CS-B enhancer (Walker et al. 1990). One possible reason for the complete loss of stimulatory activity of the 241 bp CS-B enhancer with an AF-1 mutation (5 nucleotide alteration) (Lytras et al. 1996) could be due to the creation of a binding site in the AF-1 region by mutations themselves. Although a search did not reveal that a known transcription factor can bind this mutated AF-1 site, the AF-1 mutant element was recognized by an unknown factor present in the JEG-3 nuclear extracts (data not shown). Similarly, complete loss of the CS-B enhancer activity was also observed with a mutation of the DF-1 region (5 nucleotide alteration) (Lytras and Cattini, 1994), however, the mutant DF-1 element still possessed similar binding activity to that of wild type DF-1 element, albeit with lower affinity (Lytras and Cattini, 1994). Thus, creation or alteration of the binding activities by these mutations affected CS-B enhancer activity. Further studies revealed that the AF-1 element is recognized by a major 38.0 and a minor 28.0 kDa proteins present in the placental cell nuclear extracts (Lytras et al. 1996).

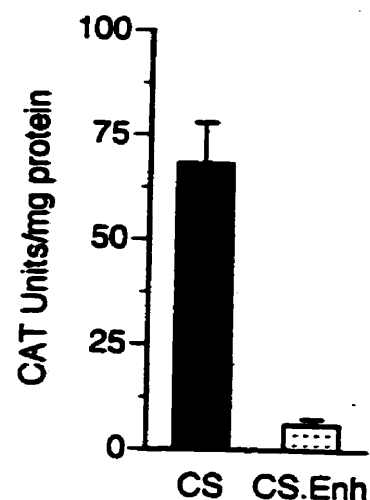
However, the placenta-specific complexes formed on the 81 bp regulatory domain of the CS-B enhancer were competed by the 22 bp TEF-1 element (Lytras and Cattini, 1994), suggesting this region is recognized by TEF-1 or TEF-1-like factors. Thus, combining these observations and results, there is a strong suggestion that the TEF-1 or TEF-1-like factors recognize multiple elements on the CS-B enhancer. This multiple element binding events appear to be responsible for high stimulatory activity of the CS-B enhancer in placental cells. The CS-B enhancer possesses at least six potential TEF-like sites (Jacquemin et al. 1997; Jiang et al. 1997). This could be one of the reasons for the weaker enhancer activity associated with CS-A enhancer-like sequence because this region bears sequence differences with CS-B enhancer region, these differences exist in either TEF-like sites or in the adjacent sequences which were shown to be involved in TEF binding (Jacquemin et al. 1997; Jiang et al. 1997). Interestingly, Jiang et al (1997) suggested that although an enhancer with a mutation in 22 bp TEF element was devoid of stimulatory activity, a construct containing two copies of this mutated enhancer restored enhancer activity in placental cells. This observation provides further evidence that multiple

TEF sites are essential for enhancer activity. However, in nuclease protection assays, similar protein-DNA interactions were observed on CS-A and CS-B enhancer sequences both with the placental and non-placental cell nuclear extracts (Jacquemin et al. 1994a; Jiang and Eberhardt, 1994). This suggests that the enhancer DNA-binding proteins are ubiquitous and further these factors could resist subtle nucleotide differences and still be able to bind enhancer DNA elements. However, these studies do not explain why the *in vitro* enhancer activities do not reflect the *in vivo* expression levels of CS-A (highly expressed) and CS-B (six times less than CS-A) genes in the placenta.

4.3. The CS-B enhancer also possesses repressor activity

Interestingly, in this study, the CS-B enhancer sequences repressed CS promoter activity consistently in rat pituitary GC cells (Fig. 4.3; n=3, p=0.0011; Fig. 4.4; n= 3, p=0.0023). Furthermore, it was observed recently that the CS enhancers form a composite silencer in GC cells, *in vitro* (Jiang and Eberhardt, 1997). Down-regulation of the CS-B enhancer associated stimulatory activity was also observed in JEG-3 cells when the reporter construct was cotransfected with a GHF-1/Pit-1 expression vector (Fig. 3.23B). Paradoxically, TEF-1 is abundant in these cells and has been suggested to be responsible for enhancing or silencing effects of the CS enhancers (Jacquemin et al. 1994a,b; 1996; 1977, Jiang and Eberhardt, 1994; 1995; 1996; Jiang et al. 1995; 1997; Lytras and Cattini, 1994; Lytras et al. 1996; Walker et al. 1990).

Fig. 4.3. The CS-B enhancer is a potent repressor of CS-A promoter activity in rat pituitary GC cells. Approximately 0.5×10^6 GC cells per 100 mm culture dish were transfected with 15 μ g of CS-Ap.CAT (CS) or CS-Ap.CAT.Enh (CS.Enh) constructs. Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. CS-A promoter activity was significantly repressed in the presence of 241 bp CS-B enhancer ($n=3$, $p=0.0011$). The mean value in CAT units per milligram of protein is shown and was derived from at least three determinations. Error bars indicate standard error of the mean. (A DNA fragment containing nucleotides from -496 to +1 of the CS-A gene was used as CS-Ap and a 240 bp fragment was used as an CS-B enhancer).

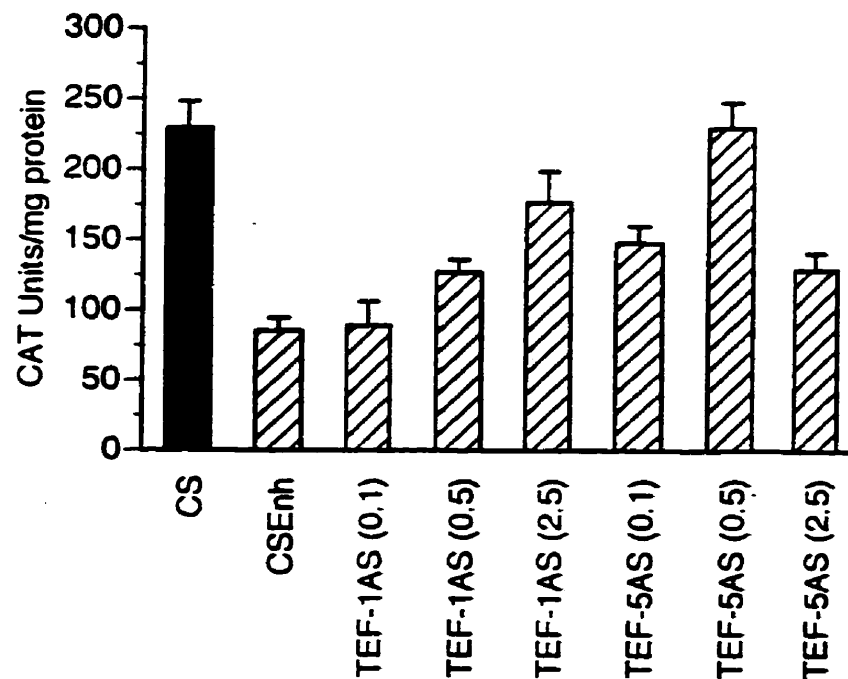


4.3.1. CS-B enhancer associated rat pituitary GC cell specific repression can be relieved by overexpression of antisense TEF-1 or TEF-5

The repression associated with the CS-B enhancer in GC cells *in vitro* suggests that TEFs may play a role in this function. To validate this assumption, antisense expression vectors of TEF-1 and TEF-5 cDNAs were constructed. The TEF-1 cDNA was isolated as an *EcoRI/BglII* fragment and was ligated into *BamHI* and *EcoRI* sites of the pCDNA-3 plasmid resulting in a TEF-1 antisense over expression vector. Similarly, the TEF-5 cDNA isolated as an *EcoRI/XhoI* fragment was initially blunted at *XhoI* site and this fragment was ligated into *BamHI* (blunted) and *EcoRI* sites of pCDNA-3, generating the TEF-5 antisense overexpression vector. These vectors (0.1, 0.5 and 2.5 μ g/100 mm culture dish) were co-transfected with CS.CAT.Enh (10 μ g/100 mm culture dish) into GC cells. Subsequently, CAT gene expression analysis revealed that the antisense TEFs could derepress the CS-B enhancer associated repression in a dose dependent manner (Fig. 4.4; $n=3$, $p<0.05$). A

more pronounced effect was observed when 0.5 μ g of antisense TEF-5 was co-transfected. These preliminary results indicated that TEFs in GC cells participate in a negative regulation of gene expression.

Fig. 4.4. Derepression of the repressor activity associated with the CS-B enhancer by overexpression of antisense TEF-1 and TEF-5 in rat pituitary GC cells. Approximately 0.5×10^6 GC cells per 100 mm culture dish were transfected with 15 μ g of CS-Ap.CAT (CS) or CS-Ap.CAT.Enh (CSEnh) constructs. Along with CS-Ap.CAT.Enh different amounts (0.1, 0.5 and 2.5 μ g) of either TEF-1 or TEF-5 antisense constructs were also transfected into GC cells. Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. The CS-B enhancer-associated pituitary-specific repression was relieved significantly in the presence of excess amounts of TEF-1- or TEF-5-specific antisense RNAs ($n=3$, $p<0.05$). The mean value in CAT units per milligram of protein is shown and was derived from at least three determinations. Error bars indicate standard error of the mean. Amounts of antisense constructs used in transfections are indicated in parenthesis. (A DNA fragment containing nucleotides from -496 to +1 of the CS-A gene was used as CS-Ap and a 240 bp fragment was used as an CS-B enhancer).



However, the CS enhancer was initially characterized as a non-tissue-specific enhancer, because stimulatory activity was also (Rogers et al. 1986) observed in a human pituitary 18-54, SF cell line (Wyche and Noteboom, 1979). The CS-B enhancer also acts positively in monkey kidney COS-1 cells as a strong enhancer (Jiang and Eberhardt, 1995), and in human cervical HeLa cells as a weak enhancer (Jiang and Eberhardt, 1997; Lytras and Cattini, 1994). The SV40 enhancer, with which the CS enhancer shows similarity in possessing multiple GTTIC or sph elements (Jiang and Eberhardt, 1994), also acts as a repressor in rat pituitary GC cells (Nachtigal et al. 1993a). Therefore, these observations explain the differences observed in the activity associated with the CS-B enhancer between the cells of rodent and primate origin. However, these differential enhancer activities could also be due to differential interactions between the TEF-1/TEF-like and certain transcriptional co-factors which act as positive or negative regulators. These co-factors might be species- or cell-specific, as TEF-1 was shown to be highly conserved among several species (Blatt and DePamphilis, 1993; Majumder et al. 1997; Swartz et al. 1998; Kaneko and DePamphilis, 1998). Therefore, characterization of TEF-1 or TEF-1 related factors is likely to prove very useful in our understanding of these variations in transcriptional outcomes.

4.4. Characterization of Transcriptional Enhancer Factor-1 (TEF-1)

As stated earlier, although the CS-B enhancer is very active in placental cells, the possible mechanisms responsible for this are still not clear. Disruption of a TEF-1 site in the CS-B enhancer resulted in a complete loss of activity, thus establishing the involvement of TEF-1 in the CS-B enhancer activity (Walker et al. 1990). TEF-1 protein is also present in placental cells (Jacquemin et al. 1994a,b; 1995; 1996a; b; Jiang and Eberhardt, 1994, 1995, Jiang et al. 1997; Walker et al. 1990). Although the TEF-1 protein was shown to have higher affinity for the elements in CS-B enhancer (Jiang et al. 1997), involvement of a 30 kDa protein, chorionic somatomammotropin enhancer factor-1 (CSEF-1), was suggested to mediate the CS-B enhancer function (Jiang and Eberhardt, 1995). CSEF-1 was reported to be distinct from TEF-1 and present in African monkey kidney COS-1 cells,

and therefore consistent with the high stimulatory activity of the CS-B enhancer in these cells (Jiang and Eberhardt, 1995). Recently, a variant of TEF-1, TEF-5 was also identified (Jacquemin et al. 1996a, 1997). TEF-5 is highly expressed in placenta, raising the possibility of a role for this factor in CS enhancer activity in placenta (Jacquemin et al. 1996a, 1997). CSEF-1 could be another variant of TEF-1, because it also recognizes the TEF-1 element. It was suggested that CSEF-1 may be a proteolytic product of TEF-5 (Jacquemin et al. 1997). Thus, it is possible that variations in the availability of TEF-1, TEF-5 and CSEF-1, might be responsible for modulating the CS-enhancer activity in placental cells.

TEF-1 is the founding member (Davidson et al. 1988; Xiao et al. 1991) of a novel family of transcription factors containing the TEA/ATTS DNA-binding domain (DBD) (Andrianopoulos and Timberlake, 1991; Burglin, 1991). Transcription factors containing the TEA DNA-binding domain were shown to play a very important role in various developmental processes in different species of living organisms including yeast. Examples include, yeast TEC1 (Laloux et al. 1990) for pseudohyphal growth (Gavrias et al. 1996), *Aspergillus nidulans* AbaA (Mirabito et al. 1989) in conidiophore development (Andrianopoulos and Timberlake, 1994), *Drosophila* scalloped (*sd*) in development of wing and central nervous system (Campbell et al. 1992; Inamdar et al. 1993), mouse TEF-1 in heart development (Chen et al. 1994).

In mammals, multiple variants of TEF factors have been reported. At least four such genes, TEF-1, TEF-3, TEF-4 and TEF-5 were isolated (Azakie et al. 1996; Davidson et al. 1988; Hsu et al. 1996; Jacquemin et al. 1996a; 1997; 1998; Kaneko et al. 1997; Xiao et al; 1991; Yasunami et al. 1995, 1996; Yockey et al. 1996). The TEFs are closely related not only in the TEA-DBD, but also in the C-terminal half of the protein. In contrast, the N-terminal region preceding the TEA domain, and the proline-rich region following the TEA domain, are more divergent (Jacquemin et al. 1996a; 1997; 1998). Interestingly, various TEF transcripts of sizes 13.0, 12.0, 8.1, 3.5, 3.2 and 1.7 kb as well as few splice variants (non-DNA binding) were reported to be present in various cells (Jacquemin et al. 1997;

Jiang and Eberhardt, 1997; Quinn et al. 1996; Xiao et al. 1991). This suggests generation of both full length and truncated forms of TEF proteins in cells. CSEF-1 could represent such a form of truncated TEF isoform.

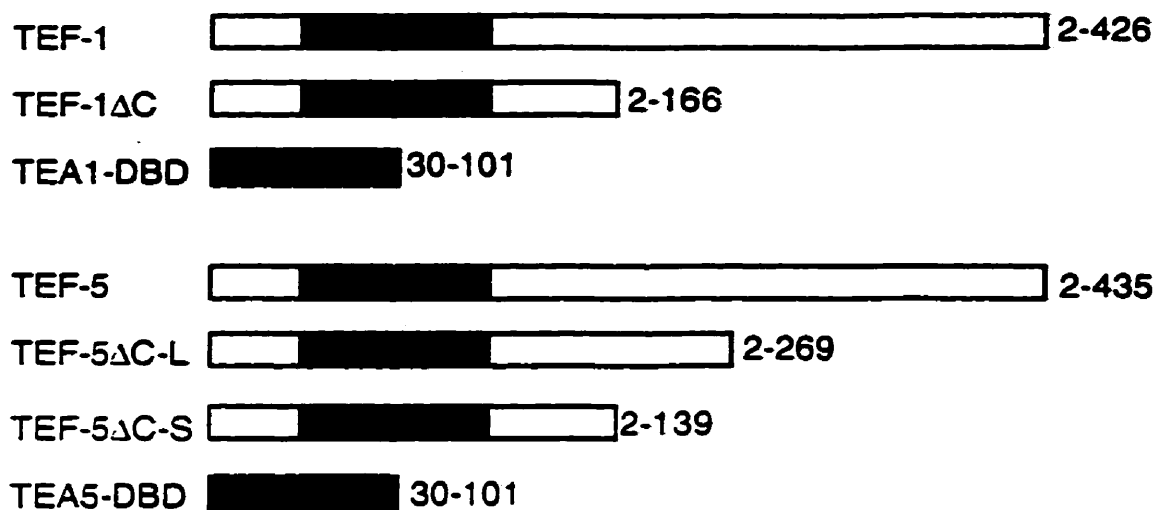
The TEF factors bind to a consensus sequence 5' - (A/T)(A/G)(A/G)NATG(C/T)(G/A)-3' or 5'-(C/T)(G/A)CATN(T/C)(T/C)(T/A)-3' containing a conserved ATG or CAT core sequence. However, other variations in the recognition sequences have been reported (Jiang et al. 1997). TEF-binding sites have been identified in many regulatory sequences apart from SV40 and CS enhancers. The TEF sites are present in cardiac and skeletal muscle genes, such as α - and β -myosin heavy chain, skeletal α -actin and cardiac troponin T and I genes (Farrance et al. 1992; Larkin et al. 1996; Mar and Ordhal, 1990; Shimizu et al. 1993) which are referred to as M-CAT elements. The keratinocyte-specific human papilloma virus-16 E6/E7 enhancer also possesses TEF sites (Davidson et al. 1988; Ishiji et al. 1992). The presence of TEF sites in these tissue-specific genes correlates with the expression of variants of TEFs in numerous tissues, but with some TEFs being tissue-specific (Jacquemin et al 1996a, 1997, 1998; Kaneko et al. 1997; Nothias et al. 1995). TEFs have not, as yet, been detected in lymphoid tissues (Davidson et al. 1988; Xiao et al. 1987).

4.4.1. Objective

TEF-1, TEF-5 or CSEF-1 all recognize the TEF element in the CS-B enhancer and have been linked to the stimulatory activity of the enhancer in placental cells. The presence or absence and variations in the amounts of these proteins in various cells (JEG-3, HeLa and GC) might be responsible for various degrees of activation or repression of the CS-B enhancer seen in these cells in *in vitro* gene transfer assays. It was hypothesized that the CS-B enhancer sequences can function as both an enhancer or a repressor of gene transcription depending on the relative levels of TEF-1 and TEF-5 or the proteolytic products of these proteins such as, CSEF-1. In order to study the possible role of truncated forms of TEFs, TEF-1 Δ C, TEF-5 Δ C (long and short), TEA1 and TEA5 were generated

and bear deletions and encode truncated forms of TEFs (Fig. 4.5).

Fig. 4.5. Different forms (deletions) of TEF-1/TEF-5 proteins. TEF-1 and TEF-5 represent full length proteins. TEF-1 Δ C, TEF-5 Δ C (long and short), TEA1-DBD and TEA5-DBD represent various truncated forms generated from respective full length proteins. Amino acid residues containing these regions or domains are indicated.



4.4.2. Experimental design

The expression vectors, carrying full length cDNAs of TEF-1 (aa 2/426) and TEF-5 (aa 2/435) genes in pJX40 plasmid were kindly supplied by Dr Irwin Davidson (France). The expression vectors TEF-1 Δ C (aa 2/166), TEF-5 Δ C (long) (aa 2/269) and TEF-5 Δ C (short) (aa 2/139) were generated from TEF-1 and TEF-5 vectors (Fig. 4.5). The TEF-1 vector was digested with *Bam*HI and *Bgl*II to release the ~ 780 bp fragment coding the region aa 167/426. The vector with remaining TEF-1 cDNA was isolated and religated to generate TEF-1 Δ C. The TEF-5 Δ C (long) was constructed by digesting the TEF-5 vector with *Bgl*II and *Xho*I which releases a ~ 500 bp fragment keeping 1-808 bp of TEF-5. To

generate TEF-5ΔC (short), the TEF-5 vector was digested with *Pst*I and *Xho*I, releasing a vector with 1-417 bp of TEF-5 sequence. Vectors from both digests were isolated, and the ends were blunted by either trimming or filling reactions using Klenow enzyme. These two vectors were self-ligated to generate the long and short forms of TEF-5ΔC expression vectors.

Two primers for the TEA domain of TEF-1 and TEF-5 were designed to isolate the TEA-DBD of TEF-1 and TEF-5. An initiation codon, ATG sequence to the TEA (forward) primer (5'-gtcgaattcATGGATGC[AG]GA[AG]GG[GC]GT[CG]TGGAGC-3') and a terminator codon, TGA sequence to the TEA (reverse) primer (5'-gtcgaattcTCA[TC]TTC[CT]T[TC]C[TG][GA]GC[AT]AGAACCTG-3') as well as a *Eco*RI site, were added to the primer sequences. The PCR generated TEA1 (aa 30/101) and TEA5 (aa 30/101) sequences encoding their respective TEA-DNA binding domains were cloned into the *Eco*RI site of the pCDNA-3 vector (Invitrogen) to generate expression vectors TEA1 and TEA5 (Fig. 4.5). The hybrid CAT gene reporter construct SVp.CAT (pCAT-promoter plasmid) was obtained from Promega, and generation of SVp.CAT.Enh bearing the 241 bp CS-B enhancer fragment and SVp.CAT.TEF having the 35 bp TEF-1 element were described earlier (Lytras and Cattini, 1994). Generation of CS-Ap.CAT and CS-Ap.CAT.Enh was detailed by Nachtigal et al (1989). TKp.CAT contains the TK promoter corresponding to the region -109 to +53 bp sequences. Cell culture, gene transfer and reporter gene expression analysis were described in the Materials and Methods section.

4.4.3. Results and Discussion

4.4.3.1. Stimulatory activity related to the TEF element or CS-B enhancer sequences is repressed by overexpression of either TEF-1 or TEF-5 in both JEG-3 and HeLa cells

The 241 bp CS-B enhancer or the 22 bp TEF-1 element of the CS-B enhancer are modular in nature and work very well with both homologous and heterologous promoters

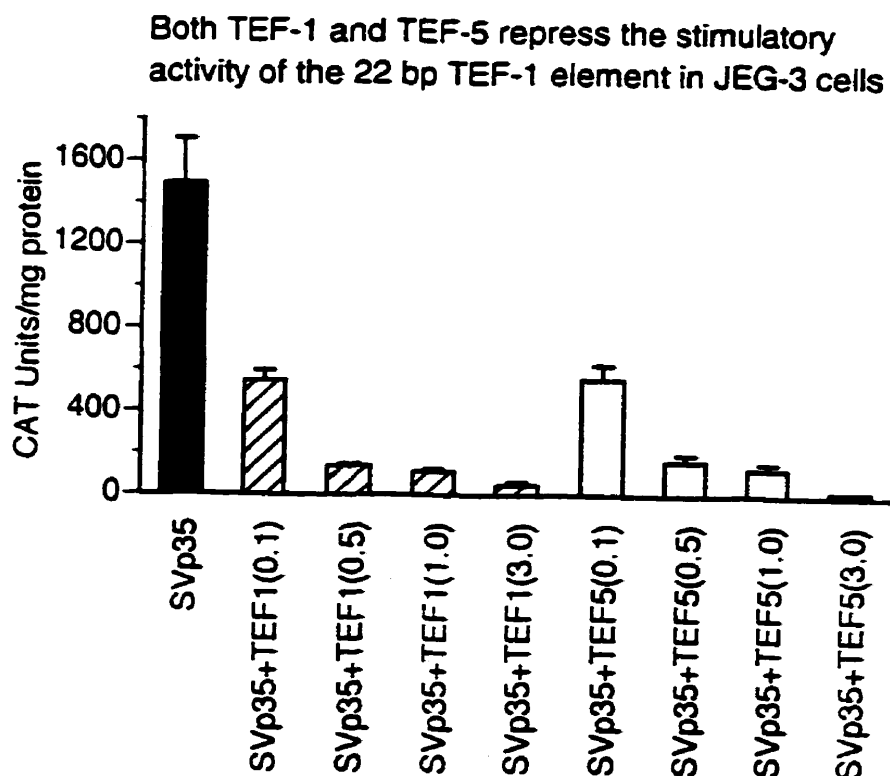
(Jacquemin et al. 1994a; Jiang and Eberhardt, 1994; Lytras and Cattini, 1994; Walker et al. 1990). TEF-1 was isolated from HeLa cells as a transactivator because of its capacity to bind to GTTIC and SphI enhancers of the SV40 enhancer, and its ability to activate *in vitro* transcription (Davidson et al. 1988). TEF-5 is not expressed in HeLa cells, whereas both TEF-1 and TEF-5 are present in placenta and placental cells (Jacquemin et al. 1996a; Quinn et al. 1996; Jiang et al. 1997). Although there was a suggestion of more TEF-5 than TEF-1 protein in the placental cells, the absolute amounts of each protein are not known (personal communication of Dr. Irwin Davidson, Institut de Genetique et de Biologie Moleculaire et Cellulaire, College de France, France to Dr. Peter A. Cattini). It is known that concentration gradients in a particular transcription factor or set of factors is very important for both gene expression and developmental processes in *Drosophila* and *Xenopus* (Drake et al. 1997; Ross et al. 1996; Ryan et al. 1996; Zecca et al. 1995). Therefore, in order to create a concentration gradient, various amounts ranging from 0.1 to 3.0 μg of the TEF-1 and TEF-5 expression vectors were co-transfected with 10 μg of each of the hybrid CAT gene constructs per 100 mm culture dish carrying 1×10^6 (JEG-3) or 0.5×10^6 (HeLa) cells to determine the influence of these proteins on target gene expression.

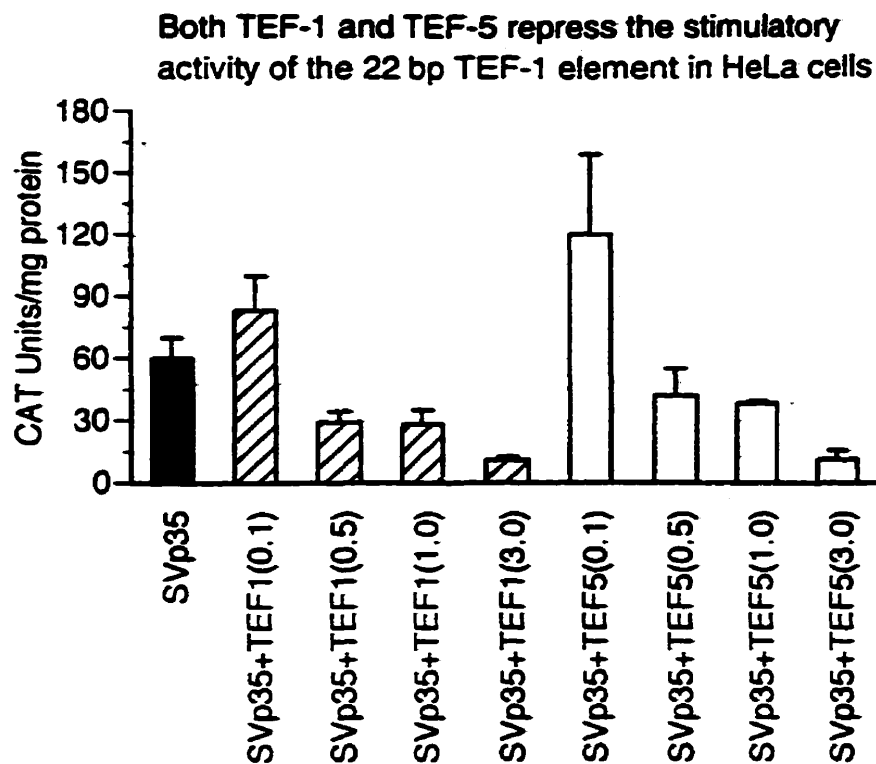
4.4.3.2. Both TEF-1 and TEF-5 repress the 22 bp TEF element associated stimulatory activity in JEG-3 and HeLa cells

Initially, the effect of overexpression of TEF-1 or TEF-5 on the 22 bp TEF element-induced stimulatory activity was determined both in placental JEG-3 and non-placental HeLa cells. Interestingly, in both cells types, the TEF element induced activity was repressed, in a dose dependent manner, by both TEF-1 and TEF-5 (Fig. 4.6A, Fig. 4.6B; $n=3$, $p<0.05$). However, more pronounced repression was observed in JEG-3 cells, whereas, in HeLa cells, at lower doses (0.1 μg) both TEF-1 and TEF-5 had a stimulatory effect. This type of transient stimulation with lower doses of TEF-1 was observed in HeLa cells in a previous study (Hwang et al. 1993). However, at higher doses, both TEF-1 and TEF-5 acted as potent repressors. These results suggest that both TEF-1 and TEF-5 have

similar functional characteristics.

Figs. 4.6 and 4.7. TEF-1 and TEF-5 repress stimulatory activity associated with 22 bp TEF-1 element as well as 240 bp CS-B enhancer both in placental JEG-3 and cervical HeLa cells in a dose-dependent manner. Approximately 0.5×10^6 HeLa or 1.0×10^6 JEG-3 cells per 100 mm culture dish were transfected with 10 μ g of SV40p.CAT.TEF-1 (SVp35) or SV40p.CAT.Enh (SVp240) along with different amounts (0.1, 0.5, 1.0 and 3.0 μ g) of either TEF-1 or TEF-5 constructs. A construct SV40p.CAT was also transfected into these cells as control. Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. Both 22bp TEF-1 element- and 241 bp CS-B enhancer-associated stimulatory activity was repressed by overexpression of either TEF-1 or TEF-5 very significantly and in a dose-dependent manner in both HeLa and JEG-3 cells ($n=3$, $p<0.05$). The mean value in CAT units per milligram of protein is shown and was derived from at least three determinations. Error bars indicate standard error of the mean. Amounts of TEF-1 and TEF-5 constructs used transfections are indicated in parenthesis.

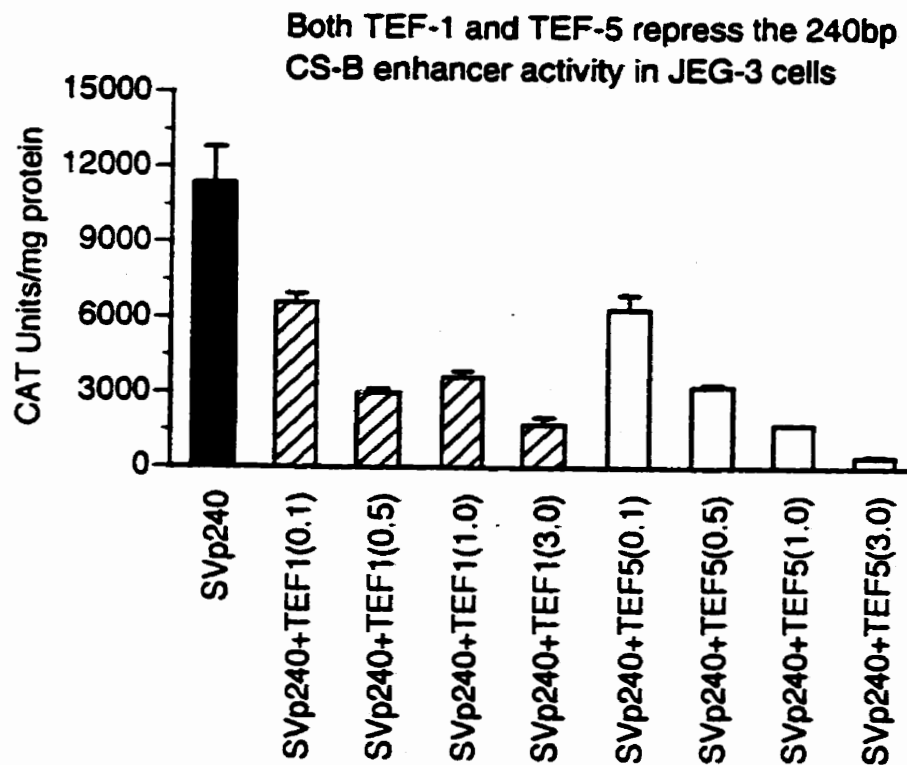


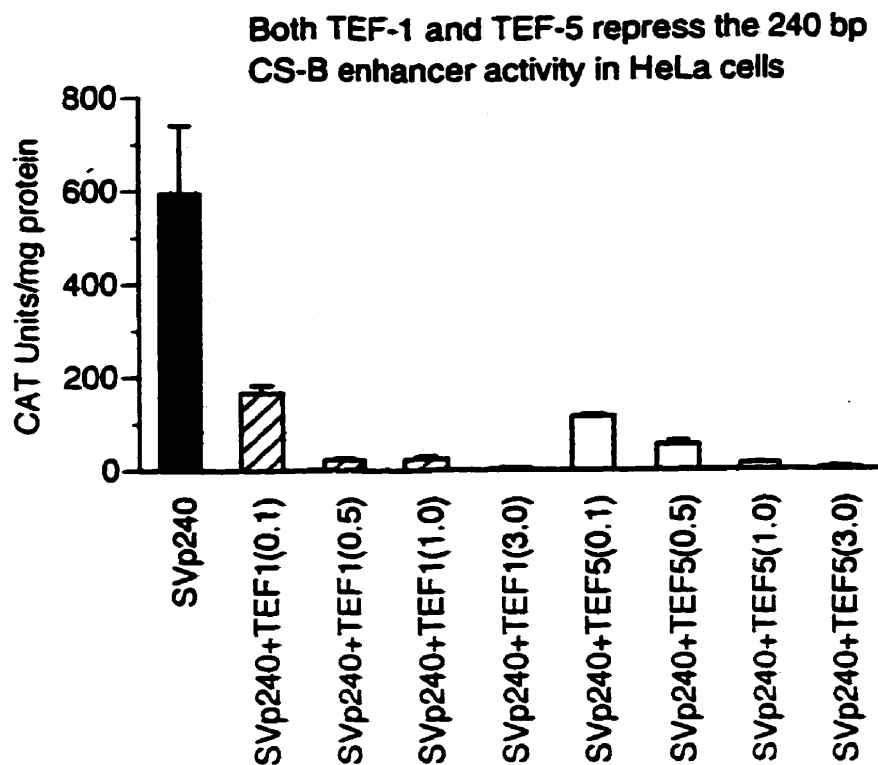


In this study the 22 bp TEF element was used, which is the major determining regulatory sequence for the CS-B enhancer activity (Walker et al. 1990). However, this sequence does not impart the full enhancer activity seen with the 241 bp CS-B enhancer fragment, suggesting the involvement of flanking sequences in the enhancer activity (Jacquemin et al. 1994a; b, 1996b; Jiang and Eberhardt, 1994; 1995; Jiang et al. 1995; 1997; Lytras and Cattini, 1994, Lytras et al. 1996). Indeed, these flanking sequences were also shown to contain multiple TEF elements, but TEF has different binding affinities for each of these TEF elements (Jacquemin et al. 1994a; b; 1996a; b; Jiang and Eberhardt, 1994; 1995; Jiang et al. 1997). Hence, it was possible that these multiple TEF elements, with different affinities for TEFs, might be necessary to reveal differences in enhancer activity related to TEF-1 versus TEF-5 binding.

4.4.3.3. Both TEF-1 and TEF-5 repress the 241 bp CS-B enhancer-associated stimulatory activity in JEG-3 and HeLa cells

Further experiments were performed using constructs containing the 241 bp CS-B enhancer. Co-transfection of TEF-1 and TEF-5 were done essentially as described above. However, from these reporter gene expression assays, TEF-1 and TEF-5 factors again acted as repressors (Fig. 4.7A, Fig. 4.7B; $n=3$, $p<0.05$), which was similar to what was seen with the previous assessment of the 22 bp TEF element. The repression occurred in a dose dependent manner as was also seen in the previous experiment (Fig. 4.6). These results indicated that the presence of multiple TEF elements did not reveal the subtle differences associated with transcriptional regulation by TEF-1 versus TEF-5.





This type of repression by overexpressing an activator of transcription is not unusual. The phenomenon, generally known as 'squenching' was first described by Gill and Ptashne (1988). Many examples of this squenching effect by various activators have been cited (Berger et al. 1990; Natesan et al. 1998; Orphanides et al. 1996). Even in work related to this thesis, overexpression of GHF-1/Pit-1 repressed the stimulatory activity of the CS-B enhancer in placental cells but not in HeLa cells (Figs. 3.23B, 3.27), although GHF-1/Pit-1 stimulated CS promoter activity in the absence of enhancer sequences in both placental JEG-3 and non-placental/non-pituitary HeLa cells (Figs. 3.23A, 3.27, 3.28, Fox et al. 1990; Ingraham et al. 1988). Furthermore, it was shown that TEF-1 down-regulates SV40 enhancer and CS-B enhancer activities after transfection (Xiao et al. 1991; Ishiji et al. 1992; Hwang et al. 1993; Jiang and Eberhardt, 1995; 1996; Takahashi et al. 1995). Although it has been suggested that TEF-5 represses CS-B enhancer activity, no data for , or about the nature of this repression was reported (Jacquemin et al. 1997).

The squelching effect was assumed to be due to titration of one or more general transcription factors (Brou et al. 1993; Ishiji et al. 1992; Hwang et al. 1993; Orphanides et al. 1996; Xiao et al. 1991). However, it was reported that TEF-1 can repress any promoter independent of the presence of its binding site, by titrating the TATA-binding protein (TBP) or TBP-associated factors (Brou et al. 1993; Jiang and Eberhardt, 1995; 1996). It was shown that the C-terminal region of TEF-1 interacts with TBP (Jiang and Eberhardt, 1996). Furthermore, many examples, including Dr1 (Yeung et al. 1994), p53 (Chen et al. 1993; Farmer et al. 1996; Chesnokov et al. 1996; Sang et al. 1997), even skipped (Um et al. 1995; Li and Manley, 1998), glucocorticoid receptor (Meyer et al. 1997), adenovirus E1A (Song et al. 1995; Parker et al. 1997), human papilloma virus E7 (Massimi and Banks, 1997), Msx-1 (Zhang et al. 1996), Kruppel (Sauer et al. 1995), human cytomegalovirus IE2 (Lang and Stamminger, 1994) and human T-cell leukemic virus Tax1 (Caron et al. 1993) were reported to exert transcriptional repression on either homo (in the presence of a binding site) or hetero (in the absence of a binding site) activators by squelching away the TBP and other general transcription factors (GTFs). How these interactions give rise to a negative regulation is still not clear. Recently, Natesan et al (1997) proposed that transcriptional squelching can be overcome by integrating the target genes into cellular chromatin, thus limiting the transrepression to only episomal target genes.

However, it was suggested that CSEF-1 was actually the CS-B enhancer binding factor and was responsible for stimulatory activity, whereas TEF-1 was the general transrepressor (Jiang and Eberhardt, 1995; 1996). Furthermore, it was also observed that CSEF-1 was present even in monkey kidney, COS-1, cells and hence, was able to stimulate CS-B enhancer activity in these cells (Jiang and Eberhardt, 1995). Therefore, given the possibility that CSEF-1 could represent a breakdown product of TEFs (Jacquemin et al. 1997), it was interesting to determine any role for truncated TEF proteins in transcriptional regulation in various cells.

4.5. Role of truncated TEF proteins in transcription

4.5.1. Identification of a HeLa cell-specific activator domain in TEF-1

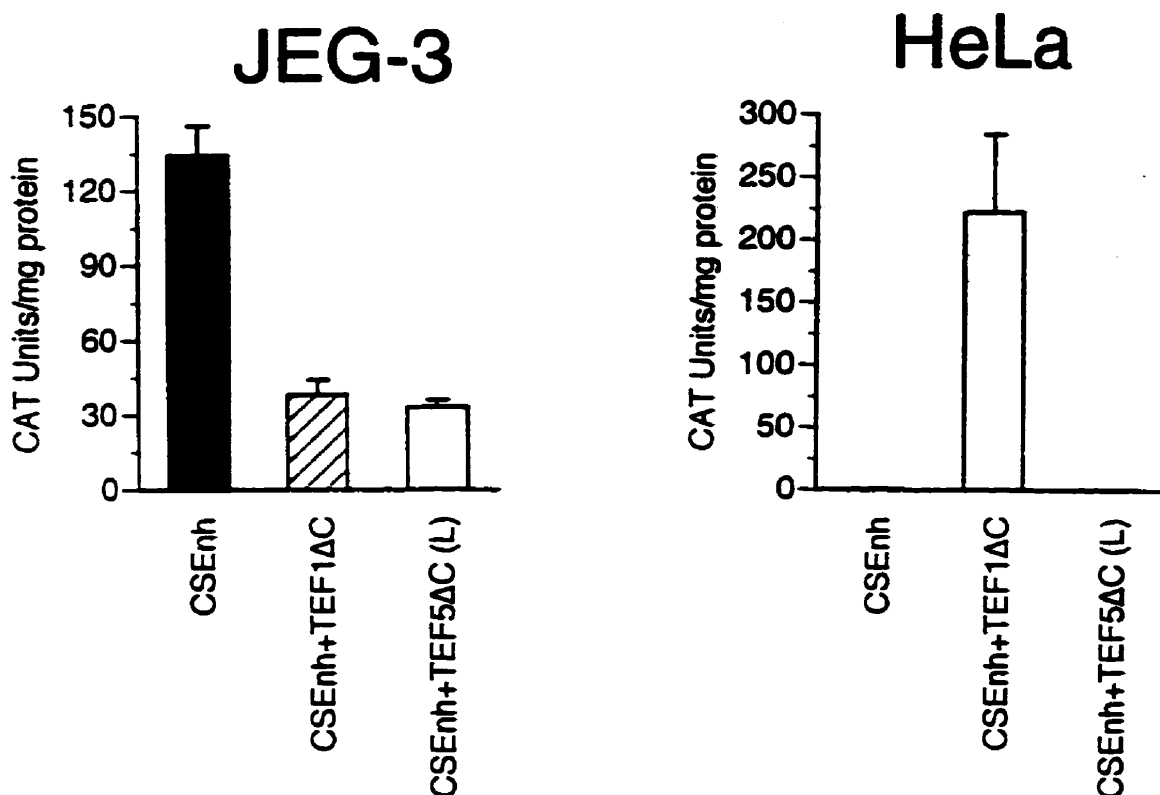
As discussed earlier, TEFs are also present in truncated forms in the cell, but their apparent functional relevance is not known except for the CSEF-1 which is yet to be isolated, characterized and to be confirmed as a TEF related protein. Truncated versions of TEF-1 and TEF-5 cDNAs, including TEF-1 Δ C, TEF-5 Δ C (long), TEF-5 Δ C (short), TEA1 and TEA5 were generated and assessed for function. The influence of these proteins on CS-A promoter with CS-B enhancer sequences was studied by transient transfections in HeLa and JEG-3.

Initially, 2 μ g of either TEF-1 Δ C, TEF-5 Δ C (long) or TEF-5 Δ C (short) expression vector along with 10 μ g of CS-Ap.CAT.Enh per 100 mm culture dish were transiently transfected into JEG-3 and HeLa cells. Analysis of CAT gene expression revealed that the C-terminus deleted TEFs also downregulated the stimulatory activity associated with the CS-B enhancer in JEG-3 cells (Fig. 4.8A; n=3-6, p<0.05). This negative regulation was similar to that seen with full-length TEFs in all cells (Figs. 4.6A, 4.7A). Interestingly, the TEF-1 Δ C protein in HeLa cells positively upregulated the CS-A promoter several fold, suggesting the presence of an activation domain between aa 2 and 166 in TEF-1, which is HeLa cell-specific (Fig. 4.8B, n=3, p<0.05). However, both the TEF-5 Δ C (long) and TEF-5 Δ C (short) did not show similar enhancement to that seen with TEF-1 Δ C (Fig. 4.8B; n=3-6).

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Fig. 4.8. Overexpression of truncated forms of both TEF-1 and TEF-5 repress the stimulatory activity associated with the CS-B enhancer in JEG-3 cells, whereas truncated TEF-1 is a potent activator of CS-A.Enh in HeLa cells. Approximately 1.0×10^6 JEG-3 or 0.5×10^6 HeLa cells per 100 mm culture dish were transfected with 10 μ g of CS-Ap.CAT.Enh (CSEnh) along with 2.0 μ g of either TEF-1 (TEF-1 Δ C) or TEF-5 (TEF-5 Δ C long (L) or short (S)) truncated constructs. In JEG-3 cells, all truncated forms TEF-1 Δ C, TEF-5 Δ C (L) or TEF-5 Δ C (S) significantly downregulated the CS-B enhancer activity (n=3-6, p<0.05). In HeLa cells, only TEF-1 Δ C enhanced the CS-Ap.CAT.Enh activity by several fold (n=3, p<0.05). Forty eight hours

after transfections, cell extracts were prepared and assayed for CAT gene expression. The mean value in CAT units per milligram of protein is shown and was derived from at least three determinations. Error bars indicate standard error of the mean. (Note: The CAT values obtained by overexpression of TEF-5 Δ C (S) were not shown as both TEF-5 Δ C (S) and TEF-5 Δ C (L) showed similar effect in JEG-3 as well as HeLa cells).

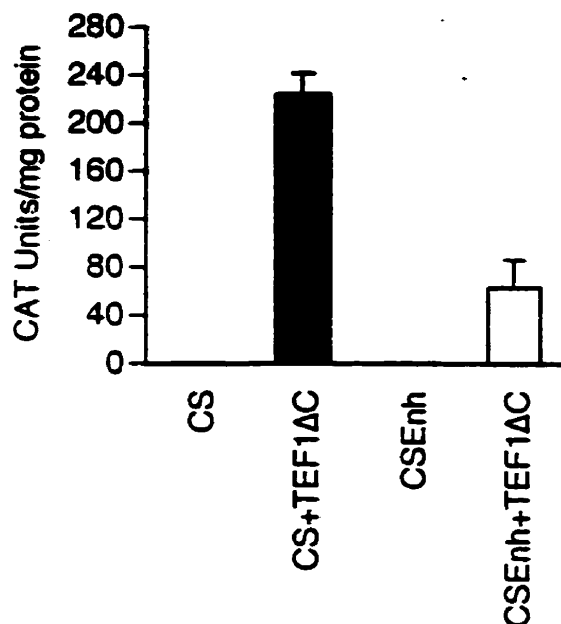


4.5.2. Differential regulation of the CS-A promoter with or without the CS-B enhancer sequences by TEF-1 Δ C in HeLa cells

The above results indicate that the C-terminus deletions of TEF-1 and TEF-5 have differential transactivation functions in HeLa cells. However, to examine whether the TEF-1 Δ C activity is dependent on the presence of TEF binding sites, further transient transfections in HeLa cells were done using CS-A.CAT and CS-A.CAT.Enh constructs along with the TEF-1 Δ C expression vector. Analysis of CAT gene expression revealed an exciting result. The inactive CS promoter was activated by several fold by over expressing

the TEF-1 Δ C (Fig. 4.9). Interestingly, the truncated TEF-1 Δ C stimulated the CS promoter almost 3.5 fold more than that of the CS promoter with enhancer sequences (Fig. 4.9; n=4, p<0.05). These results suggest that the HeLa-specific transactivation associated with the TEF-1 Δ C does not require a TEF binding site. Further experiments to characterize the TEF-1 Δ C were performed using the CS-A.CAT construct.

Fig. 4.9. Transactivation of CS-A promoter by TEF-1 Δ C in HeLa cells does not require TEF-1 (consensus) DNA elements. Approximately 0.5×10^6 HeLa cells per 100 mm culture dish were transfected with 10 μ g of CS-Ap.CAT (CS) or CS-Ap.CAT.Enh (CSEnh) along with 2.0 μ g of TEF-1 Δ C construct. Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. CS-A promoter was activated by several fold upon overexpression of TEF-1 Δ C both in the presence or the absence of CS-B enhancer sequences (n=4, p<0.05). The mean value in CAT units per milligram of protein is shown and was derived from at least four determinations. Error bars indicate standard error of the mean. (A DNA fragment containing nucleotides from -496 to +1 of the CS-A gene was used as CS-Ap and a 240 bp fragment was used as an CS-B enhancer).

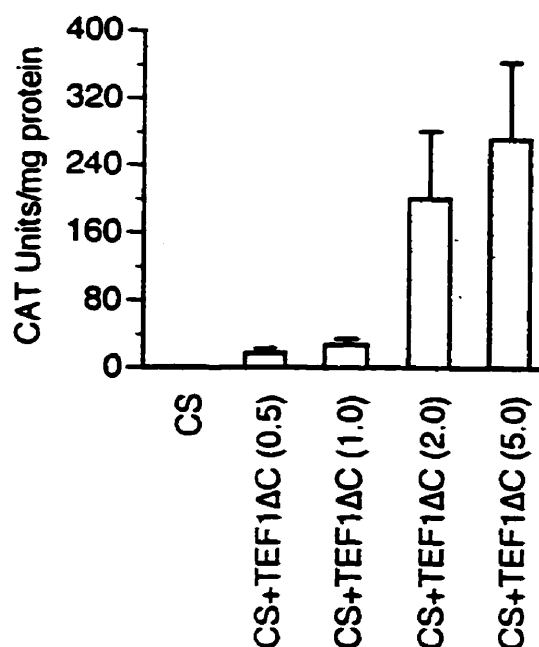


4.5.3. Dose-dependent activation of the CS-A promoter by TEF-1 Δ C in HeLa cells

Activation of the CS-A promoter to such a high level by a truncated TEF-1 protein in HeLa cells is very interesting as this promoter is either not or sometimes very weakly

active in HeLa and placental cells and needs an enhancer to be active in placental cells. In the previous experiment, expression of 2 μg of TEF-1 Δ C resulted in high levels of CS-A promoter activity in HeLa cells, which is uncharacteristic of the CS-A promoter. This prompted an investigation of whether this activation was dose dependent. Transient transfections were repeated in HeLa cells using similar conditions as described above, but using different amounts (0.5, 1.0, 2.0 and 5.0 μg) of the TEF-1 Δ C expression vector for cotransfection. As indicated in Fig. 4.10, the CS promoter activity in HeLa cell was dependent on the amount of TEF-1 Δ C ($n=3$, $p<0.05$). This further confirms the earlier result that a potential transactivation domain exists between aa 2 and 166 in TEF-1, which is HeLa cell-specific. It should also be noted that CS promoter activation was not due to subtle changes in the concentration gradient of the transcription factors assessed, based on the dose dependent activation observed.

Fig. 4.10. Dose-dependent activation of CS-A promoter by TEF-1 Δ C in HeLa cells. Approximately 0.5×10^6 HeLa cells per 100 mm culture dish were transfected with 10 μg of CS-Ap.CAT (CS) along with 0.5, 1.0, 2.0 and 5.0 μg of TEF-1 Δ C construct. Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. The mean value in CAT units per milligram of protein is shown and was derived from at least three determinations. Error bars indicate standard error of the mean. Amounts of TEF-1 Δ C construct used is indicated in parenthesis.



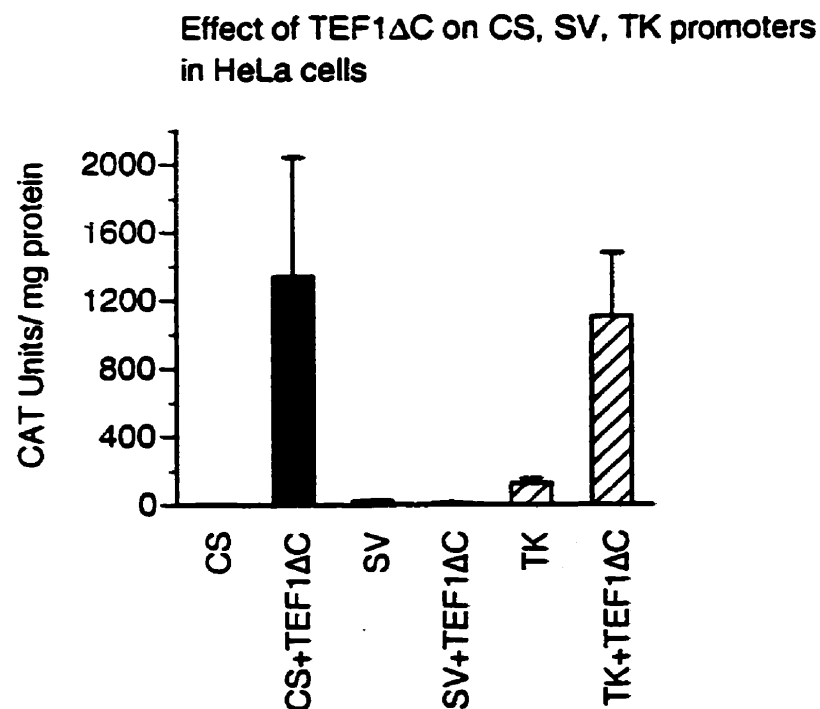
4.5.4. The TEF-1 activation domain from aa 2 to 166 possesses promoter-specificity

This surprising result of activation of the CS promoter in a TEF site independent manner might be due to generation of a non-specific transactivator in the form of TEF-1 Δ C. This positive effect could be due to the influence of truncated TEF-1 (TEF-1 Δ C) on general transcription factors (GTFs). Hence, the influence of the TEF-1 Δ C region on the transcriptional activity of SV40 and TK promoters was tested along with CS promoter in HeLa, JEG-3 and GC cells. Interestingly, only the CS and TK promoters were activated by this region in HeLa cells, whereas a down-regulation of SV40 promoter activity was observed in the presence of TEF-1 Δ C (Fig. 4.11A, n=3-6, p<0.05). However, none of these promoters were activated by TEF-1 Δ C in JEG-3 and GC cells. Indeed, all promoters were downregulated (Figs. 4.11B, C). These results suggest that there is not only a cell-specific, but also a promoter-specific effect of TEF-1 Δ C.

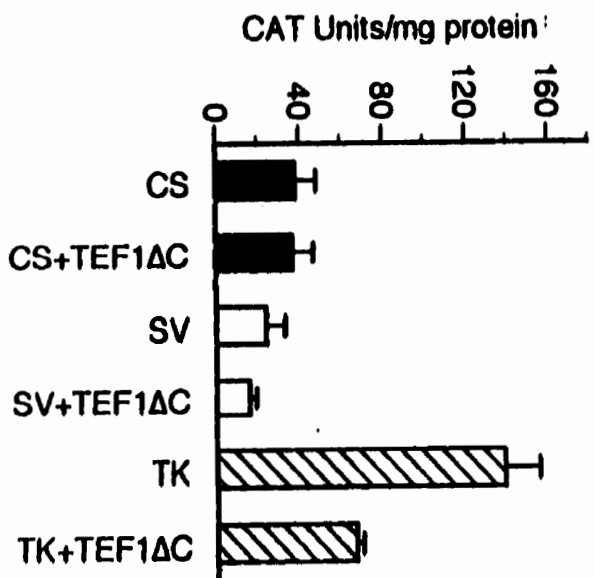
Characterization of the functionality of the different domains of TEF-1 was carried out by Hwang et al (1993). In these experiments, the influence of the different TEF-1 deletion mutants was determined in terms of their ability to interfere with the activity of the endogenous TEF-1 on the stimulated expression of a hybrid CAT gene. This hybrid CAT gene was under the influence of TK promoter with eight TEF-1 binding sites (Hwang et al. 1993). It was concluded that with the exception of the full-length and mutants containing a C-terminal region from aa 402 to 426, none of the other C-terminus deleted mutants interfered with the endogenous TEF-1 stimulatory activity (Hwang et al. 1993). Surprisingly, a mutant with deletion of the TEA-DBD, interfered with endogenous TEF-1 stimulatory activity, suggesting site-specific DNA binding by TEF-1 is not required to influence transcription (Hwang et al. 1993). Our assays using only TEA1 and TEA5 expression vectors, which encode only the TEA-DBDs of TEF-1 and TEF-5, respectively, did not show any influence on the transcription of CS, SV and TK promoters (data not shown). The TEA-DBDs of TEF-1 and TEF-5 have only one amino acid difference at amino acid position 100 and this reflects a conservative change involving lysine to arginine

residues (Jacquemin et al. 1997). However, the TEA-DBD domain was still able to bind to DNA (Hwang et al. 1993). Furthermore, it was also shown that the scalloped (sd) gene whose TEA-DBD differs by only a single amino acid from that of TEF-1, did not efficiently bind to the TEF site *in vitro*, although it was suggested that DNA binding is modulated by other regions of TEF-1 (Hwang et al. 1993). The effect of TEA1 or TEA5 on CS-B enhancer activity has not been studied.

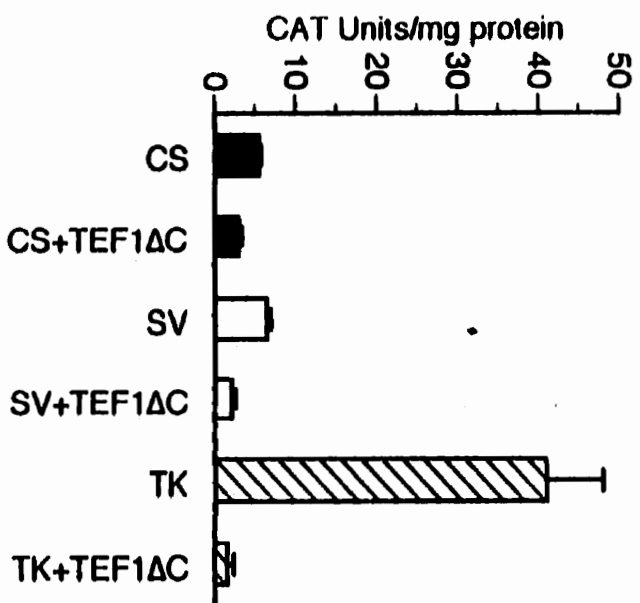
Fig. 4.11. TEF-1 Δ C transactivates CS-A and TK promoters but not SV40 promoter only in HeLa cells. Approximately 0.5×10^6 HeLa or GC and 1.0×10^6 JEG-3 cells per 100 mm culture dish were transfected with 10 μ g of CS-Ap.CAT (CS), SV40p.CAT (SV) or TKp.CAT (TK) along with or without 2.0 μ g of TEF-1 Δ C construct. Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. Both CS-A and TK promoters were transactivated by TEF-1 Δ C ($n=3-6$, $p<0.06$). The mean value in CAT units per milligram of protein is shown and was derived from at least three determinations. Error bars indicate standard error of the mean.



Effect of TEF1ΔC on CS, SV, TK promoters
in GC cells



Effect of TEF1ΔC on CS, SV, TK promoters
in JEG-3 cells



This could be one possible explanation for the difference in transcriptional influence seen between TEF-1 and TEF-5. Although, the TEF-1 and TEF-5 have a highly conserved TEA-DBD, there are amino acid sequence differences in the aa region 1-166 which could be responsible for the HeLa cell-specific and TEF-1-specific activation domain (Fig. 4.12, Jacquemin et al. 1997). Using heterologous DBD fused to different regions of TEF-1, three regions of TEF-1 were shown to contribute to its activation function, but interestingly, none of these regions were shown to function as autonomous activating domains (Hwang et al. 1993). The results of our study show that the region comprising aa 2-166 of TEF-1 can function not only as an autonomous activation domain, but also in a site-independent manner, as well as a cell-specific and probably promoter-specific manner. Recently it was suggested that transactivation function can be cell-specific and this is determined by the availability of cell-specific factors interacting with the transactivation domain (Corton et al. 1996).

The promoter-specificity of TEF-1 Δ C observed in these studies only in HeLa cells could be explained by the possible differential interaction between the basal core promoter factors and activation domains on TEF-1 Δ C. The basal core promoter composition of mammalian genes is highly variable; some contain TATA boxes, some contain initiator (Inr) elements, and others contain both or neither of these basal elements. TATA-containing and Inr-containing core promoters direct transcription initiation by similar mechanisms. They both use the TFIID complex, which contains TBP and TAFs (reviewed by Roeder, 1996). In spite of this similarity, there are striking differences in relative strengths of each type of promoter and their response to different activators (Emami et al. 1995). Furthermore, different activation domains in the activators stimulated the TATA-containing and Inr-containing core promoters differentially, suggesting that the core promoter structure found in a given gene may reflect a preference of the regulators of that gene (Emami et al. 1995). However, the transcription initiation complex has now been shown to exist as a 'holocomplex', containing as many as 42 polypeptides, which include RNA polymerase II, the GTFs, co-activators, negative regulators and chromatin remodelling factors (reviewed by Bjorklund and Kim, 1996). One of the TAFs in *Drosophila*, dTAF_{II}150, recognizes the

Inr element (reviewed by Verrijzer and Tjian, 1996). However, Inr element recognition is facilitated by other DNA-binding proteins, including YY1 (Seto et al. 1991), TFII-I (Roy et al. 1993), and USF (Du et al. 1993), suggesting their possible role in differential modulation of a particular gene core promoter. The proximal elements, including the TATA box and the Inr element located between nucleotide -15/+1 of the CS promoter are necessary for full basal and CS enhancer-associated stimulatory activity (Jiang et al. 1995). Interestingly, this Inr element was shown to be recognized by different factors in a tissue-specific manner (Jiang et al. 1995). A 70 kDa protein was found in placental cells, whereas a minor 70 kDa and two major 30 and 20 kDa proteins were bound to the Inr element in GC cells (Jiang et al. 1995), indicating a differential role for these factors in regulating the CS promoter activity in these two cell types. Although we do not know the nature of HeLa cell-specific proteins recognizing these elements, we can speculate, in view of the present results obtained with the truncated TEF-1 (TEF-1 Δ C) activation domain, that there may be a tissue-specific factor recognizing the Inr element, and this protein may be responsible for this TEF-1 Δ C-associated stimulation of transcription seen with the CS-A and TK promoters.

In the TEF-1 protein, the region containing aa 1 to 45 is very rich in serines (9/45) and acidic (12/45) residues, whereas the region between aa 143 and 204 contains 16 prolines making it a proline-rich domain (Table. 4. 1)

Table. 4.1. Structural features of TEF-1 protein

a a residues	Structural feature
2-45	Serine rich region
21-44	Acidic region
30-97	TEA-DBD
55-121	Basic region
143-204	Proline rich (16/42) region
167-426	Transactivating domain
300-328	Serine/threonine and tyrosine rich region
402-419	Putative Zinc-finger domain

The equivalent regions from TEF-1 and TEF-5 possess amino acid differences, particularly in the number of serines and acidic amino acids (Fig. 4.12). Certain amino acid-rich regions, such as glutamine-rich, acidic (aspartic- and glutamic acid-rich) domains and proline rich-regions in many transcription factors were shown to contribute to activation function. There are many classical transcription factors that fall into these categories. These include, glutamine-rich regions in Sp1 (Courey et al. 1988, 1989), Oct-1 and Oct-2 (Tanaka and Herr, 1990), OTF-2 (Gerster et al. 1990), HNF-1 (Raney et al. 1991); acidic domains in GAL4 (Gill and Ptashne, 1987), glucocorticoid receptor (Danielsen et al. 1987), JUN (Struhl, 1988), VP16 (Cress and Trienzenberg, 1991), Oct-2 (Tanaka and Herr, 1990), PU.1 (Fisher et al. 1998; Klemsz and Maki, 1996); and proline-rich residues in NF-1/CTF (Mermoud et al. 1989), AP-2 (Williams and Tjian, 1991), GHF-1/Pit-1 (Theill et al. 1989), Oct-2 (Tanka and Herr, 1990) and HNF-1 (Raney et al. 1991). Therefore, the proline-rich and acidic regions in the TEF-1 protein may contribute to the transcriptional activation function of TEF-1. Furthermore, the presence of serine-rich residues, which are potential sites for phosphorylation, may also play an important role. The phosphorylation state of TEF-1 was not characterized, but phosphorylation of many transcription factors such as AP-1 and CREB, was shown to play a crucial role in transcription (reviewed by Karin and Smeal, 1992). Proline-rich regions of NF-1/CTF were shown to interact with histone H3 and/or oligomers of histones H3 and H4 both in transiently or stably transfected mammalian cells, and this interaction was up-regulated by TGF-beta *in vivo*, thus regulating gene expression (Alevizopoulos et al. 1995). Sometimes synergistic activation of transcription in a cell-specific manner occurs through cooperative interaction between two different activation domains present on the same transcription factor (Tanaka et al. 1994). Furthermore, it was suggested that a species-specific interaction of the activation domain of an activator with the TBP correlates with the ability of the activator to activate transcription (Emili et al. 1994). However, a homopolymer of as many as 10 to 30 glutamines or about 10 prolines is sufficient to activate transcription to a maximum in cell transfections (Gerber et al. 1994).

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Fig. 4.12. Amino acid sequence comparison between TEF-1 and TEF-5 factors. (A) Region N-terminal (a a residues 1/29); (B) region C-terminal (a a residues 102/166 for TEF-1, 102/172 for TEF-5) to the TEA-DBD were shown. (:::) denotes similarity in sequence and (---) is introduced to enhance the similarity. Acidic (Glu and Asp), Ser and Pro residues in TEF-1 factor are underlined.

A. Amino acids 1-29

TEF-1	Ile <u>Glu</u> <u>Pro</u> <u>Ser</u> <u>Ser</u> Trp <u>Ser</u> Gly <u>Ser</u> <u>Glu</u> <u>Ser</u> <u>Pro</u> Ala
TEF-5	::: Ala Ser Asn ::: ::: Asn Ala ::: Ser ::: ::: Gly
TEF-1	<u>Glu</u> Asn Met <u>Glu</u> Arg Met <u>Ser</u> Asp <u>Ser</u> Ala Asp Lys <u>Pro</u>
TEF-5	::: Ala Arg ::: Asp Glu Pro Glu Gly Leu ::: ::: Gly
TEF-1	Ile <u>Asp</u> Asn
TEF-5	Leu ::: :::

B. Amino acids 102-166/172

TEF-1	<u>Ser</u> Arg <u>Asp</u> Phe His <u>Ser</u> Lys Leu Lys --- --- --- ---
TEF-5	Val ::: Glu Tyr Gln Val Gly Ile :::
TEF-1	<u>Glu</u> Gln Thr Ala Lys <u>Asp</u> Lys Ala Leu Gln His Met Ala
TEF-5	::: ::: Val Ser ::: ::: ::: ::: ::: ::: Ser ::: :::
TEF-1	Ala Met <u>Ser</u> <u>Ser</u> Ala Gln Ile Val <u>Ser</u> Ala Thr Ala Ile
TEF-5	Ser ::: ::: ::: ::: ::: ::: ::: ::: ::: Ser Val Leu
TEF-1	His Asn Lys Leu Gly Leu <u>Pro</u> Gly --- Ile <u>Pro</u> Arg <u>Pro</u>
TEF-5	Gln ::: ::: Phe Ser Pro Pro Ser Pro Leu ::: Gln Ala
TEF-1	Thr Phe <u>Pro</u> Gly Ala <u>Pro</u> Gly Phe Trp <u>Pro</u> Gly --- Met
TEF-5	Val ::: Ser Thr Ser Ser Arg ::: ::: Ser Ser Pro Pro
TEF-1	Ile Gln Thr Gly Gln <u>Pro</u>
TEF-5	Leu Leu Gly Gln ::: :::

Using the truncated form of TEF-1, a potential activator function was observed, which is a novel phenomenon, strongly suggesting the participation of acidic and serine-rich and part of the proline-rich regions in this function. A region of TEF-1 containing the sequences from aa 135 to 426 was shown to contain the strongest capacity to bind TBP (Jiang and Eberhardt, 1996) and this binding seems to be responsible for squelching of TBP and subsequent transrepression by TEF-1 (Jiang and Eberhardt, 1996). However, according to Hwang et al. (1993) the mutant TEF-1 protein from aa 1 to 402 did not self-interfere with endogenous TEF-1 stimulated transcriptional activity, suggesting the presence of a putative inhibitory domain in the region containing aa 403-426 of TEF-1. Interestingly, a region from aa 343-426 of TEF-1 did not bind to TBP (Jiang and Eberhardt, 1996) suggesting this region may not be responsible for negative transcriptional regulation. Thus, these two studies did not explain the possible mechanisms involved in transrepression by TEF-1. However, although these studies were performed with TEF-1, Hwang et al (1993) used HeLa cells whereas Jiang and Eberhardt (1996) used placental cells, raising the possibility of tissue-specific regulatory mechanisms associated with TEF-1 function.

We know some of the target genes that are transcriptionally regulated by TEFs, but there may be more genes that respond to TEFs in a cell, and some of these target gene products may in turn regulate TEF activity. This regulation might not exist in a "normal" situation, but is only recapitulated when the equilibrium is disturbed by overexpressing the exogenous TEFs. These changes seems to have an adverse effect only on naked DNA templates and are independent of a DNA binding site. This mechanism could be applicable to all activators which have been shown to have a squelching effect. The squelching was observed only when higher doses of activator-expression vectors were used and the transrepression was specific-binding element independent (Hwang et al. 1993). All vectors used so far in transient transfections to express the TEFs in cells possess very strong promoters of viral origin. Hence, transrepression was observed following overexpression of either TEF-1 or TEF-5 in JEG-3, JAR, HeLa and GC cells. A possible mechanism responsible for the HeLa cell-specific transcriptional activation by TEF-1 Δ C is not clear at

present. However, it is possible, that in each of the cells there is a specific target gene regulating the function of an activator, but that this inhibitor is expressed only at certain conditions such as overexpression of the activator. Once these inhibitor proteins are expressed, they may bind to different regions of the activator and inhibit both the endogenous and the exogenous activator proteins. This inhibition is reflected in negative regulation of the activator-dependent transcription on a naked DNA template. In the case of HeLa cells, the inhibitor protein might be binding to the C-terminal region of TEF-1. However, TEF-1 Δ C, which itself is a potent activator, may not be able to upregulate the inhibitor in HeLa cells. Alternatively, activation of an inhibitor by TEF-1 Δ C still occurs, but the inhibitor may not bind to TEF-1 Δ C, because the C-terminal domain is deleted. Recently, a mechanism for the regulation of transcriptional response to heat shock was proposed (Shi et al. 1998). In heat shock, the heat shock factor 1 (HSF1) transcription factor is converted to an active form and this results in elevated expression of the heat shock proteins (HSP70 and HDJ1). These proteins in turn bring rapid attenuation of this response by binding directly to the activation domain of HSF1, resulting in inactivation. (Shi et al. 1998). In parallel, repression was observed with TEF-1 in all cells, and this effect could be due to the presence of an inhibitor protein. It is possible that like HSPs, which are targets of HSF1, the inhibitor could be a target gene for TEF-1. In fact, a HeLa cell factor (NEF-1) was identified which negatively regulates transcriptional activation *in vitro* by TEF-1 (Chaudhary et al. 1995). However, the nature and actual identity of NEF-1 is yet to be determined.

4.6. Possible implications of transcriptional activators like TEF-1 Δ C

Chromosomal translocations of genes and especially transcription factors and cell cycle regulators (BCL-1, BCL-2, cyclin D1, ETS, MYC, PAX, ERB2, FGFR1, MOZ and CBP) are characteristic of neoplasia of many cell types and particularly in leukaemias (reviewed by Heerema, 1998 and references therein; Roth, 1996; Borrow et al. 1996). These translocations result in the generation of completely new types of fusion proteins possessing regulatory domains of transcription factors. These fusion proteins with newly

acquired transcriptional characteristics may lead to aberrant expression of several genes including oncogenes and, hence tumorigenesis. An example of this mechanism is the in-frame MOZ-CBP fusion transcripts combining MOZ zinc finger motifs and a putative acetyl transferase domain with a largely intact CBP, suggesting the MOZ-CBP fusion protein could mediate leukaemogenesis via aberrant chromatin acetylation (Borrow et al. 1996). It is tempting to speculate that the truncated form of TEF-1, the TEF-1 Δ C, with its high transcriptional activation function, might play a role in tumorigenesis, similar to that of MOZ-CBP. The TEF-1 Δ C may aberrantly transactivate some of the oncogenes. Furthermore, the transactivation function of TEF-1 Δ C is HeLa cell-specific and these cells were originally isolated from a human cervical epithelial cell carcinoma. Further studies to decipher the mechanism of this transactivation associated with TEF-1 Δ C and detection of the transcriptional activators interacting with the TEF-1 Δ C protein in HeLa cells could be very informative. Another interesting aspect would be to identify the endogenous target genes, including GH/CS genes, for their activation in HeLa cells transfected with TEF-1 Δ C. This will help to answer the question about the role of TEF-1 Δ C at the chromatin level as well as reveal whether this kind of activator can overcome chromatin-associated repression.

4.7. Naturally occurring truncated forms of transcription factors

Many naturally occurring truncated or shorter versions of several transcription factors have been identified. Examples include Spl (Briggs et al. 1986; Persengiev et al. 1995), NF-1B (Liu et al. 1997), nuclear receptors such as xFF1rA (Ellinger-Ziegelbauer et al. 1995), Nurr1 (Castillo et al. 1998), and Stat6 (Patel et al. 1998). However, many of these truncated proteins, although exhibiting similar DNA binding affinities, possess less activation potential, or formed complexes with their full length counterparts and had a negative effect on transcription. At present we do not know whether TEF-1 Δ C is a naturally occurring truncated form of TEF-1 factor. Identification of different splice variants of TEFs in several cell types (Xiao et al. 1991; Jacquemin et al. 1996a, Jiang and Eberhardt, 1997) suggests the possible existence of truncated forms of TEFs in a cell.

CHAPTER 5

5.1. CHARACTERIZATION OF FAR-UPSTREAM AND FAR-DOWNSTREAM SEQUENCES OF THE HUMAN GROWTH HORMONE GENE FAMILY ON CHROMOSOME 17

5.1.1. Introduction

The human growth hormone/chorionic somatomammotropin (GH/CS) locus consists of five genes spanning 46,728 base pairs (bp) (Chen et al., 1989) on chromosome 17q22-24 (George et al., 1981). These five genes are arranged in the same transcriptional orientation and are, from 5' to 3', pituitary growth hormone (GH-N), chorionic somatomammotropin-like (CS-L), chorionic somatomammotropin-A (CS-A), placental growth hormone variant (GH-V) and chorionic somatomammotropin-B (CS-B). The GH/CS genes share the same genomic organization with five exons and four introns as well as high nucleotide sequence similarity (92-98%) in their immediate flanking, intervening, and coding sequences (Seeburg, 1982). It is predicted that this gene cluster has evolved through a recent duplication of an ancestral GH gene in primates (Barsh et al., 1983). The gene cluster is interspersed with as many as 48 repetitive Alu sequences (Chen et al., 1989) suggesting a possible role of Alu sequences in the duplication process. All other mammals possess only one GH gene. The significance of this duplication in primates is still not clear. Despite the high degree of homology and conserved genomic organization, the GH-N gene is expressed specifically in the pituitary while the GH-V and CS genes are expressed in the placenta (Chen et al., 1989). It should be noted that the CS genes are peculiar to primates, and should not be confused with the structurally distinct placental lactogens found in other mammals, which show more similarity to PRL than GH-N (Soares et al., 1991).

Efforts to elucidate the mechanisms underlying tissue-specific expression of the

GH/CS genes have focused largely on regulatory elements located in the proximal flanking sequences of the GH-N and CS-B genes. It is now clear that regulation of gene expression at the chromosomal level can involve DNA elements located at a distance of tens of thousands of base pairs (bp) from its transcription initiation site. These remote sequences may be important for delineating the boundaries of a gene locus and/or participate in exposing regulatory elements located more proximally to the promoter, such as those responsible for tissue-specificity and enhanced or repressed activities (Grosveld et al. 1998). Thus, these remote sequences would be expected to play an important role in the spatial and temporal expression of a gene or locus. These remote sequences, representing locus control regions (LCRs), are characterized by the presence of altered chromatin structure resulting in deoxyribonuclease I hypersensitive sites (HSs) (Elgin 1988). Although these sequences may exist in intergenic spaces, further analysis revealed that they can also reside in the exonic or intronic sequences of adjacent genes (Vyas et al., 1992; Jones et al., 1995). More recently, nuclease sensitivity assays of chromatin reflecting both upstream and downstream flanking sequences of human GH/CS or rat GH-N genes, revealed a series of HSs (Aizawa et al., 1995; Jones et al., 1995; Kazahari et al., 1997a). These HSs were shown to either have enhancer activity or comprise a LCR controlling pituitary-specific expression of the hGH-N gene (Aizawa et al., 1995; Jones et al., 1995). Characterization of the upstream regions flanking the GH-N genes led to the identification of the skeletal muscle-specific sodium channel α -subunit (SCN4A) gene and B-lymphocyte-specific CD79b gene both in the human (Bennani-Baiti et al. 1995; 1998a) and rat (Kazahari et al. 1997a; Nakazato et al. 1998) genomes. Furthermore, the HSs of the hGH/CS gene LCR were contained in or associated with these genes (Jones et al. 1995; Bennani-Baiti et al. 1995; 1998a). Clearly identification of gene loci that are adjacent to a particular gene locus of interest can provide essential information. The presence of sequence information in one gene influencing the expression of a gene immediately upstream or downstream, suggests a physical linkage between these genes.

The hGH/CS LCR is comprised of five (I-V) HSs of which HS I and II are pituitary-specific and HS IV is placenta specific, whereas HS III and V are present in both

tissues (Fig. 1.8; Jones et al. 1995). Although the role of the LCR, specifically of the HS I and II regions in the pituitary-specific regulation of expression of GH-N gene as an enhancer was characterized *in vivo* using transgenic mice, no sequence information including the DNA-elements and their corresponding factors responsible for the creation of HSs in a pituitary-specific manner was made available (Jones et al. 1995). The sequence information of this region is very valuable and necessary to understand the underlying mechanism to study the enhancer activity associated with these HS regions. Furthermore, the role of placenta-specific HS IV as well as ubiquitous HS III and V regions and their sequence information was not available. However, approximate locations of these HS regions were determined (Jones et al. 1995). The HS I and II were localized to a 1.6 kb *Bgl*/II fragment which coordinates at -14.5 and -15.4 kb, whereas HS III to V were localized at coordinates -27.5 and -32.5 kb relative to GH-N transcription start site (Jones et al. 1995). Further studies from the same laboratory revealed that the HS I and II were in the vicinity of 5'-flanking sequences of the B-lymphocyte-specific CD79b gene (Bennani-Baiti et al. 1998a), whereas HS III to V were localized to the intronic sequences of the skeletal muscle-specific sodium channel α -subunit (SCN4A) gene (Bennani-Baiti et al. 1995).

In contrast to upstream sequence, those downstream of the GH-N gene have received relatively less attention. Duplication of the GH-N gene in primates resulted in the insertion immediately downstream of four placenta-specific genes (GH-V, CS-A, B and L) (Barsh et al. 1983). A placenta-specific HS was also identified 24 kb downstream of the CS-B gene, but was not characterized (Cooke and Liebhaber, 1995). In the equivalent regions downstream of the rat GH-N gene, less cell-specific HSs were observed (Kazahari et al. 1997a). Further analysis of these HS regions led to the identification of BAF60b and Sug-1/TRIP-1 genes downstream of the rat GH-N gene (Kazahari et al. 1997b; Nomoto et al. 1997).

5.1.2. Objective

With a view to characterize the sequences both far-upstream and far-downstream of the human GH/CS locus, P1 clones containing hGH/CS genomic sequences as well as both upstream and downstream sequences were isolated and analyzed. It was hypothesized that the sequence information obtained by characterizing these clones will help to determine the mechanisms involved in LCR function as well as the role of hGH/CS downstream sequences.

5.1.3. Isolation and characterization of P1 clones

5.1.3.1. Isolation of the P1 clone containing GH sequences

A P1 library containing human genomic DNA was screened by polymerase chain reaction (PCR) using a 160 bp fragment generated with the primers, 5'-CAGCCTCTGATCTCAAGG AAG-3' (corresponds to nucleotides 2037/2057; 8651/8671 and 45890/45910 of the hGH/CS locus sequences according to Chen et al. 1989) and 5'-GTGGGGTTGAGGACGATCAC-3' (corresponding to nucleotides 2177/2196; 8791/8810 and 46030/46049 of hGH/CS genes). Three P1 clones (P1-1, P1-2 and P1-3) were obtained (Genome Systems Inc., St. Louis, MO, USA).

5.1.3.2. Determination of boundaries of the human genomic inserts in the P1 clones

P1 DNA was prepared according to suppliers instructions as described in Materials and Methods. Partial sequencing of the P1 clone was done using T7 (5'-CCGCTAATACGACTCACTATAGGG-3') and SP6 (5'-GGCCGTCGACATTTAGGTG ACAC-3') primers which correspond to sequences flanking the *Bam*H1 site containing the genomic (cloned) fragment. The sequences obtained were searched for similarity in the GenBank using the BLAST program (Altschul et al., 1990) and the boundaries were

determined. Furthermore, the P1 clone DNAs were digested with *Bam*HI or *Eco*RI and blotted onto nitrocellulose membrane and probed with the hGH-N cDNA probe as described in Materials and Methods. These results further confirmed the GH/CS genomic sequences in the inserts.

It was generally believed that P1 clone genomic libraries contain approximately > 70 to 80 kb inserts. As shown in Fig. 5.1, the P1-1 clone contains all hGH/CS genes except the CS-B gene with one end of sequences ending after GH-V gene sequence, but the sequence of the other end of the P1-1 genomic insert revealed a novel region. However, Southern analysis of the *Bgl*II digested P1-1 DNA with a 1.6 kb *Bgl*II fragment corresponding to the HSs I and II sequences revealed that this region of the hGH/CS gene LCR (Fig. 5.2) and therefore, the CD79b gene is present in the insert.

The P1-2 clone genomic insert starts from nucleotide 9581 of GH/CS gene locus (according to the nucleotide sequence designation in Chen et al. 1989) thus, comprising CS-L, CS-A, GH-V and CS-B genes and extending tens of kbs downstream (Fig. 5.1).

Finally, the P1-3 clone includes sequences from exon 9 (nucleotide 135) of the SCN4A gene and extends into the enhancer sequences in the 3'-flanking region of the CS-B gene, thus, comprising all components of hGH/CS gene LCR (HSs I to V) and CD79b and GH/CS genes and their known regulatory elements (Fig. 5.1).

For further characterization of LCR sequences as well as to isolate the HSs regions, the P1-3 clone was used, whereas to map the genes downstream of the CS-B gene and to determine a possible mechanism involved in the formation of the GH/CS gene downstream placenta-specific HS (3'-HS), the P1-2 clone was used.

Fig. 5.1. Mapping of genomic DNA inserts in the P1 clones. The P1 clone (P1-1, P1-2 and P1-3) plasmid DNA was prepared and sequenced using T7 and SP-6 primers. The partial sequences obtained from each clone were BLAST searched in GenBank Database for sequence similarity. This information was used to map the genomic inserts.

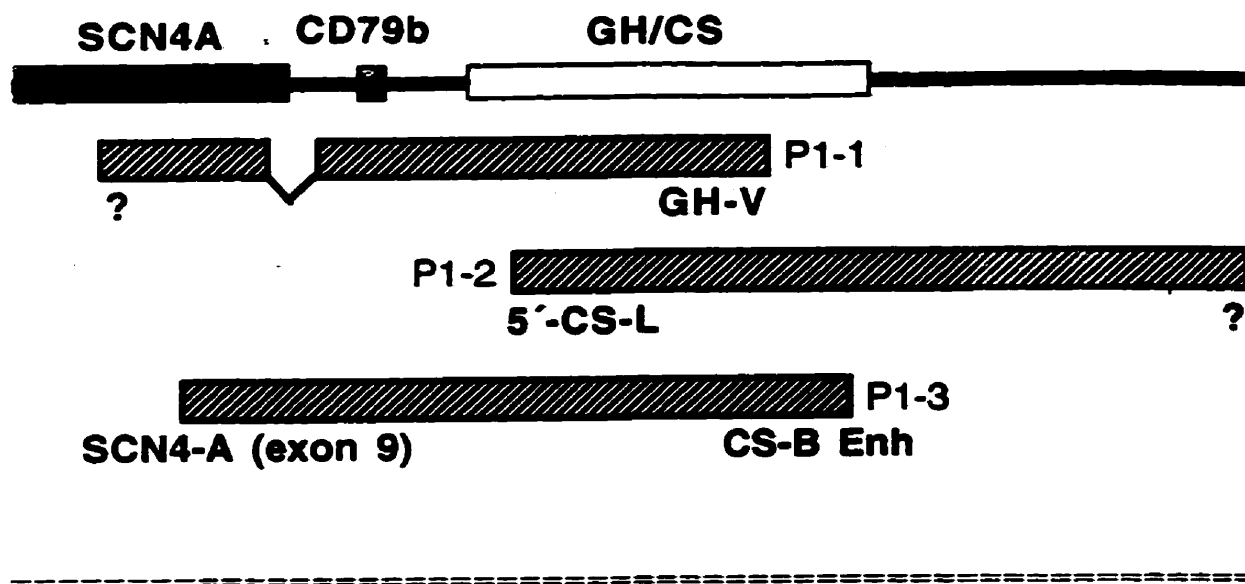
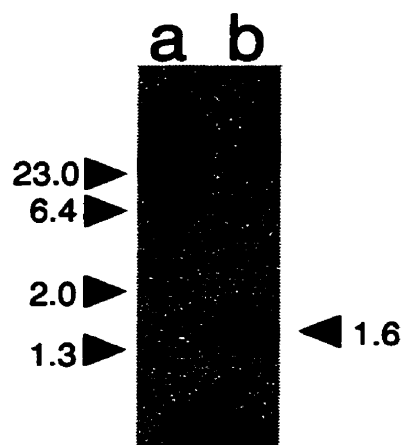


Fig. 5.2. Detection of 1.6 kb *Bgl*II fragment containing hypersensitive sites (HS) I and II of the GH/CS locus control region (LCR) in P1-1 clone genomic insert. P1.1 Clone DNA was digested with *Bgl*II, the DNA fragments were separated in a 1% agarose gel and transferred onto a nitrocellulose membrane. The membrane was probed with a radiolabelled 1.6 kb *Bgl*II DNA fragment containing HSs I and II of GH/CS LCR. Lanes: a) undigested P1-1 DNA and b) *Bgl*II digested P1-1 DNA. λ -*Hind*III digested DNA molecular markers (in kilo bases) and the 1.6 kb fragment were indicated as arrow heads.



5.2. THE HUMAN GH GENE FAMILY IS PHYSICALLY LINKED TO TWO UBIQUITOUSLY EXPRESSED GENES, THE BAF60B GENE, A SUBUNIT OF SWI/SNF COMPLEX AND THE THYROID HORMONE RECEPTOR INTERACTING PROTEIN-1 (TRIP-1) GENE ON CHROMOSOME 17

5.2.1. Introduction

As stated earlier, in rat and human genomes, a physical linkage among GH-N, CD79b and SCN4A genes was observed (Bennani-Baiti et al. 1995; 1998a; Kazahari et al. 1997a; Nakazato et al. 1998). Based on the location of the GH-N and SCN4A genes on the same chromosome 11 in mouse (Ambrose et al. 1992; Jackson-Grusby et al. 1988) it was thought that these genes are physically linked among several species (Bennani-Baiti et al. 1995). Recently, the BAF60b and Sug-1/proteasome p45 genes were mapped downstream of the GH gene in the rat and thus suggested that these genes are also physically linked (Kazahari et al. 1997b; Nomoto et al. 1997). Thus, the SCN4A, CD79b, GH-N, BAF60b and Sug-1/proteasome p45 genes are tandemly arranged on the same chromosome with Sug-1 being in opposite transcriptional orientation to that of all other genes and are physically linked (Kazahari et al. 1997a, b; Nakazato et al. 1998; Nomoto et al. 1997). However, in primate species, duplication of the GH-N gene resulted in the insertion immediately downstream of four placenta-specific genes (GH-V, CS-A, B and L₁) (Barsh et al. 1983). Thus, it was interesting to analyze whether the insertion of the placental GHs in human genome had any effect on the localization of the BAF60b and Sug-1 genes downstream of the CS-B gene on chromosome 17. In addition, such a study would inevitably characterize sequences in the vicinity of the downstream placenta-specific HS, which was shown to be 24 kb downstream of CS-B gene (Cooke and Liebhaber, 1995).

5.2.2. Objective

Mapping of genes downstream of the hGH/CS gene locus was done by

characterizing the P1-2 clone. To further extend the sequences downstream of the CS-B gene, from nucleotide 66,495 (Chen et al. 1989), sequencing of the P1-2 clone was performed with a primer corresponding to the 3'-end of the GH/CS locus.

5.2.3. Experimental design

5.2.3.1. Designing of primers for extension of sequence downstream of the GH/CS locus

Primers were designed to extend the sequence of the GH/CS locus downstream and included: GHP1, 5'-GCACTGCAGGATGGAATTC-3'; GHP2, 5'-GGGCGGACACAA GGCAAGAG-3'; GHP3, 5'-GCCAATGACTCTGGTGAGGA-3'; and GHP4, 5'-GGCA TGGTGATGCGTGCCT G-3'. PCR sequencing was performed using the fmol sequencing kit (Promega).

5.2.3.2. Designing of primers for extension of sequence downstream of the TRIP-1 gene and to identify the BAF60b gene

Partial sequencing of the P1-2 clone to extend the sequence downstream of the TRIP-1 gene was done using the following primers: TPP4 (5'-GAAGTGAGTGGACAGC CTTTG-3', nucleotides 1233/1253 in the TRIP-1 cDNA (Lee et al. 1995) TPP5 (5'- CCTCAGGCCATGCTAGAGAA-3'), TPP6 (5'-CCAGCAGCAAGGCTGGGCTTGTAG-3') and TPP7 (5'-GGGGGGCAGAGGAGGCATCTGC-3').

5.2.3.3. Cell lines and cell culture

Growth and culturing conditions to maintain the rat anterior pituitary tumor GC, human cervical carcinoma HeLa, African green monkey kidney COS-7 cells are described in the Materials and Methods section.

5.2.3.4. Isolation of genomic DNA

Genomic DNA was isolated according to established protocols (Nickel and Cattini, 1991). Briefly, cells were washed once with phosphate buffered saline and then harvested. The cells were pelleted, resuspended in lysis buffer (1 ml per 10^7 cells; 10 mM Tris.Cl, pH 8.0, 10 mM EDTA, 100 mM sodium chloride (NaCl), 0.5% sodium dodecyl sulfate (SDS) with 100 µg/ml Proteinase K) and incubated at 55 °C for 16 hours. Genomic DNA was precipitated after phenol, chloroform and isoamyl alcohol extraction. Finally the pellet was dissolved in 10 mM Tris.Cl pH 8.0, 1 mM EDTA buffer and the concentration of DNA was determined.

5.2.3.5. Polymerase chain reaction

Primers were designed based on the sequence information available for the TRIP-1 cDNA (Lee et al., 1995a, b). These included: TPP1 (5'-GTGACACCCAATTGCCGGGT-3', nucleotides 340/360 in TRIP-1 cDNA), TPP2 (5'-CTTGGGCTGAGCAATGCCCA-3', nucleotides 551/571 in TRIP-1 cDNA), TPP3 (5'-AAAGGCTGTCCACTCACTTC-3', nucleotides 1233/1253 in TRIP-1 cDNA). The primers, BAF1 (5'-GAGCGGGAGTACATCAACTGC-3', nucleotides 1305/1325 in the BAF60b cDNA) and BAF2 (5'-ATGTGCCTGCCTACTGCTTCC-3', nucleotides 1792/1772 in the BAF60b cDNA) were designed based on the sequence information available for the BAF60b cDNA (Wang et al. 1996). Polymerase chain reactions (PCR) were done using human genomic (100 ng) and P1-2 DNAs, and Pwo DNA polymerase enzyme (Boehringer Mannheim, Laval, PQ). Initial denaturation was for 4 minutes at 94 °C, followed by 30 cycles with each cycle consisting of 90 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 2 minutes, as well as a final step of 72 °C for 8 minutes.

5.2.3.6. First strand synthesis

Isolation of RNA from cells or tissues was described in the Materials and Methods

section. First strand synthesis or reverse transcription of RNA was done using the Gibco-BRL kit. For reverse transcription, approximately 1 µg of total RNA in 1 µl was mixed with 2 µl of 5 x RT buufer, 1 µl of 0.1 M DTT, 1 µl of Moloney murine leukemia virus reverse transcriptase (200 U/µl) (MMLV-RT), 2 µl of 2.5 mM dNTPs, 1 µl of random hexamer (pdN6; Pharmacia), 1µl of 0.1% BSA, 0.5 µl of RNA guard (30 U/µl; Pharmacia) and incubated for 2 h at 37 °C. Canine tracheal smooth muscle RNA treated with reverse transcriptase was a generous gift from Dr. N.L. Stephens (University of Manitoba, MB). These samples were amplified by PCR as described above, and the products were analyzed in 1-1.5% agarose gels.

5.2.3.7. DNA (Southern) blotting

DNA (Southern) transfer was described in Materials and Methods. Membranes were probed with either the GH-N cDNA (Martial et al., 1979) or a 1.8 kb TRIP-1 gene-specific probe generated by PCR using TPP1 and TPP3 with P1-2 DNA, or a 1.1 kb BAF60b gene-specific probe generated by PCR using primers BAF1 and BAF2 with P1-2 DNA. Probes were radiolabelled by random priming (Prime-a-gene; Promega Inc) to a specific activity of 10^3 - 10^9 cpm/µg of DNA. Hybridizations were carried out in 50% formamide solutions at 42 °C for 16 hours. The membranes were washed with 0.1X SSC/0.1% SDS (20x SSC, 3.0 M NaCl, 0.3 M sodium citrate) at 65 °C for 10-15 minutes and the washings were repeated (2x), before autoradiography.

5.2.3.8. RNA (northern) blotting

Fifty micrograms of total RNA was fractionated in a 1.5% formaldehyde-agarose gel and transferred onto nitrocellulose membrane as described in the Materials and Methods section. A 231 bp fragment of the human TRIP-1 cDNA was generated as a probe by PCR, using TPP1 and TPP2 primers with P1-2 DNA, and radiolabelled to a specific activity of 1×10^9 cpm/µg DNA. Hybridization and washing conditions were as described above.

5.2.3.9. Pulse-field gel electrophoresis

P1-2 DNA was prepared according to suppliers instructions. The DNA was digested with restriction endonucleases for 16 hours at 37 °C. The restricted fragments were separated in a 1% agarose gel in 0.1 M Tris, 0.1 M boric acid and 2 mM ethylenediaminetetraacetic acid (EDTA) at 200 mA with 5 second pulses for 20 hours at 8 °C.

5.2.4. Results

5.2.4.1. The P1-2 clone contains part of the GH gene locus and the TRIP-1 gene

Partial sequence from both ends of the cloned insert in P1-2 was obtained using SP6 and T7 primers, respectively. These primers complement sequences flanking the cloning site in the pAD10SacBII (P1) vector. The sequence obtained with the SP6 but not the T7 primer consisted of a region of the GH/CS locus. As a result, the upstream end of the cloned P1-2 insert corresponded to nucleotide 9,581 using the numbering system for the GH/CS locus reported by Chen et al., (1989). Previously, Chen et al. (1989) reported the sequence (nucleotides 1-66,495) containing the five members of the GH/CS gene family. A primer sequence at the 3'-end of the GH/CS locus corresponding to nucleotides 66,477/66,495 was selected to confirm the presence of this DNA in the P1-2 clone and, at the same time, extend the sequence of the locus. Additional primers were synthesized based on the sequence generated and, as a result, the 3'-end of the locus was extended by 1,120 bp to nucleotide position 67,615 (Fig. 5.3; GenBank Accession #AF037360).

Fig. 5.3. Sequence of the DNA downstream of CS-B gene. P1-2 plasmid DNA was prepared and sequenced using primers GHP1-4. A total of 1120 bases was submitted to GenBank Database (Accession #AF037360).

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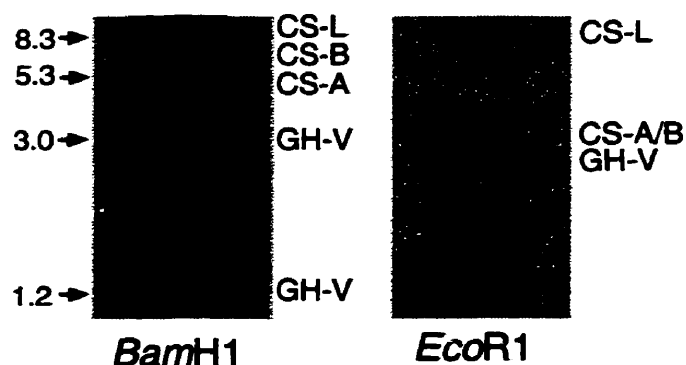
1      tttgcccacc tgagttttct ttaagaaggt ctcaattcca ctgggtcctg
51     ggtctgggca agtagtgctg cagttgacca cgcactgcag gatggaattc
101    tttgcccacc tgagttttct ttaagaaggt ctcaattcca ctgggtcctg
151    ggtctgggca agtagtgctg cagttgacca ctagggactg tccaaactcc
201    accagggcct gacttggcca aatagaaacc tcaaacaact cttccgggac
251    ccctgaaata tgaaccacat cggccaggcg cgggtggctca cgcctgtaat
301    ccccagcact ttgggaggcc gaggcgggcg gacacaaggc aagagatcaa
351    gaccatcctg gccaacatga tgaaaccccg tctctattaa aagtactaaa
401    ataagctggg tgtagtggcg cacgctgtaa tcccagctac tcgggaggct
451    gaggcaggag aatcgttgaa ccagggaggc agagggttgca gtgagccggg
501    attgtgccac tgcactccag cctggcaaca gagccaggac tccatctcaa
551    aaaaaaaaaa aaaaaaaaaa gaaccacata tgccaatgac tctggtgagg
601    aaccagagac ccaaataaat acagaaccta ttctatctag ttccagggtta
651    gagaacagga aacctgccct gaaaaaaaaa gttcagacaa ccagacagga
701    caaaagacct gtcaattggc cgggcggggg ggttcacgct gtcatcctcc
751    ccacttggga ggccaagggt ggcggtatcac ccgagggttg aagttagaga
801    ccagcctgac caatatggag aaaccctgtc ctactaaaaa tacaaaatta
851    gccaggcatg gtgatgcgtg cctgtaatcc agctactcag gaggctgagg
901    cagagaatca cttgaacca ggaggcagag gttgcagtga gttgagattg
951    tgccattgca ctccatcctg ggcaacaaga gtgaaactcg actcaaacaa
1001   acaaacaaaa aaaaactgta gttaacaagt tagctctgat ttcaccacta
1051   ttccctccac ctatcatctt tcttgagaac ttgaattctc tattagtatc
1101   acatggaatt atggagttct

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Since sequencing suggested that the P1-2 insert originated at nucleotide position

9,581 and extended, presumably, beyond the downstream boundary of the GH/CS locus, it would be expected to contain the CS-L, CS-A, GH-V and CS-B, but not the pituitary GH-N gene. To test this assumption, P1-2 DNA was digested with *Bam*HI and *Eco*RI, blotted and probed with the GH-N cDNA probe. This probe will detect GH-N, CS-L, CS-A, GH-V and CS-B sequences with equal sensitivity due to the 92-98% homology shared by these genes (Seeburg, 1982). These digests were used because they were shown previously to reveal distinctive patterns reflecting the presence of each individual member of the GH/CS gene family in human genomic DNA (Nickel and Cattini, 1991). The pattern generated by *Bam*HI digestion showed the presence of four but not five GH/CS genes; the 3.8 kb band associated with the GH-N gene was absent (Fig. 5.4). Similarly, *Eco*RI digestion would be expected to release two fragments of 2.9 kb containing the CS-A and CS-B genes, and two fragments of 2.6 kb containing the GH-N and GH-V genes as well as a single 9.5 kb fragment containing the CS-L gene (Nickel and Cattini, 1991). The intensity of the CS versus GH bands suggested that only one of the GH genes was present in the P1-2 clone (Fig. 5.4).

Fig. 5.4. Characterization of P1-2 clone DNA for the presence of human GH/CS gene family members. P1-2 DNA was digested with *Bam*HI or *Eco*RI, resolved by electrophoresis, transferred to nitrocellulose membrane and probed with a radiolabelled human GH-N cDNA. The fragment sizes and their (GH/CS gene) identity are indicated. Note, the expected 3.8 kb fragment corresponding to the GH-N gene after *Bam*HI digestion is absent, and the intensity of 2.6 kb GH bands are reduced relative to the 2.9 kb CS bands after *Eco*RI digestion.



Comparison of the P1-2 sequence obtained using the T7 primer with the contents of EMBL/GenBank Data Libraries revealed a striking homology with the human TRIP-1 (Lee et al., 1995a,b) and the porcine TBP-10 cDNAs (Leeb et al., 1996) as well as with other SUG-1 homologues from rat (Kazahari et al., 1997b) and mouse (vom Baur et al., 1996) (Fig. 5.5). The human TRIP-1 gene was localized recently to chromosome 17 (Hoyle et al., 1997). Based on a comparison of the P1-2 and human TRIP-1 cDNA sequences, the TRIP-1 gene is located in the reverse transcriptional orientation relative to each member of the GH/CS gene cluster on the long-arm of chromosome 17 (Surabhi et al. 1998).

Fig. 5.5. Comparison of P1-2 clone DNA sequence with human TRIP-1/SUG-1 cDNA and other homologous genes from different species. P1-2 clone DNA was sequenced using the T7 primer, and the partial sequence obtained was used to search for similarity in GenBank using the BLAST program. Identical nucleotides are represented by (:).

P1-2 sequence	TGTCTTCAGA TGGAGCTGGA GGAGGGGAAG GCAGGCAGCG GACTCCGCCA	144
h. TRIP-1 mRNA	:::: :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::	89
pig 26S p45 DNA	:::::::::: :::::::::: A:::::::::: :::::::::: T: ::::::::::	1350
pig 26S p45 mRNA	: :::::::::: A:::::::::: :::::::::: T: ::::::::::	50
rat SUG/p45 DNA	:::: ::::A:::: A:::::::::: :::::::::: T: :::::::::: T::	1202
rat SUG/p45 mRNA	:::: ::::A:::: A:::::::::: :::::::::: T: :::::::::: T::	86
mouse SUG mRNA	:::: ::::A:::: A:::::::::: :::::::::: T: :::::::::: T::	77
P1-2 sequence	ATATTATCTG TCCAAGATTG AAGAACTCCA GGTGAG	205
h. TRIP-1 mRNA	:::::::::: :::::::::: :::::::::: :C:::	118
pig 26S p45 DNA	G:::::::::: :::::::::: :::::::::: :A:::	1384
pig 26S p45 mRNA	G:::::::::: :::::::::: :::::::::: :C:::	76
rat SUG/p45 DNA	:::::::::: :::::::::: :::::::::: :A:::	1238
rat SUG/p45 mRNA	:::::::::: :::::::::: :::::::::: :T	115
mouse SUG mRNA	:::::::::: :::::::::: :::::::::: :T:::	106

5.2.4.2. Partial sequencing of the P1-2 clone revealed the presence of BAF60b gene immediately downstream of the TRIP-1 gene

A primer (TPP4) corresponding to the 3'-end of the TRIP-1 cDNA and encompassing the stop codon (Lee et al. 1995a), was used to extend the sequence in the

P1-2 clone, downstream of TRIP-1 gene and into the intergenic region that lies between the CS-B and TRIP-1 genes on chromosome 17 (Surabhi et al. 1998). Based on the sequence obtained, three additional primers, TPP5, TPP6 and TPP7, were designed and 975 bp of sequence was generated (Fig. 5.6). These sequences were used to search the GenBank database using the BLAST program (Altschul et al. 1990) and homology to the BAF60b cDNA was detected. Further analysis revealed that the BAF60b gene is in the reverse transcriptional orientation relative to the TRIP-1 gene and, thus, in the same direction as the GH/CS genes. The distance between the polyadenylation sites of BAF60b and TRIP-1 was determined to be only 93 bp (Fig. 5.6). The distance between these 2 sites in the rat genome is 92 bp (Fig. 5.6; Nomoto et al. 1997). An examination of the previously reported sequence for the porcine TRIP-1 (TBP-10) gene on chromosome 12 (Leeb et al., 1996), revealed the presence of the BAF60b gene, again, 93 bp downstream of the TRIP-1 gene (Fig. 5.6). Thus, this sequence analysis of the P1-2 clone has not only helped to map the human BAF60b gene precisely between the GH/CS gene cluster and TRIP-1 gene locus on chromosome 17, but also the porcine BAF60b gene on chromosome 12 (Surabhi et al. in press).

5.2.4.3. Partial restriction endonuclease mapping of the TRIP-1 and BAF60b genes in P1-2

Although the genomic organization of the porcine TRIP-1 homologue, the TBP-10/26S proteasome subunit gene, is known, this is not the case for the human TRIP-1 gene. Based on the available sequence information and comparisons with TRIP-1 homologues from different species (yeast SUG-1 and porcine TBP-10), two primers, TPP1 and TPP2, which could amplify a conserved 231 bp fragment (corresponding to exon 6 in the porcine TBP-10 gene and nucleotides 340/571 in the human TRIP-1 cDNA) were synthesized. A third primer, TPP3, was generated to sequences present in the human TRIP-1 cDNA but in the 3'-untranslated region. The TPP1 primer was used in combination with TPP2 or TPP3 to compare the genomic organization of TRIP-1 sequences in human genomic versus P1-2 clone DNA by PCR, and identical patterns were obtained (Fig. 5.7).

Amplification with TPP1 and TPP2 resulted in a 231 bp fragment, whereas TPP1 and TPP3 gave rise to a 1.8 kb product (Fig. 5.7). To obtain a fragment containing all the TRIP-1 coding sequences available in the P1-2 clone, the TPP3 and TPP2 primers were used for PCR in combination with the T7 primer corresponding to vector sequences. Products of 3.9 kb and 2.4 kb were observed with the TPP3 and TPP2 primers, respectively (Fig. 5.7).

Fig. 5.6. Partial sequence of the human BAF60b gene and comparison of the region containing the BAF60b and TRIP-1 polyadenylation sites in the human, porcine and rat genomes. A region (975 bp) of the P1-2 genomic clone insert was sequenced and shown to contain both BAF60b and TRIP-1 gene sequences (nucleotides numbered from position 1 to 975). The direction of transcription for the BAF60b and TRIP-1 genes are indicated with an arrow. The polyadenylation sites for both BAF60b and TRIP-1 are identified and underlined. Identical and mismatched nucleotides between human (Hum) and porcine (Pig) or rat sequences are indicated by uppercase and lowercase characters, respectively. Colons (:) were inserted in the sequences to increase their similarity. The numbering for the porcine and rat sequences are as previously reported by Leeb et al. (1996) and Nomoto et al. (1997), respectively. GenBank accession number for human sequences is AF068245.

TRIP-1 transcription →		TRIP-1 polyA			
Hum-GAAGTGAGTGGACAGCCCTT:GTGTGTAT:CTCTCCAAATAAGCTCTGTG			48		
Pig-GAAGTGAGGcaggTGTCTTCTGTGTGATCCTCAATATAAGCTCTGCA			4986		
Rat-GAAGTGAGGcgtgtgtccttt:GTGTGTAT:CTCTCAATATAAGCTCTGTG			9774		
Hum-GGCCAAGTCTCTAGGACTCCAGTCTGTAAATGAG:GGCTGTGCTGTTC			9		
Pig-GGatGAGCCTTTGAGGACTtgAGTCTGTAAATAGCGCTacCTGTTC			5036		
Rat-cGatAAA::CTCCAGAGCTCCAGTCTGTATGatGcATGcatGTTC			9726		
		BAF60b polyA			
Hum-AGGACCAGGTCCAGGCACCACCAATAGACAAAAGGATTTATTTGAAATT			147		
Pig-AACACTGGGTCTGGGACCACCAATAAACAAA:GATTTATTTGAAATT			5085		
Rat-AEGACCAGGTCTGGGACCACCAATAAACAAAAGATTTATTTGAAATT			9676		
Hum-TCCAAACCTGCCAGATGTGGCACTCGCCAAAGCCCTAGGCCACCC:::T			193		
Pig-TCCAAACCTGCCAGAGTGGCACTCGCCAAAGCCCTAGGCCACCCaccct			5135		
Rat-TCCAAgttTGCCAGAGTGGCACTCGCCAAAGCCCTAGGCCACCC:ctct			9627		
Hum-CCTCACCAGCT:CCAAAGGG:CCAACACCAAGTCTCCAGGCTCCCA:			240		
Pig-CCTTACCgAGCT:CCAAAGGG::CAACagCAGTCTCTCCaCaTggaag:			5181		
Rat-caagACCA:CTgCAAAAGGtaCAACAG:A:T:CT:CCAGCTTCCCC:			9583		
TTCACCTTAC	CCTGGCCTCC	ACATGCACAT	ACCCCATACC	TCAGGCCATG	290
CTAGAGAACT	GGAGTGTCCC	CTCCCCGGCC	AGCCCGCTGA	GAGGCAGCCT	340
CTGGCATCCA	GGAGATGATG	GGGCCAGAG	CCGTTCGATA	GATTGAGGAA	390
AGAAAGAAG	GAGGCACCTA	ACAGGCTCCC	TGAAAACCCC	CAACTTACCC	440
CTGTACAAA	AAAGTGGCTC	CCACATAGAA	AACTTCTCTC	CAAAATAGTG	490
CAAAATACCA	AAGGAAGGA	AAAAACCAGC	AGCAAGGCTG	GGCTTGTAGG	540
AATGGCAAG	AACCTTGCTT	TAGAAAGGCT	AGGAAGAGTC	TGGCTGCAC	590
CTCTCTTCC	TAGCCCAAGT	CCCCAGAGCC	AAGCTCTGAC	CCTGCCCAGG	640
GGGAGTCTGA	AACATGCAAA	CCCAGCAGCC	TTTGGTTCCG	AAATGTCTTA	690
CAATGTCAAA	GCACAGCCTC	CAGCAGTCT	ACTTGGGCTT	GGCTGCAGGG	740
GGGCAGGGA	GGCATCTGCT	GAACCAATTA	AAGCAATCGA	AAAGTATAA	790
GGCTTTGGGG	ACCAAGCAAC	AAGGAATCCT	ATCACTACAT	TTATAAGACT	840
AAAGCTGAG	AGTGAGGGTG	ACAGCAGACA	CTCTCACCCC	AGCTTACGTG	890
TCTGCGGCC	CAGCAAGGAC	CCAGAGGGTG	GTCTCCCTCC	AGGTCCAGGG	940
CTGGGAAGAA	AGATCTGAC	GAGTTAGGTC	AGGGG		975
		← BAF60b transcription			

Similarly, the relationship between the human and rat BAF60b cDNAs suggested by their 97% homology at the amino acid level (Nomoto et al. 1997), raised the possibility that this similarity would extend to the exon-intron organization between the genes. To examine this possibility, two primers, BAF1 (5'-GAGCGGGAGTACATCAACTGC-3', nucleotides 1305/1325 in the hBAF60b cDNA) and BAF2 (5'-ATGTGCCTGCCTACTGC TTCC-3', nucleotides 1792/1772 in the hBAF60b cDNA) were designed, which would map to sequences in exons 8 and 12 in the rat BAF60b gene, respectively (Nomoto et al. 1997). Based on rat BAF60b gene sequences, the BAF1 and BAF2 primers would be expected to amplify a 1.1 kb product from rat DNA by PCR. Assessment of P1-2 clone and human genomic DNA both resulted in a 1.1 kb product (Fig. 5.7), suggesting common exon-intron organization between the human and rat BAF60b genes, at least for those exons and introns located between exons 8 and 12. These results also indicated that the sequences contained between exons 8 and 12 in the P1-2 clone are also present in human genomic DNA without any gross rearrangement.

Both TRIP-1 specific (1.8, 2.4, and 3.9 kb) and BAF60b specific genomic (1.1 kb) fragments generated by PCR were cut with several restriction endonucleases (Fig. 5.8). This information was used for further analysis of the P1-2 clone and genomic DNA and an estimate of the distance between the GH/CS, BAF60b and TRIP-1 gene loci.

5.2.4.4. Both the BAF60b and TRIP-1 genes are mapped to a region +30 to +40 kb downstream of the CS-B gene

We were unable to generate a single DNA band which could be recognized by both GH-N cDNA, BAF60b and TRIP-1 probes after DNA blotting of the P1-2 DNA, since use of restriction endonucleases, which are known to cut genomic DNA very rarely, gave more than one band (Fig. 5.8). DNA (Southern) blotting analysis of human genomic and P1-2 clone DNAs digested with *Bam*HI, *Bgl*II, *Hind*III, *Xba*I and *Xho*I was carried out using either the GH-N cDNA, 1.1 kb BAF60b (PCR-generated) or 1.8 kb TRIP-1 (PCR-generated) genomic fragment as probes. Some identical bands were detected in P1-2 and

Fig. 5.7. PCR analysis of TRIP-1 and BAF60b genomic sequences. PCR was performed with different combinations of the primers TPP1-3 as well as BAF1 and BAF2 using either human genomic (JEG-3 or JAR cell) or P1-2 clone DNA. The PCR products were gel electrophoresed and photographed. The primer pairs used, the source of DNA, and the fragment sizes are indicated. A mixture of λ HindIII and ϕ X 174/HaeIII digested DNAs were used as markers.

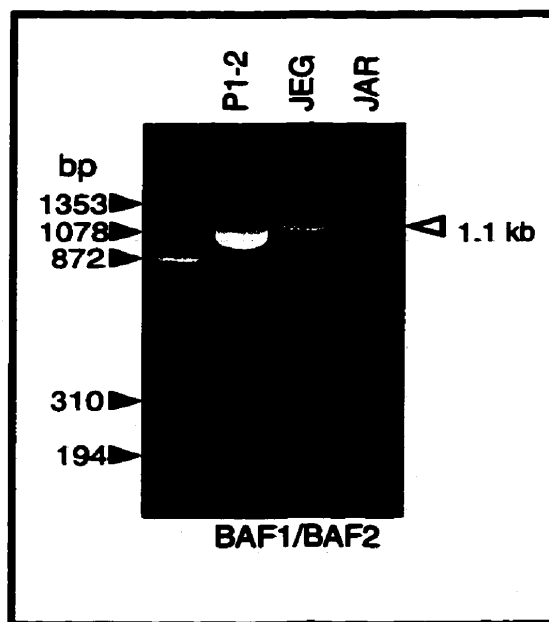
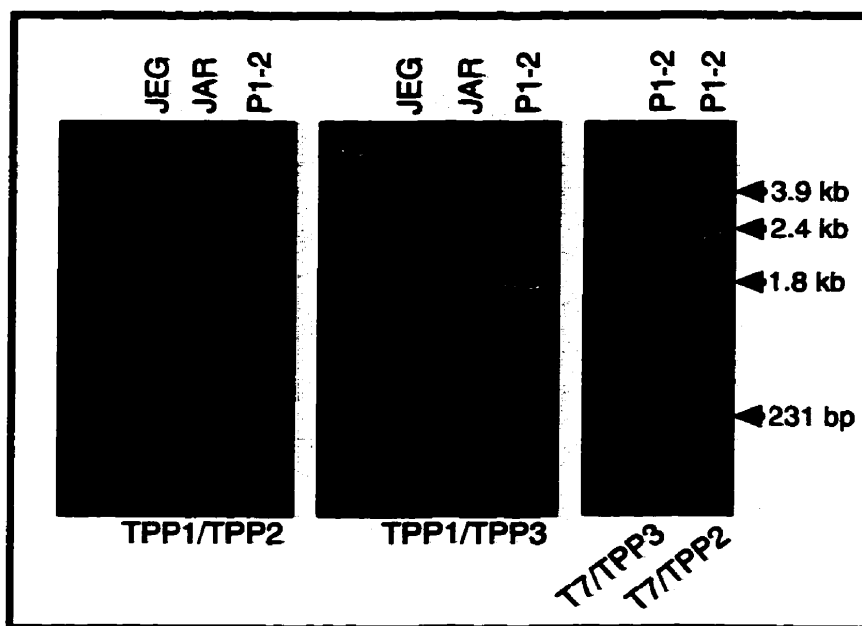
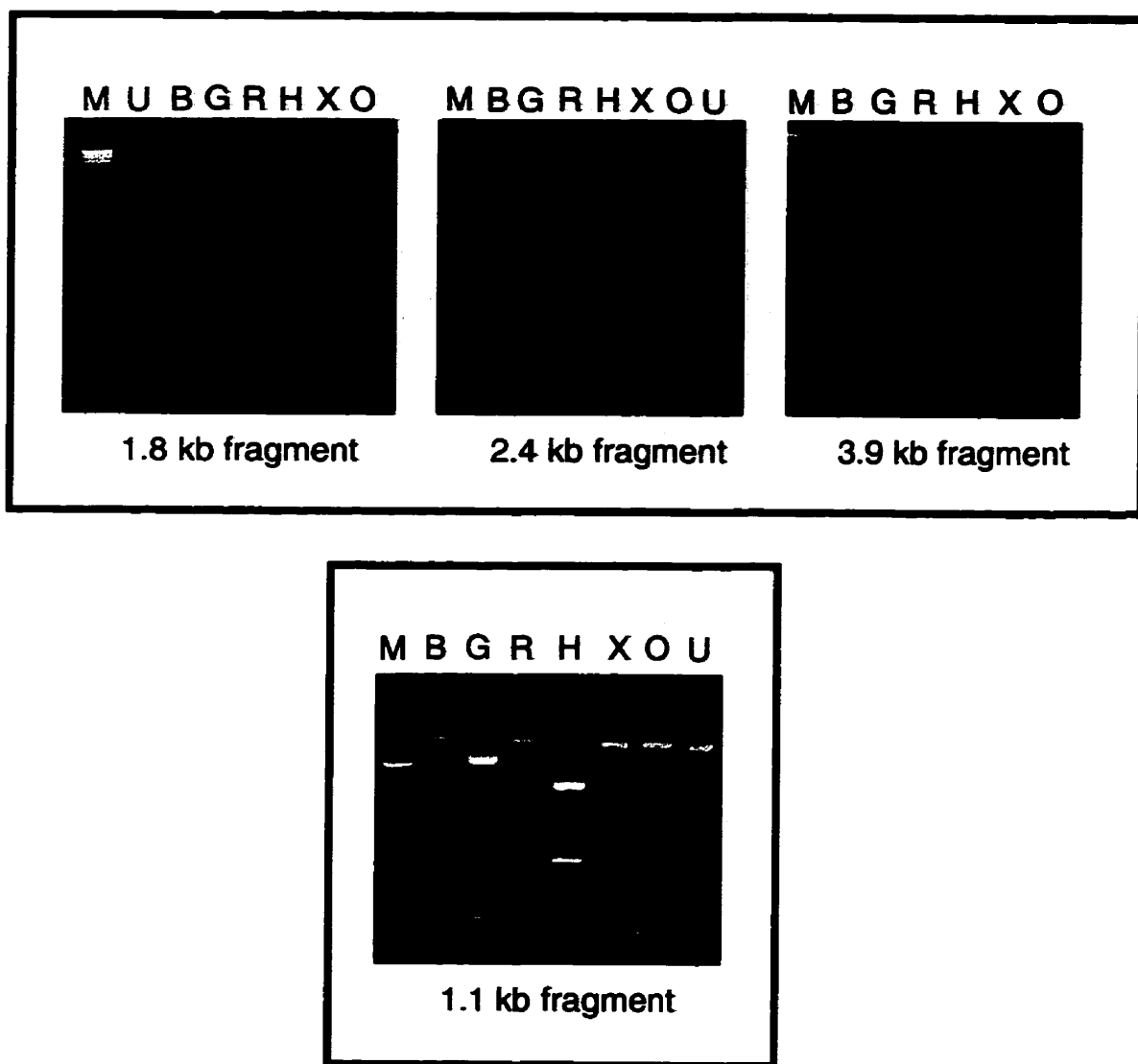


Fig. 5.8. Partial restriction endonuclease mapping of BAF60b and TRIP-1 genomic DNA fragments. PCR generated, TRIP-1 (1.8 kb, 2.4 kb and 3.9 kb) and BAF60b (1.1 kb) genomic DNA fragments were digested with *Bam*HI (B), *Bgl*II (G), *Eco*RI (R), *Hind*III (H), *Xba*I (X) and *Xho*I (O). After digestion, the fragments were separated in electrophoresis and were photographed. Undigested (U) fragments are also shown. A mixture of λ /*Hind*III and ϕ X174/*Hae*III digested DNAs were used as markers.

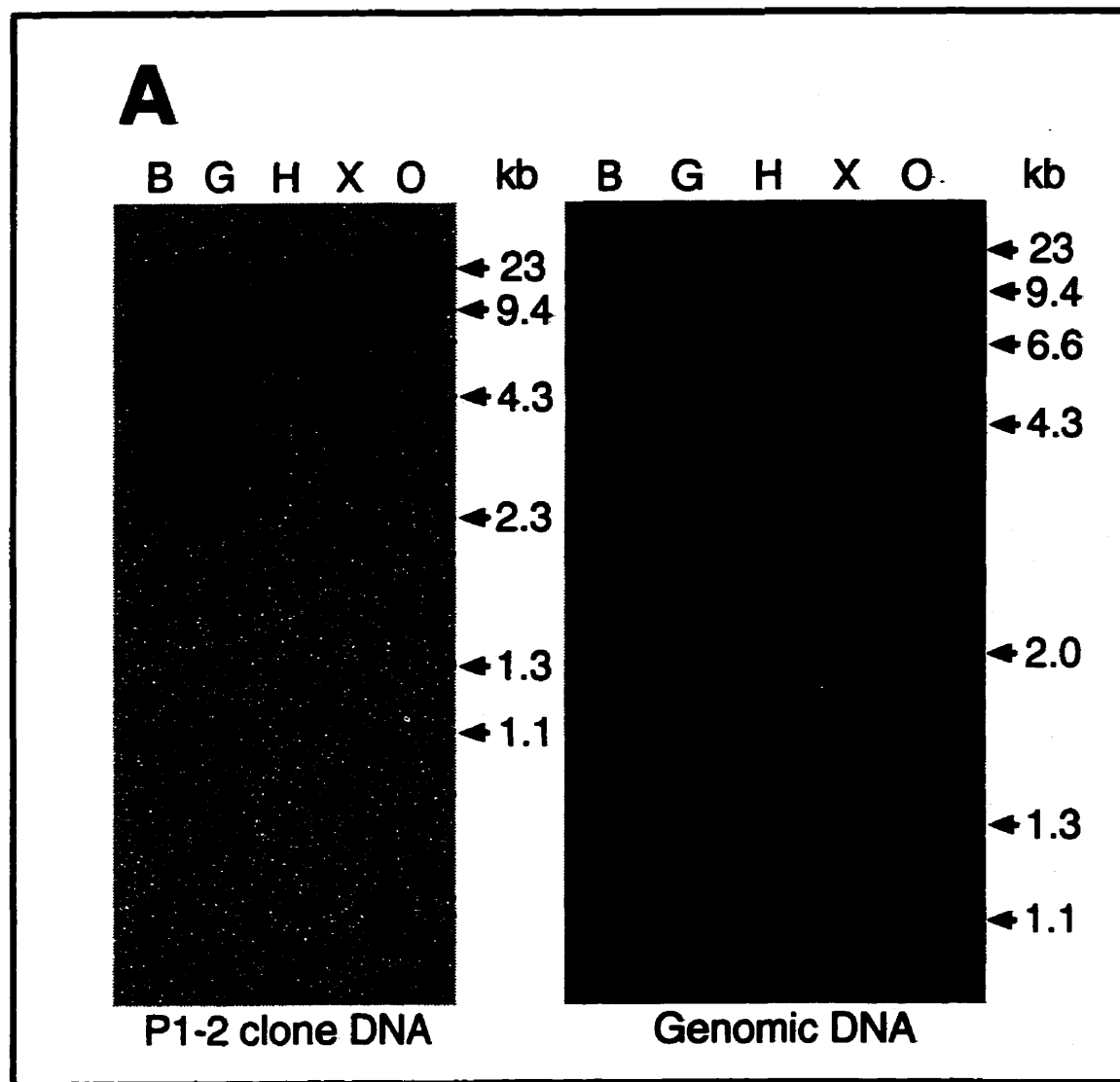


genomic DNA with the GH-N probe with all digests. Furthermore, the 1.1 kb BAF60b or the 1.8 kb TRIP-1 probe detected similar bands after digestion with *Bgl*II, *Hind*III, *Xba*I and *Xho*I or *Bgl*II and *Xba*I, respectively, suggesting the integrity of the GH/CS, BAF60b and TRIP-1 genes in the P1-2 clone (Fig. 5.9). The detection of different fragment lengths after digestion with *Bam*HI, with the BAF60b probe, or *Bam*HI (shorter in P1-2 DNA) and *Xho*I (longer in P1-2 DNA) with the TRIP-1 probe, reflects the presence of these sites in the P1 vector.

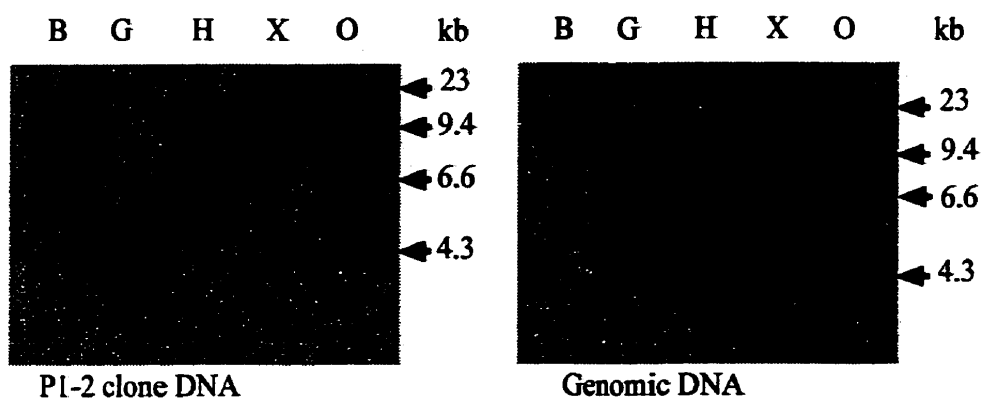
*Sal*I or *Not*I digestion of P1-2 clone DNA resulted in 3 (72.5, 20.0 and 10.0 kb) or 2 (82.5 and 20.0 kb) fragments, respectively (Fig. 5.10). Subtraction of the P1 vector size (17 kb) results in an approximate size of 85.5 kb for the genomic insert. Given the location of the GH/CS genes in this fragment based on sequence information, we estimate that the TRIP-1 gene is located about 40.0 kb downstream of the CS-B gene and thus placing BAF60b gene immediately in the vicinity of the TRIP-1 gene.

However, further Southern analysis was done with these fragments using GH-N cDNA and BAF60b as probes to unravel the distance between the CS-B and BAF60b genes. The GH-N cDNA probe hybridized with the 82.5 (*Not*I) and 72.5 kb (*Sal*I) bands (Fig. 5.10). The 1.1 kb BAF60b specific probe detected 20.0 kb and 20.0 as well as 10.0 kb in *Not*I and *Sal*I digests, respectively (Fig. 5.10). Restriction digestion of BAF60b PCR generated 1.1 kb genomic fragment with *Not*I and *Sal*I revealed the presence of a *Sal*I site (not shown), which would explain why two bands were detected in *Sal*I digested DNA with the BAF60b specific probe. The presence of the other *Sal*I site in the BAF60b gene was confirmed by analyzing the BAF60b cDNA sequence (Wang et al. 1996). As stated earlier, a strong possibility of common genomic organization exists between the human and rat BAF60b genes, and thus, the sizes of the both genes could be same. As the size of the rat BAF60b gene was reported to be approximately 9.5 kb (Nomoto et al. 1997), this localizes the human BAF60b gene at 30 kb downstream of CS-B gene.

Fig. 5.9. Integrity of genomic insert in the P1-2 clone. Both human genomic and P1-2 clone DNAs were digested with *Bam*HI (B), *Bgl*II (G), *Hind*III (H), *Xba*I (X) and *Xho*I (O), electrophoresed and blotted to nitrocellulose membrane. Membranes were probed with either radiolabelled human GH-N cDNA (A) or 1.8 kb TRIP-1 genomic DNA fragment (B) or 1.1 kb BAF60b genomic DNA fragment (C).



B



C

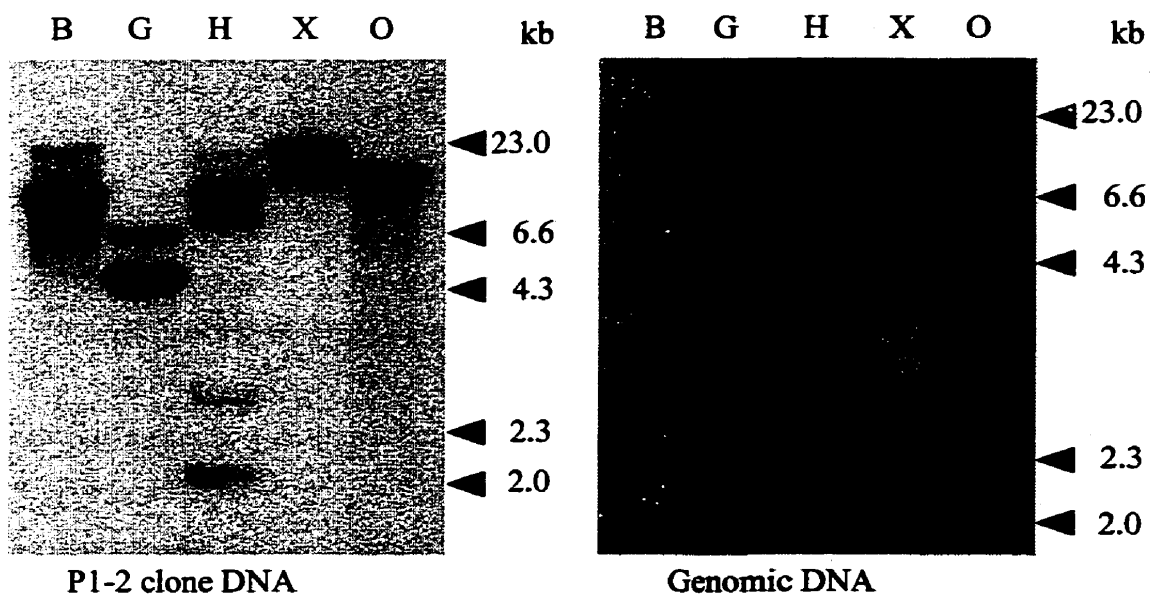
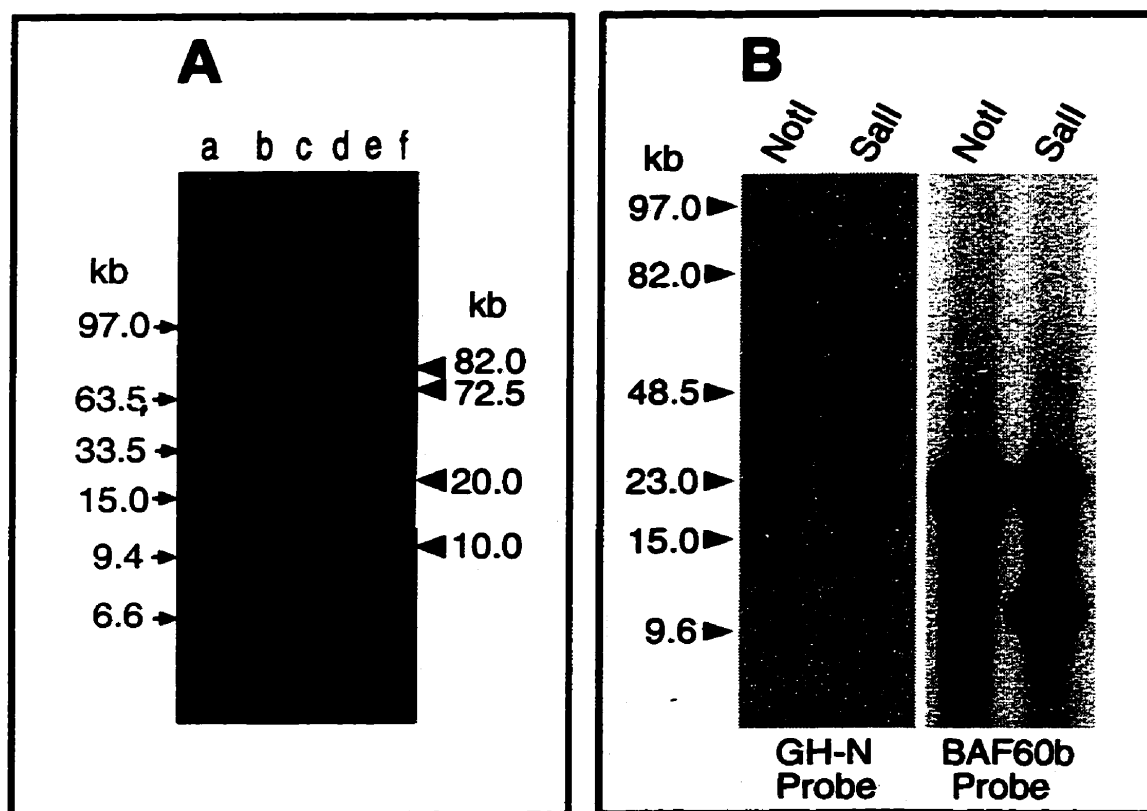


Fig. 5.10. Estimation of genomic insert size and detection of BAF60b and GH/CS genes in the P1-2 clone by pulse-field gel electrophoresis and DNA blotting. Estimates of genomic insert size and distance from the CS-B gene were obtained by digesting the P1-2 clone DNA with *NotI* and *Sall*, and separating the fragments by pulse-field gel electrophoresis (A). Lane: a, MidRange PFG marker I (New England Biolabs Inc. Mississauga, ON, Canada); b, λ /HindIII marker DNA; c, control P1 vector DNA/*NotI*; d, control P1 vector DNA/*Sall*; e, P1-2 clone DNA/*NotI*; and f, P1-2 clone DNA/*Sall*. After electrophoresis, the DNA fragments were blotted to nitrocellulose membrane. Membranes were probed with a radiolabelled human GH-N cDNA or BAF60b-specific (1.1 kb) probe as indicated (B). The "marker" sizes (arrowheads) correspond to the migration of a MidRange PFG marker I (New England Biolabs Inc. Mississauga, ON, Canada).



5.2.4.5. Assessment of expression of TRIP-1 and BAF60b genes

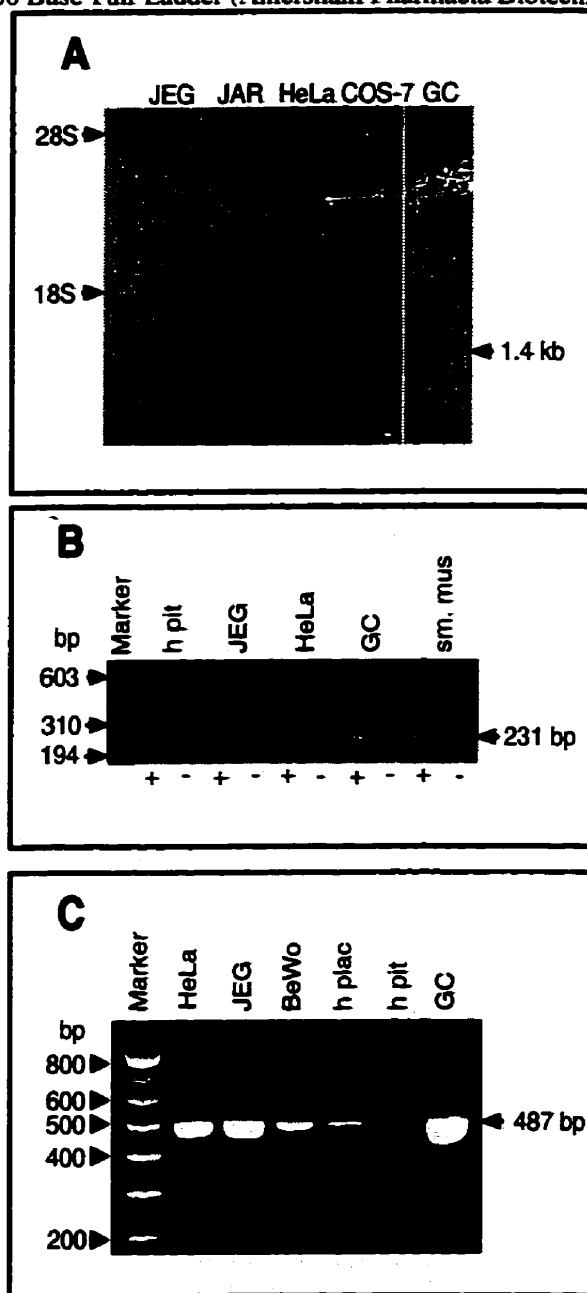
RNA (50 µg) from pituitary (GC), placental (JEG-3, JAR) or non pituitary/placental (HeLa and COS-7) cells was electrophoresed, transferred to nitrocellulose and probed with the 231 bp TRIP-1 exon 6 fragment. A transcript of the predicted size, 1.4 kb, was detected in all samples (Fig. 5.11).

Reverse transcriptase-PCR (RT-PCR) as well as RNA blotting was done to examine the pattern of TRIP-1 gene expression and determine any tissue- or species-dependence. More specifically, given the predominantly pituitary and placental expression of members of the GH/CS family, we assessed whether the BAF60b and TRIP-1 genes were active in pituitary (human, rat GC), placental (human JEG-3 and/or JAR) or non pituitary/placental (human HeLa, canine tracheal smooth muscle and/or monkey COS-7) cells. RT-PCR was performed using the primers BAF1/BAF2 or TPP1/TPP2. A BAF60b gene specific 487 bp or TRIP-1 gene specific 231 bp fragments were observed with all samples tested (Fig. 5.11). The TRIP-1 gene specific 231 bp product was absent from those samples not treated with reverse transcriptase, and reflects no of genomic DNA contamination (Fig. 5.11).

5.2.5. Discussion

The human GH/CS gene cluster, including 5' to 3', GH-N, CS-L, CS-A, GH-V and CS-B, was localized to chromosome 17q22-24 (George et al 1981) and was one of the first large human genomic DNA regions to be sequenced (Chen et al. 1989). Although the TRIP-1 gene was mapped to chromosome 17q24-25 (Hoyle et al. 1997), its fine localization 40 kb downstream of the CS-B gene and the extension of *Alu* repeats into their intergenic sequences, was made possible with the analysis of a P1 (P1-2) clone performed in this study.

Fig. 5.11. Detection of TRIP-1 and BAF60b RNA in pituitary, placental and non pituitary/placental cells. (A) Total RNA (50 μ g) was fractionated under denaturing conditions and transferred onto nitrocellulose membrane. The membrane was probed with radiolabelled 231 bp TRIP-1 specific PCR fragment and a 1.4 kb transcript was detected. The source of total RNA and electrophoretic mobilities of the 28S and 18S ribosomal RNA bands are indicated. (B) PCR was performed using TPPI/TPP2 primer pairs to amplify a 231 bp fragment from RNA samples treated with (+) or without (-) reverse transcriptase and isolated from human pituitary tissue (h pit), JEG-3 (JEG), HeLa, GC and canine tracheal smooth muscle (sm.mus) cells. (C) PCR was done using BAF1/BAF2 primer pairs to amplify a 487 bp fragment from human cervical HeLa, placental JEG-3, placental BeWo, human placenta (h plac), human pituitary (h pit) and rat GC cell/tissue samples. The "marker" sizes indicated by arrowheads correspond to the migration of a 100 Base-Pair Ladder (Amersham Pharmacia Biotech, PQ, Canada).



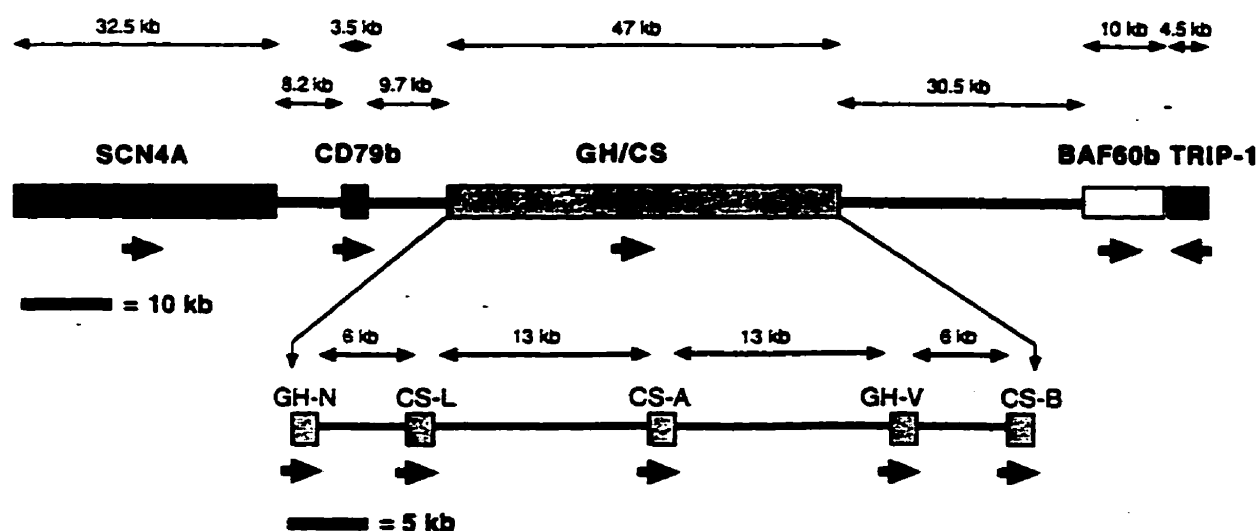
The data presented also show that the TRIP-1 gene is present in the reverse transcriptional orientation relative to all the GH/CS genes (Surabhi et al. 1998). Similarly, the sodium channel α subunit, SCN4A, gene located on chromosome 17q23.1-25.3 (George et al. 1991), was accurately mapped to a region 21.5 kb upstream of the GH-N transcription start site by analyzing yeast artificial chromosome and cosmid clones (Bennani-Baiti et al. 1995). Subsequently, further analysis of these clones led to the identification of the B-lymphocyte specific, CD79b gene, placed equidistantly, between GH/CS and SCN4A gene loci (Bennani-Baiti et al. 1998). Extension of sequences from the 3'-end of the TRIP-1 gene in the genomic insert of the P1-2 clone revealed the presence of the human BAF60b gene, placing it between the GH/CS gene cluster and TRIP-1 gene locus (Fig. 5.6). Our data also show that the BAF60b gene is present in the reverse transcriptional orientation relative to the TRIP-1 gene and, thus, in the same direction as all the GH/CS genes (Fig. 5.6). The "tail to tail" arrangement between the TRIP-1 and BAF60b genes is such that their polyadenylation sites are separated by only 93 bp. This arrangement appears to be conserved between species. When we examined the sequences reported for the porcine TRIP-1 (TBP-10/Sug-1/p45) gene (Leeb et al. 1996), we found evidence for the BAF60b gene 93 bp upstream of the TRIP-1 gene (Fig. 5.6). Recently, it was reported that a similar arrangement also occurs between these two genes in the rat genome (Nomoto et al. 1997). Thus, these data provide evidence for a physical linkage between the GH-N gene (GH/CS locus in humans), BAF60b gene and the TRIP-1 gene based on their mapping to the same chromosome in the human, porcine and rat genomes (Hoyle et al. 1997; Leeb et al. 1996; Nomoto et al. 1997; Surabhi et al. 1998; Surabhi et al. *in press*; Thomsen et al. 1990).

5.2.5.1. Linkage of nine genes on human chromosome 17

Genome analyses and chromosomal mapping of genes adjacent to GH-N gene revealed the existence of synteny between the SCN4A, CD79b, GH/CS, BAF60b and TRIP-1 gene loci in humans (Bennani-Baiti et al. 1995; Surabhi et al. 1998), and precisely maps nine genes in a 150 kb region of human chromosome 17, in the order: SCN4A,

CD79b, GH-N, CS-L, CS-A, GH-V, CS-B, BAF60b and TRIP-1 (Fig. 5.12). The relatively recent duplication of the GH-N gene in primates, which resulted in the CS-L, CS-A, GH-V and CS-B genes (Barsh et al. 1983), would predict a five gene arrangement (SCN4A, CD79b, GH-N, BAF60b and TRIP-1) in other vertebrates. Indeed, this pattern of genes is seen in the rat genome (Kazahari et al. 1997; Nomoto et al. 1997; Nakazato et al. 1998). The extent of the evolutionary conservation of this gene arrangement is further suggested by the common chromosome allocation for at least 3 of the 5 genes to chromosome 11 in the mouse (SCN4A, Ambrose et al. 1992; GH-N, Jackson-Grusby et al. 1988; TRIP-1, Hoyle et al. 1997) and chromosome 12 in the pig (GH-N, Thomsen et al. 1990; BAF60b, Fig. 5.6; TRIP-1; Leeb et al. 1996). The presence of the CS-L, CS-A, GH-V and CS-B genes between the GH-N gene and BAF60b gene in humans, and presumably other primates, indicates that the GH-N gene duplication (Barsh et al. 1983), did not adversely affect this arrangement.

Fig. 5.12. Schematic representation of the GH/CS locus on human chromosome 17 and the relative position of adjacent genes including those coding for SCN4A, CD79b, BAF60b and TRIP-1. The mapping is based on the present study and previous reports by Bennani-Baiti et al. (1995; 1998a) and Surabhi et al. (1998; *in press*). The large arrowheads indicate the direction of transcription for each of the 9 genes, and intergenic distances are also shown.



5.2.5.2. BAF60b and TRIP-1 genes are highly conserved across species

Unlike members of the the GH/CS family where synthesis is restricted largely to cells of the pituitary and placenta, we confirmed that BAF60b and SUG-1/TRIP-1 RNA expression occurs in many mammalian cells and tissues, generating a transcript of about 1.4 kb (Fig. 5.11; Lee et al., 1995a; Kazahari et al., 1997b). The detection of the BAF60b gene-specific 487 bp and TRIP-1 gene specific 231 bp (likely exon 6) fragment by RT-PCR and 1.4 kb TRIP-1 transcript across species (human, monkey, dog and rat) also suggests that these genes are highly conserved. Indeed, a gene from the tobacco hackmoth which shares 92% sequence similarity with human TRIP-1 was shown to be expressed in all tissues examined (Sun et al., 1996). BAF60b genes from human, mouse and rat are highly similar (Wang et al. 1996; Nomoto et al. 1997). Our data also suggest that the genomic organization of these genes is very similar in many species, since generation of a 1.4 kb transcript in case of the TRIP-1 gene, and 487 bp RT-PCR product as well as 1.1 kb genomic fragment in the case of the BAF60b gene agrees well with the available porcine (TRIP-1) and rat (BAF60b) genomic organization (Leeb et al., 1996; Nomoto et al. 1997).

5.2.5.3. BAF60b and TRIP-1 genes possess properties of housekeeping genes

The requirement for SUG-1 in yeast is well documented (Swaffield et al., 1992) and the same may also be true for other eukaryotes based on its structural/sequence conservation. Functional data suggest that human TRIP-1 can substitute for SUG-1 in yeast as a transcriptional modulator (Lee et al., 1995b). TRIP-1, takes its name from its ability to interact with the thyroid hormone receptor, a nuclear receptor/DNA binding protein (Lee et al., 1995a). The amino acid sequence of TRIP-1 reveals a highly conserved ATPase domain, which would be expected to participate in the ATP-dependent gene activation process (Fraser et al. 1997; Kim et al. 1994; Weeda et al. 1997). In the case of the TRIP-1 homologue in the tobacco hackmoth, there was an upregulation of this gene both at the mRNA and protein levels in tissues or cells that were undergoing apoptosis

(Sun et al., 1996). With these properties and its ubiquitous nature, TRIP-1 may be considered a "housekeeping" gene, which would be consistent with a possible open chromatin structure in both the pituitary and placenta (Fig.6.11).

The BAF60b gene was initially identified as a gene coding for a subunit of the SWI/SNF multi-protein complex (Wang et al. 1996). The role of SWI/SNF complex in remodeling chromatin structure is now well established (Wilson et al. 1996). The data also confirms that the BAF60b gene is widely expressed using RT-PCR and samples of human pituitary, placenta, cervical carcinoma tissue/cells (Fig. 5.11; Wang et al. 1996). This is consistent with the observation of ubiquitous BAF60b expression in the rat (Nomoto et al. 1997). The level of rat BAF60b expression was correlated with Sp1 levels, as the TATA-less promoter of the BAF60b gene was suggested to depend mostly on this transcription factor (Nomoto et al. 1997). Also, a 487 bp BAF60b-specific RT-PCR product was detected with both rat and human RNA using the same primers, corresponding to sequences in exons 8 and 12, which indicates the conservation of this gene across both species (Fig. 5.11). A 1.1 kb product was observed by PCR using the exon 8 and exon 12 primers with either human genomic or P1-2 clone DNA. This is also consistent with conservation of the genomic organization of the human and rat BAF60b genes, since an examination of the published rat BAF60b gene sequences reveals that a 1.1 kb fragment would also be generated with these primers and rat genomic DNA (Nomoto et al. 1997).

5.2.5.4. Possible functional significance of the presence of BAF60b. and TRIP-1 genes downstream of GH/CS locus

The physical linkage observed between the SCN4A, CD79b, GH-N (GH/CS in humans), BAF60b and TRIP-1 genes suggests this arrangement has functional significance. This idea is reinforced by the presence of HSs both upstream and downstream of the GH-N (GH/CS) gene locus in human and rat chromatin (Jones et al. 1995; Kazahari et al. 1997a). An examination of deoxyribonuclease I HSs associated with regions upstream of the human GH/CS locus revealed both pituitary- and placenta-specific as well

as ubiquitous HSs (Jones et al. 1995). Furthermore, regions of the human genome containing these HSs were shown to function *in vivo* as a LCR in transgenic mice (Jones et al. 1995). Subsequently, the two ubiquitous sites (HS III and V) and a placenta-specific site (HS IV) were localized to an intron of the skeletal muscle specific SCN4A gene, and the two pituitary-specific sites (HS I and II) were localized to the 5'-flanking DNA of the B-lymphocyte specific CD79b gene (Bennani-Baiti et al. 1995 and 1998a). Similarly, 5'-HSs were also detected in distal sequences upstream of the rat GH-N gene (Kazahari et al. 1997a), placing them in the vicinity of the recently mapped rat SCN4A and CD79b genes (Kazahari et al 1997a; Nakazato et al 1998). With regard to the presence of HSs downstream of the human GH/CS locus, a placenta-specific site was reported 24 kb from the CS-B gene (Cooke and Liebhaber, 1995). The results presented here would suggest that this 3'-HS is located in the 5'-flanking DNA of the ubiquitous BAF60b gene. Interestingly, less cell-type specific and scattered HSs were detected in regions 22/27 kb and 42/43 kb downstream of the rat GH gene locus, where the rat BAF60b and TRIP-1 (sug-1/p45) genes are located (Kazahari et al. 1997a,b; Nomoto et al. 1997). Given the absence of placenta-specific CS genes in the rat GH-N locus, the relevance of these sites to GH-N gene expression, at least in the pituitary, is unclear. The placement of regulatory regions in adjacent genes is not peculiar to the GH-N locus. The HSs associated with elements controlling α -globin gene transcription were also shown to be located in the sequences of the adjacent, "-14 gene" that is expressed ubiquitously and is constitutively active (Vyas et al. 1992 and 1995). Taken together, these observations suggest that the HSs harboring transcriptional control elements for a gene locus can reside in adjacent tissue-specifically or ubiquitously expressed genes.

There is increasing evidence that the members of GH/CS gene family can be expressed outside the pituitary or placenta, albeit at low levels, in lymphocytes, ovary, testis and mononuclear cells (Lytras et al. 1993; Schwarzler et al. 1997; Untergasser et al. 1997; Melen et al. 1997). Similar extra-pituitary expression of the GH-N gene has been reported in other species (Weigent et al. 1988; Lacroix et al. 1996; Yoshizato et al. 1998). A necessary component of this expression would be that the genes are not completely

closed down in an inactive chromatin structure. The presence of euchromatin and heterochromatin structures are associated with decondensed (active or poised) genes and highly condensed (inactive genes), respectively (Eissenberg et al. 1995). The DNA in heterochromatin was shown to consist almost entirely of repetitive sequences, and encode relatively few genes (Spofford, 1976). In *Drosophila* and mammals, closely linked repeats or arrays of three or more transgenes induced formation of heterochromatin due to pairing of repeats (Dorer and Henikoff, 1994; Garrick et al. 1998). The human GH/CS locus harbors 5 highly (94%) homologous genes, generated by gene duplication, and 48 Alu repeat sequences (Chen et al. 1989) and, thus, would be a candidate to form an inactive heterochromatin structure. However, based on nuclease sensitivity data, the chromatin containing the placenta-specific CS genes and pituitary-specific GH-N gene appeared equally "open" in the pituitary (Nickel and Cattini, 1996). Thus, unlike the GH-N gene that is active, the CS genes would be poised. There is evidence to support the idea that the chromatin structure in one region can influence its conformation in an adjacent region. In yeast as well as *Drosophila*, genes juxtaposed to heterochromatin are frequently inactivated due to spreading of heterochromatic state to the adjacent regions along the chromosome (Wilson et al. 1990). Although the opposite effect, it is possible that the presence of actively expressed genes upstream (GH-N) and downstream (BAF60b and TRIP-1), possessing an "open" chromatin structure, have a negative influence on any heterochromatic tendency of the GH/CS locus. Alternatively, the CS genes are open in the pituitary because their chromatin falls under the influence of the mechanism controlling the GH-N locus. In both instances, a mechanism would have arisen to maintain the CS genes in a poised state, and transcriptional repressors have been proposed that could serve this function (Nachtigal et al. 1993; Lytras and Cattini, 1994; Jiang and Eberhardt. 1997). In the human placenta, an independent mechanism to allow placental expression of the CS but not GH-N genes appears to have arisen, based on the presence of placenta-specific HSs among the CS genes as well as associated with flanking genes (SCN4A and BAF60b sequences; Jimenez et al. 1993; Jones et al. 1995; Cooke and Liebhaber 1995). However, it was reported recently that the pituitary-specific GH-N gene was expressed in the placenta of a woman with a total deletion of the CS-A, GH-V and CS-B genes (Alsat et al. 1997).

Although the extent of this deletion into downstream sequences is unknown, it is possible that the placenta-specific HS was brought into proximity of the GH-N gene and contributed to its expression. Alternatively, this positive effect on the GH-N expression could result from a change in the constitutively open configuration in the neighboring regions harboring ubiquitously expressed BAF60b and TRIP-1 genes, which would be brought into close proximity. An extension of this latter possibility, is that the open chromatin organization (euchromatin) associated with the BAF60b and TRIP-1 genes might influence expression of the GH-V and CS genes in the intact genome. Thus, the reported expression of GH/CS genes outside the pituitary and placenta could reflect the presence of nearby HSs, possibly in widely expressed genes, like BAF60b and TRIP-1, that could exert local perturbations in the chromatin structure, thereby influencing the regulation of expression of nearby genes. However, to go beyond this poised state and be expressed, these genes would still require activation by local transcription factors.

The placenta-specific 3'-HS could also be acting as an insulator. The placenta-specific CS-B enhancer element is present in the 3'-flanking sequences of the CS-B gene (Rogers et al. 1986). "Classical" cis-acting enhancers are known to influence the expression of a gene independent of their orientation and distance (including distances greater than 85 kb; reviewed by Blackwood and Kadonaga, 1998). Thus, the CS-B enhancer might also influence the neighboring BAF60b gene in the placenta. However, it is possible that the influence of the enhancer on gene expression is limited to the GH/CS genes alone and the presence of the 3'-HS in the vicinity of the BAF60b gene may aid in this limitation by functioning as a boundary element or an insulator. These possibilities are yet to be investigated.

In summary, the BAF60b gene and the TRIP-1 gene are located downstream of human GH/CS gene locus and, more specifically, in the region +30 to +40 kb 3' to the CS-B gene. The BAF60b gene exists in the same transcriptional orientation relative to GH/CS genes and in the reverse direction compared to the TRIP-1 gene. Thus, the human GH/CS gene locus is flanked by a muscle-specific, SCN4A, and a B-lymphocyte specific, CD79b,

genes and two widely expressed, BAF60b and TRIP-1, genes within a 150 kb stretch of DNA on chromosome 17. The conservation of this arrangement, 5'-SCN4A, CD79b, GH gene(s), BAF60b, TRIP-1-3' in the human and rat genomes as well as common chromosomal assignment for different pairs of these genes in the pig and mouse, indicates physical linkage of these genes. Also, the ability of regions associated with these flanking genes, identified by HSs, to influence GH-N gene expression, indicates a functional significance for this arrangement.

5.3. ISOLATION AND CHARACTERIZATION OF SEQUENCES CONTAINING HYPERSENSITIVE SITES I AND II AS WELL AS III AND V OF THE GH/CS GENE LCR

5.3.1. Introduction

The human GH gene was shown to be regulated by a multicomponent LCR (Jones et al. 1995). The GH/CS gene LCR consists of five hypersensitive sites (HSs), I to V, which are localized between -14.6 and -32.5 kb upstream from the transcription initiation site of the GH-N gene (Jones et al. 1995; Bennani-Baiti et al. 1998b). The HSs I and II are characterized as pituitary-specific, whereas HS IV is placenta-specific in nature (Jones et al. 1995). The remaining HSs III and V were observed with chromatin from both placenta and pituitary nuclei (Jones et al. 1995). Further studies to localize these HSs resulted in mapping of HSs I and II in the 5'-flanking sequences of the B-lymphocyte-specific CD79b gene (Bennani-Baiti et al. 1998a), whereas the HS III to V were localized to intronic sequences of the skeletal muscle-specific SCN4A gene (Bennani-Baiti et al. 1995). Furthermore, the function of HSs I and II as a pituitary-specific enhancer for the GH-N gene was well established using transgenic mice (Jones et al. 1995; Bennani-Baiti et al. 1998). When a fragment including the HSs III and V region was fused to the 2.6 kb GH-N structural gene (with 0.5 kb of 5'-flanking sequences of GH-N gene) and used as transgene, the GH-N gene was expressed in the transgenic mouse pituitary, albeit 100 times less than that of a transgene that included HSs I and II (Jones et al. 1995).

Interestingly, in the same transgenic mouse, very high levels of GH-N gene expression were observed in the kidney (Jones et al. 1995). However, none of the transgenic mouse which included placenta-specific HS IV sequences resulted in GH-N expression in mouse placenta (Jones et al. 1995). This could be due to anatomical differences between the placentas of human and mouse. Also the GH-N gene is not expressed in the placenta and the GH-related CS/GH-V genes are peculiar to primates. However, no sequence information about these HS regions was made available in these studies (Jones et al. 1995).

5.3.2. Objective

Isolation and subsequent sequencing of the HS regions has the potential to reveal elements involved in both the generation and the functional activity of HSs *in vivo*. Furthermore, *in vitro* characterization of these regions could increase the understanding of transcriptional mechanisms involved in GH and CS gene expression in their respective tissues.

5.3.3. Experimental design

A 1.6 kb *Bgl*II (1.6G) fragment containing the HSs I and II regions and a 10 kb *Xba*I (10X) fragment which includes the sequences of HSs III to V were isolated from the P1-3 clone by agarose gel electrophoresis as described in the Materials and Methods section. The 1.6G fragment was subcloned into the *Bgl*II site of pSP73 and the 10X fragment was subcloned into the *Xba*I site of pBS.

The complete sequencing of the 1.6G fragment was done in our laboratory. Furthermore, nuclease protection assays on subfragments of 1.6G fragment revealed a large pituitary-specific DNaseI protected region of 133-136 bp corresponding to nucleotides 1344/1476 of the 1.6G. From this 136 bp sequence (Fig. 5.13), three overlapping fragments corresponding to nucleotides 1343/1424, 1377/1411 and 1413/1461 containing AT-rich elements were designed.

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Fig. 5.13. Sequence of the nuclease protected region (nucleotides 1343/1478) of the 1.6G fragment. Putative GHF-1/Pit-1 sites were double underlined.

atgggcctcaagctgacctcaggtgatgttttatatttctgagctgtttattccatgaa
ctgaacatctgacagcttttcagagaaatgttttttcatttggaacatctggaaacaaga
aagaacatctggggctgcc

Oligonucleotide 1343/1424

atgggcctcaagctgacctcaggtgatgttttatatttctgagctgtttattccatgaa
ctgaacatctgacagcttttcaga

Oligonucleotide 1413/1461

cagcttttcagagaaatgttttttcatttggaacatctggaaacaagaa

Oligonucleotide 1377/1411

tttctgagctgtttattccatgaactgaacatctg

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These oligonucleotides were characterized for their ability to bind GHF-1/Pit-1 by gel mobility shift as well as "supershift" assays with pituitary GC nuclear protein.

5.3.3.1. Gel mobility and supershift assay

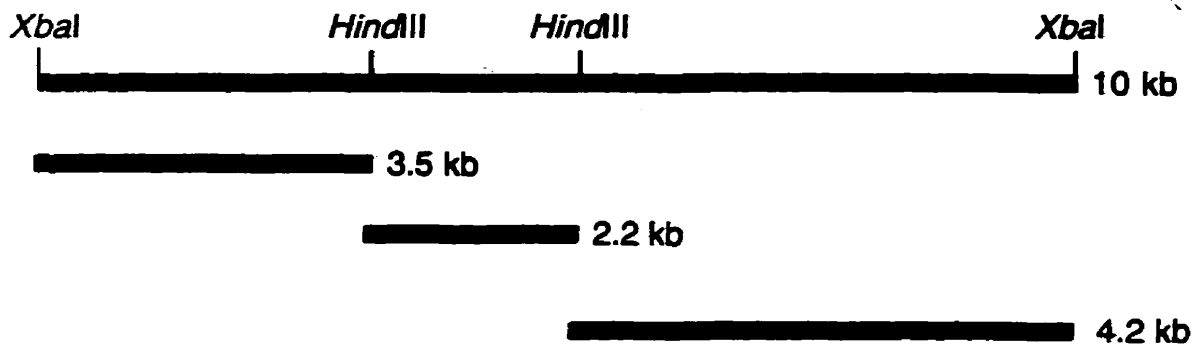
Initial binding reactions for gel mobility shift assay were done by incubating 5 μ g of pituitary GC nuclear protein with 2 μ g of poly dIdC, 7.2 mM MgCl₂ and ³²P-labeled DNA fragments (100-250 pg; 5-7x10⁴ cpm). Reactions were done in binding buffer (20 mM HEPES-KOH pH 7.9; 20% glycerol; 100 mM KCl; 0.2 mM EDTA; 0.5 mM DTT) for

15 minutes at 4 °C, followed by 5 minutes at room temperature. For competition, corresponding double stranded oligonucleotides were added along with radiolabelled DNA. The “specific” GHF-1/Pit-1 competitor, corresponding to the proximal site in the GH-N gene promoter, and “non-specific” RF-1 competitor, corresponding to a region in the 3'-flanking region of the CS-B gene were used. For supershift assays, after the initial incubation, 3 µl of either rabbit GHF-1 antiserum or normal rabbit serum was added to the binding reaction (23 µl final volume) and incubated for 2 h at 4 °C. Antibodies to the carboxy terminal region (amino acids 274-285) of GHF-1 (lot # 5032-2 and # 5033-3) were kindly provided by Drs M. Karin and C.Caelles (University of California, San Diego, La Jolla, California). The DNA-protein complexes were resolved in non-denaturing 4% polyacrylamide gels as described in the Materials and Methods section.

5.3.3.2. Generation of hybrid CAT gene constructs containing sequences of the HSs III-V

The isolated 10X fragment was digested with *Hind*III and the resulting *Xba*I-*Hind*III (3.5 and 4.2 kb) and *Hind*III-*Hind*III (2.2 kb) fragments (Fig. 5.14) were gel isolated and subcloned into the respective sites of pUC19. The 10X and *Xba*I-*Hind*III as well as *Hind*III-*Hind*III fragments were also subcloned into the respective sites of CS-Ap.CAT or CS-Ap.CAT.Enh constructs. Growth and maintenance of cell lines, transient transfections, reporter gene analysis and PCR sequencing were detailed in the Materials and Methods section.

Fig. 5.14. Restriction map and subfragments of the 10X fragment. The 10 kb *XbaI-XbaI* (10X) fragment containing the HSs III-V of the GH/CS gene LCR and the resulting subfragments with digestion of *HindIII* (H) are shown. These fragments were ligated into the respective sites of CS-Ap.CAT or CS-Ap.CAT.Enh constructs, generating 10X.CS-Ap.CAT, 10X.CS-Ap.CAT.Enh, 3.5X-H.CS-Ap.CAT, 3.5X-H.CS-Ap.CAT.Enh, 2.2H.CS-Ap.CAT, 2.2H.CS-Ap.CAT.Enh, 4.2H-X.CS-Ap.CAT and 4.2H-X.CS-Ap.CAT.Enh. These constructs were used in transient transfections along with CS-Ap.CAT and CS-Ap.CAT.Enh to determine the role of HSs III-V of the GH/CS gene LCR.



5.3.4. Results and Discussion

5.3.4.1. GHF-1/Pit-1 recognizes the AT-rich sequences present in the 1.6G (HSs I and II) fragment

The 1.6G fragment was characterized as a pituitary-specific enhancer in the transgenic mouse (Jones et al. 1995; Bennani-Baiti et al. 1998). Initial studies by Cooke et al (1997) localized the HSI and II activity to 3'-half of the 1.6G fragment. Although, our laboratory had first submitted the complete sequence of the 1.6G fragment to the GenBank Data Library (Accession Number: AF010280), recently Bennani-Baiti et al (1998b) also published the same sequence (Accession Number: AF039413) and localized the enhancer activity to a 400 bp region in the 3'-sequences of the 1.6G fragment. Furthermore, the functional characterization using transient transfection assays led to localization of the

enhancer activity to a 264 bp subfragment (nucleotides 1342/1605) in our laboratory (Y. Jin and P.A. Cattini, unpublished observation). Furthermore, DNase I protection assays revealed a large region of 133-136 bp (nucleotides 1344/1478) protected with pituitary GC cell nuclear extracts. Examination of this region revealed three AT-rich sequences at positions 1375/1367, 1388/1396 and 1430/1438 (Fig. 5.13), suggesting the possibility of GHF-1/Pit-1 binding in the the large pituitary-specific protected region (nucleotides 1344/1478).

Three overlapping fragments corresponding to nucleotides 1343/1424, 1377/1411 and 1413/1461 containing AT-rich elements were designed. These oligonucleotides were used as probes as well as competitors in gel mobility shift assays with pituitary GC nuclear extracts. Complexes were also competed with a (consensus) GHF-1/Pit-1 element from the hGH-N proximal promoter (nucleotides -100/-60; 5'-CGTCAGTGGCCCCATGCATAAA TGTACACAGAAACAGGTG-3') (Lefevre et al. 1987) or unrelated RF-1 element from the CS-B enhancer 241 bp region (nucleotides 14/36; 5'-CTCATCAACTTACGTCGGAC GGC-3') (Walker et al. 1990; Lytras and Cattini, 1994). For the region 1343/1424, which contains two putative GHF-1/Pit-1 sites at nucleotides 1367/1376 (TGATGTTTAT) and 1388/1398 (TTTATTCCATG), 6 specific complexes (a1-6) corresponding to both low and high mobility bands, and reflecting both "larger" and "smaller" complexes were observed and competed effectively with non radiolabeled 1343/1411 fragment (Fig. 5.15). Competition of complexes a1 and a2 was also seen with the GHF-1/Pit-1 element but none of the complexes were competed with unrelated RF-1 element. A major (b1) and minor (b2) complex was observed with the fragment corresponding to nucleotides 1377/1411 (Fig. 5.15). The sequences 1377/1411 are contained within the 1343/1424 fragment but include only one of the two putative GHF-1/Pit-1 sites at position 1388/ 1398. Complex b1 was specific and competed with unlabeled 1377/1411 fragment and, to a greater extent, by the longer 1434/1424 fragment. In contrast, there was little evidence of competition with either the GHF-1/Pit-1 element or the 1413/1461 fragment containing putative GHF-1/Pit-1 site (Fig. 5.15), as well as the RF-1 element (Fig. 5.15). For the region 1413/1424, which contains a putative GHF-1/Pit-1 site at nucleotides 1430/1441 (TTTTTTCATTT), at least

ten complexes (c1-10) ranging in mobility/size were detected (Fig. 5.15). Competition was observed with unlabelled 1413/1424 but not 1377/1411 or the RF-1 element. In contrast, competition of c3-5 was observed with the GHF-1/Pit-1 oligonucleotide. The inability of any fragment other than the 1413/1461 region to compete effectively for c7 suggests this is a highly specific as well as high affinity interaction.

Gel mobility shift assays used in combination with a GHF-1/Pit-1 element as a competitor indicated the presence of GHF-1/Pit-1 binding sites in fragments corresponding to nucleotides 1343/1424 and 1413/1461. Antibodies to GHF-1 were used to obtain a more direct assessment of the participation of GHF-1/Pit-1 in complexes formed at these locations in the 1.6G fragment. More specifically, complexes formed with nucleotide regions 1343/1424, 1377/1411 and 1413/1461 were assessed for their ability to be 'supershifted' with antibodies to GHF-1 in a gel mobility shift assay. Supershifted bands (open arrowheads), observed in the presence of GHF-1 antibodies but not normal rabbit serum, were detected with fragments corresponding to nucleotides 1343/1424 and 1413/1461 but not 1377/1411 (Fig. 5.16).

These results suggest that GHF-1/Pit-1 is involved in the *in vivo* enhancer-like activity of the HSs I and II regions. Furthermore, GHF-1/Pit-1 binding to these regions may also account for the generation of pituitary-specific HSs. As stated earlier, our laboratory localized the enhancer activity associated with 1.6G fragment to a 264 bp subfragment. However, using both 5'- and 3'-deletions of the 1.6G as transgenes, the pituitary-specific activity associated with 1.6G was localized to a 3'- region of 406 bp fragment (nucleotides 1198/1602) in 'transient transgenic' experiments (Bennani-Baiti et al. 1998b). This 406 bp fragment includes the 264 bp subfragment identified in our laboratory. However, no structural characterization was done on this 406 bp fragment and from their sequence analysis of 1.6G fragment the authors concluded that the 406 bp fragment does not contain any GHF-1/Pit-1 related elements (Bennani-Baiti et al. 1998b).

Fig. 5.15. Multiple complexes are associated with A/T-rich regions in the 136 bp nuclease protected domain. Pituitary GC cell nuclear extract (2 μ g) was incubated with 2 μ g of poly (dI-dC) and 32 P-labelled DNA probes (250 pg). For competition assays, competitor double stranded oligonucleotides were added with the radiolabelled fragment. (A): 1344/1425 probe, (a); probe/pituitary protein extract (b) in the presence of 50 (c), 100 (d) and 200 (e) pM excess 1344/1425 fragment, or 50 (f), 100 (g) and 200 (h) pM excess GH-N Pit-1/GHF-1 oligonucleotide, or 50 (i) and 100 (j) pM excess RF-1 element. (B): 1378/1412 probe, (a); probe/pituitary protein extract (b) in the presence of 50 (c) and 100 (d) pM excess GH-N Pit-1/GHF-1 oligonucleotide, or 200 (e) and 400 (f) pM excess 1344/1425 fragment, or 100 (g) and 200 (h) pM excess 1378/1412 fragment, or 50 (i) and 100 (j) pM excess 1416/1455 fragment. (C): 1416/1455 probe (a); probe/pituitary protein extract (b) in the presence of 50 (c) and 100 (d) pM excess GH-N Pit-1/GHF-1 oligonucleotide, or 200 (e) and 400 (f) pM excess 1344/1425 fragment, or 100 (g) and 200 (h) pM excess 1378/1412 fragment, or 50 (i) and 100 (j) pM excess 1416/1455 fragment, or 50 (k) and 100 (l) pM excess RF-1 element. Multiple complexes generated with each of the fragments and detected after gel electrophoresis and autoradiography are indicated.

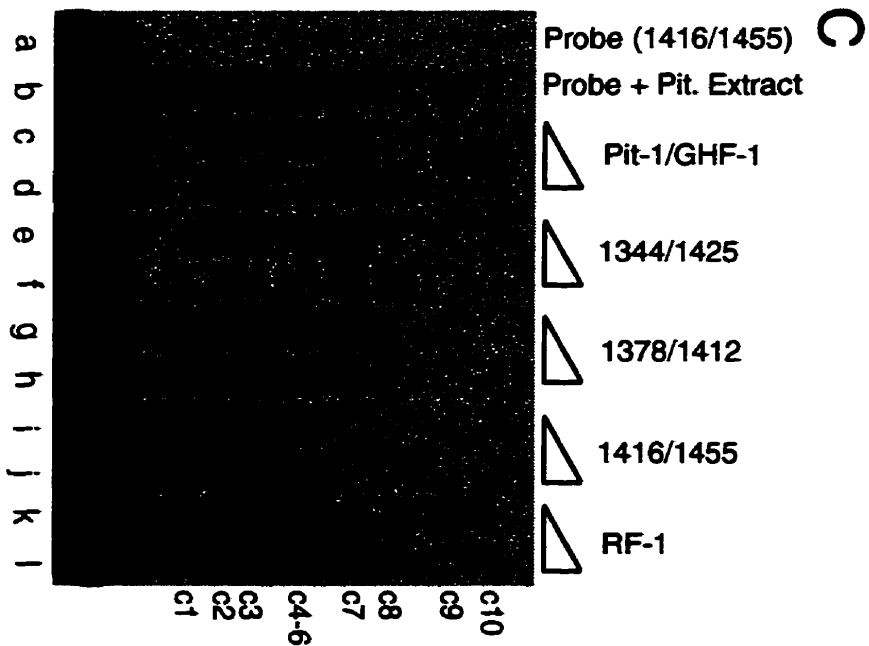
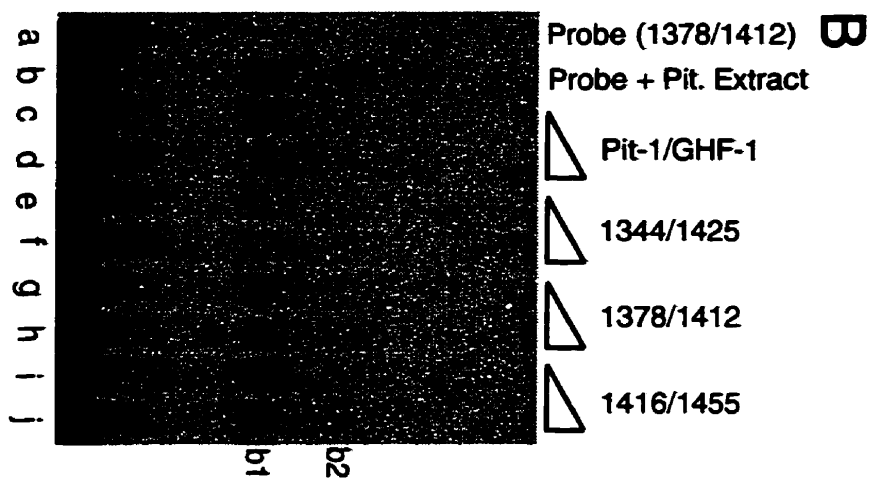
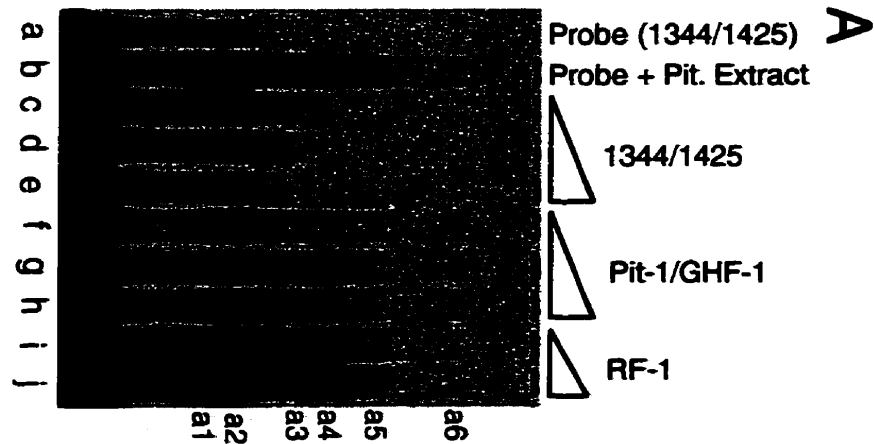
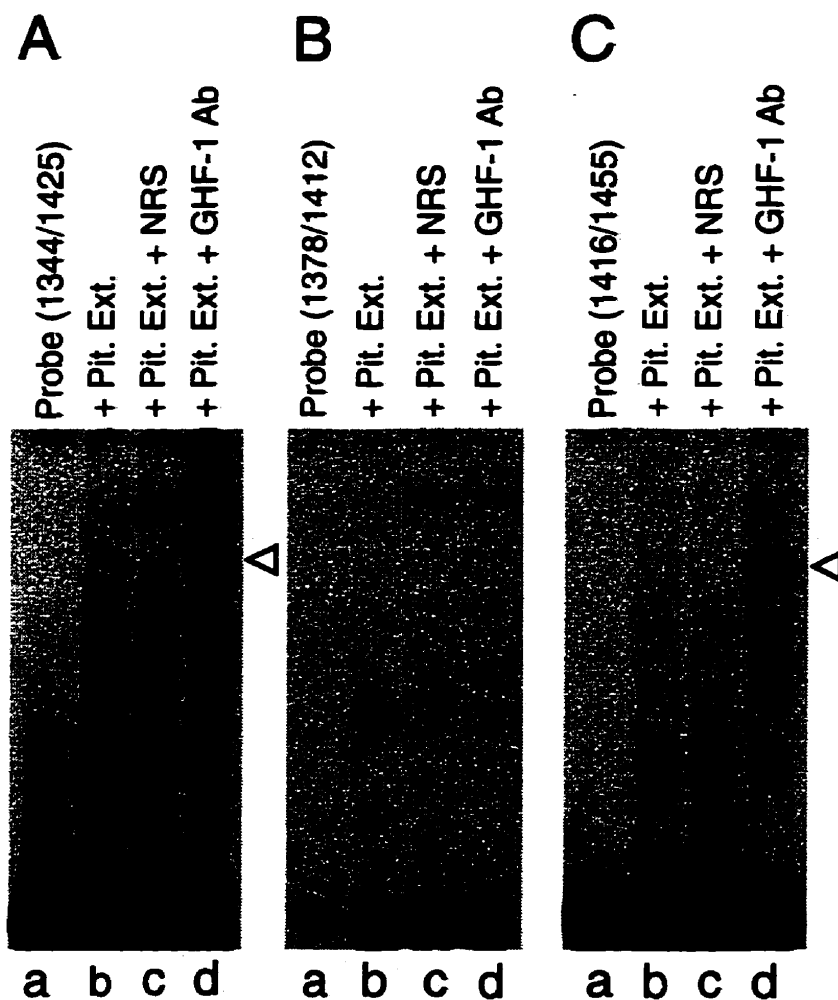


Fig. 5.16. Pit-1/GHF-1 is associated with A/T-rich regions in the 136 bp nuclease protected domain. Radiolabelled DNA probes (250 pg; 1×10^4 cpm) corresponding to (A) 1344/1425, (B) 1378/1412 and (C) 1416/1455, were incubated with 2 μ g of poly (dI-dC) in the absence (a) or presence (b) of pituitary GC cell nuclear extract, with (c) normal rabbit serum (NRS) or (d) GHF-1 antiserum (Ab). "Supershifted" bands detected with GHF-1 antibodies after gel electrophoresis and autoradiography are indicated with open arrowheads.



Although, Bennani-Baiti et al (1998b) failed to identify GHF-1/Pit-1 elements in this region, the activation of reporter transgene expression (used human GH-N gene as reporter) with constructs consisting of HSs I and II was observed between embryonic days 15.5 and 16.5 in the transient transgenic mouse pituitary (Bennani-Baiti et al. 1998b). This expression pattern coincides with the GHF-1/Pit-1 activation of endogenous mouse GH gene in the pituitary. Thus, these results strongly supports the present finding that AT-rich elements in the HSs I and II region are recognized by GHF-1/Pit-1 protein.

5.3.5. Functional characterization of HSs III, IV and V regions

The HSs III to V were located within a 10 kb *Xba*I fragment (Bennani-Baiti et al. 1995; Jones et al. 1995). Although transgenic mice with transgenes bearing these HSs could support low-level pituitary as well as high-level kidney expression of reporter GH-N gene, none of the transgenics expressed GH-N gene in their placentas even though the human placenta-specific HS was also present in the transgene (Jones et al. 1995). However, it is not known whether this HS was reestablished in mouse placenta; reestablishment of HS I, II and III, but not HS V was observed in transgenic mouse pituitary (Jones et al. 1995).

Hence, the functional relevance of human placenta-specific HS IV was studied using human placental choriocarcinoma JEG-3 and JAR cells. Although it is known that HSs will function only in *in vivo* or in stably integrated form, it was shown that the stimulatory activity associated with HS I and II of GH/CS gene LCR could be studied in transient transfections (unpublished results from our laboratory; Bennani-Baiti et al. 1998b). Similarly the enhancer-like functions of the HS2 and HS3 of the β -globin LCR (reviewed by Hardison et al. 1997) and the HSs present within -2.0 kb of rat GH gene (Aizawa et al. 1995) were also studied in transient transfections. Thus, transient transfection assays were chosen to study the functionality of HS III, IV and V in human placental JEG-3 and JAR cells. As indicated earlier, different CS-Ap.CAT and CS-Ap.CAT. Enh constructs consisting of the 10X fragment or various 10X subfragments

(Fig. 5. 14) were used in transient transfections. However, preliminary transient transfection assays did not reveal any functional activity associated with these fragments in the presence or absence of CS-B enhancer elements (data not shown). Furthermore, several attempts to stably integrate the 10X.CS-Ap.CAT or 10X.CS-Ap.CAT.Enh constructs into JEG-3 and JAR cells resulted in the detection of G418 resistant clones. Two clones, one for each construct, 10X.CS-Ap.CAT or 10X.CS-Ap.CAT.Enh, was positive for CAT gene on Southern analysis of the genomic DNA from these clones (data not shown). However, none of these clones expressed reporter CAT gene (data not shown).

Further studies are required to detect the DNA-protein interactions associated with these regions. This will help to identify the elements and their respective DNA-binding proteins. This in turn will help unravel the *in vivo* kidney- and pituitary-specific transcriptional mechanism associated with the HS III and V regions. It is surprising to note that this region did not support the expression of the transgene in the mouse placenta and this might be due, perhaps, to anatomical differences between the mouse and human placenta. However, the human placental chorionic gonadotropin gene family when used as a transgene was expressed in mouse placenta (Strauss et al. 1994) suggesting the mouse trophoblasts can support expression of a human placental gene. Further studies by Jones et al (1995) using a 15 kb fragment containing the CS-A structural gene accompanied by -5.4 kb 5'- and +7.2 kb 3'-flanking sequences as a transgene, resulted in placenta-specific expression of the CS-A gene in mouse placenta. The transgene contains the CS-A proximal promoter, the pituitary-specific repressor PSF sequences (Nachtigal. et al. 1993) in the 5.4 kb 5'-flanking as well as the enhancer element (Jacquemin et al. 1994a) in the 7.2 kb 3'-flanking sequences. However, the expression of the CS-A gene in different lines of transgenic mice with varying copy numbers was not copy number dependent (due to different integration events). Thus, these results indicate that the proximal elements were insufficient to drive the target gene in a reproducible manner (Jones et al. 1995). Similar conclusions were also drawn with transgenes containing the GH-N structural gene accompanied by either -0.5 kb or -7.5 kb of 5'-flanking sequences, although there was pituitary-specific expression of the GH-N gene in the transgenic mouse (Jones et al. 1995).

Initial trials to generate transgenic mice with either rat or human GH-N genes along with their flanking sequences resulted in no expression of GH-N transgene in the mouse pituitary (Hammer et al. 1984; Palmiter and Brinster, 1986). However, it was revealed later that the proximal sequences of the rat GH gene as short as -145 bp were sufficient to target expression of transgenes to the anterior pituitary gland in the mouse (Lira et al. 1993). However, when the -145 target gene (transgene) was fused with rat GH gene upstream sequences (-1.7 kb/-146 bp), enhanced expression of the transgene in the mouse pituitary was observed (Lira et al. 1993). In this study, it was also noted that the transgene expression was not copy number dependent in some transgenic lines (Lira et al. 1993). This type of position effect variegation was also observed with the β -globin LCR (only when individual HSs were deleted) in transgenics (Grosveld et al. 1998). It was thought that heterochromatin had an effect on LCR activation leading to severe reduction in expression (Grosveld et al. 1998). Previously, it was shown that the HS2 and full LCR of β -globin are equivalent: function as classical enhancers in transient transfection assays, increase the number of expressing clones when stably integrated in cell lines, and confer position independence and high level expression in transgenic mice (Pawlik and Townes, 1995; reviewed by Reik et al. 1998). These observations are in contrast to the results obtained with the LCR deletion transgenics, where the transgene expression was sensitive to site of integration even in the presence of an intact HS2 (Grosveld et al. 1998). In contrast to the earlier notion that the β -globin LCR sequences are necessary for keeping β -globin genes in an open chromatin structure, it was shown recently that the open chromatin structure can still be maintained in the absence of HSs 2 to 5 of the β -globin LCR (Reik et al. 1998). Furthermore, it was shown by transgenic studies, that developmental regulation of human γ - and β -globin genes could still be observed even in the absence of the LCR sequences (Starck et al. 1994). These observations indicate that both the LCR and adjacent sequences may form a euchromatin structure that are necessary for β -globin gene expression. Indeed, erythroid-specific transcripts from LCR sequences were detected (Ashe et al. 1997; Tuan et al. 1992). Thus, these possibilities also agree well with the suggested influence of euchromatin (in the form of presence of constitutively active

BAF60b and TRIP-1 genes) on the CS/GH-V gene expression in the placenta. Interestingly, the footprinted region of the HSs I and II of the GH/CS gene LCR also contain a TATA element (Fig. 5.13) and this was recognized by GHF-1/Pit-1 (Fig. 5.13). The B-lymphocyte-specific CD79b gene is located downstream of these sequences (Bennani-Baiti et al. 1998a). Furthermore, both pituitary- and placenta-specific transcripts were detected that were initiated from the GH-N gene promoter sequences (Courtois et al. 1990; Labarriere et al. 1995).

It is well known that the transcriptional regulation of pituitary-specific expression of GH-N gene is highly conserved in vertebrates (Argenton et al. 1993; Yowe and Epping, 1995). Interestingly, the rat GH-N gene is also flanked both upstream and downstream by similar genes to those observed in the hGH/CS gene locus (Bennani-Baiti et al. 1995; 1998a; Kazahari et al. 1997a; b; Nakazato et al. 1998; Nomoto et al. 1997; Surabhi et al. 1998; Surabhi et al. *in press*). However, as seen with hGH-N gene, no HSs were noticed in the vicinity of the CD79b or in the intronic sequences of SCN4A genes (Kazahari et al. 1997a), except for the presence of HSs within a 2.0 kb region upstream of the GH-N gene transcription initiation site (Aizawa et al. 1995). These HSs could be mapped to a region 1.5 kb downstream of CD79b gene, as in the rat genome. The intergenic distance between the CD79b and GH-N genes is 3.3 kb (Nakazato et al. 1998). Interestingly, as stated earlier, 1.7 kb of upstream flanking sequences containing these HSs were shown to enhance the rat GH-N transgene expression in the mouse pituitary (Lira et al. 1993).

Thus, at present it is not known whether the region consisting of HSs I and II have any effect on the adjacent B-lymphocyte-specific gene. It is also not clear why the human GH/CS gene locus acquired a LCR as the homologous regions of rat genome do not possess HSs/LCR (Kazahari et al. 1997a). Furthermore, as stated above, the transcriptional regulation of pituitary-specific GH gene expression in vertebrates is highly conserved (Argenton et al. 1993; Yowe and Epping, 1995). It is yet to be demonstrated clearly that the GHF-1/Pit-1 sites present in the HS I and II region are responsible for the

enhancer activity. It would be interesting to investigate whether other primate GH/CS gene clusters possess a similar LCR. Interestingly, a 60 bp region (nucleotides 946/1005) 5' to the HS I and II in the 1.6G fragment is a highly conserved sequence (90-98%) in many human chromosomes. Although this region is not needed for pituitary-specific enhancer activity (unpublished results from our laboratory; Bennani-Baiti et al. 1998), its ubiquitous presence indicates it may have a role *in vivo*. However, unlike the human β -globin LCR, the factors recognizing the HSs in hGH/CS gene LCR are still not known. Although much is known about the human β -globin LCR, it is still not clear how the LCRs activates these genes. It has been suggested that activators such as NF-E2 bound to the LCR sequences may interact with TBP and TAFII30 and mediate long-range activation of the α - and β -globin gene loci (Amrolia et al. 1997). Furthermore, the LCR may interact with individual promoters, interacting one gene at a time (Grosveld et al. 1998). Thus, further studies are required to understand the role of HSs and LCRs in gene expression.

CHAPTER 6

FINAL COMMENTS AND FUTURE PERSPECTIVES

The mechanism of transcriptional control of a gene expression is often presented in a very simple manner with the necessary information for this process localized in the proximal sequences upstream of the transcription initiation site of the gene. Much of the research work has focused on these sequences. This assumption holds good not only *in vitro* but also in the *in vivo* situation for most cases of gene expression. Similar observations were also made with the hGH/CS promoters described in this thesis. Although, the minimal -133/+1 CS-A promoter with one GHF-1/Pit-1 element was not active *in vitro* in GC cells, it was sufficient to support the pituitary-specific transcription of a reporter gene when the gene was integrated into GC cell chromatin. These results also corroborated observations by others that activated transcription of a gene in its chromatin structure occurs at several fold higher levels than that of a gene in its naked form.

In vitro studies using the proximal promoter sequences of a gene have been very useful in the identification of regulatory elements, and subsequently this has led to isolation of the sequence-specific DNA-binding proteins. However, several laboratories are trying to understand the role of these transcription factors in gene expression at the chromatin level. Many of these studies have identified protein complexes which contain both general and sequence-specific transcription factors as well as mediators of transcription, chromatin remodeling and modifying enzymes. Identification of such complexes suggest that a gene can be regulated either positively or negatively depending on the specific transcription factor and other proteins present in the complex. This specific complex formation appears to be modulated by the cell environment and external signals.

In this thesis, attempts were made to isolate the PSF-sequence-specific factors which are responsible for the pituitary repression of the human placental GH genes. Recently the pituitary-specific GHF-1/Pit-1 factor was suggested to form both negative and

positive transcriptional complexes. Our laboratory had made an observation the GHF-1/Pit-1 and PSF factors participate in common complexes. These observations are very interesting and it is possible that the complex containing the GHF-1/Pit-1 and PSFs is a negative regulating complex. This thesis work suggests that the PSFs are NF-1-like factors and NF-1-like factors were shown to possess even a negative regulatory effect in pituitary cells, especially on GH-N gene expression. Recently, we had obtained the NF-1 cDNA clone from Dr Tjian, University of California, Berkeley. Thus, part of future studies will be to understand the mechanism of interaction between GHF-1/Pit-1 and NF-1 as well as identify complexes which contain histone deacetylase activity. This will allow an increased understanding of CS/GH-V gene repression in a pituitary environment.

However, it is well known now that far-upstream as well as far-downstream sequences play an important role in gene expression. The factors binding to the far-upstream sequences or LCRs appear to interact with factors recognizing the proximal promoter sequences by DNA looping and thus affect expression of that particular gene. A LCR region for the hGH/CS gene family was reported recently. This region was isolated and partially characterized as part of this thesis. The suggestion from this thesis work is that the sequences associated with pituitary-specific HSs I and II of the LCR are recognized by GHF-1/Pit-1. This is very exciting and enhances one's understanding about the transcriptional regulation of hGH/CS gene family. It is known that the GHF-1/Pit-1 is expressed in human placenta and placental cell lines. This observation suggests that GHF-1/Pit-1 may not be the sole factor involved in the generation of the pituitary-specific HSs I and II. It is possible that in a placental environment, GHF-1/Pit-1 is not forming a protein complex which contains the chromatin remodeling enzymes, thus the HSs I and II are not generated in placenta. Thus, further work is needed to be done to characterize the GHF-1/Pit-1 protein complexes in the placenta. It is also necessary to identify other factors recognizing the LCR sequences.

Furthermore, it is also known that the expression of a gene is influenced by the neighboring chromatin structure. One of the possible mechanisms suggested for LCR

function in the erythroid-specific expression of the β -globin genes is that the LCR and the intergenic sequences between the ϵ -gene and LCR are transcribed in an erythroid-specific manner. Under these circumstances, these sequences may form an open chromatin structure in the vicinity of the β -globin genes. This structure may be responsible for generation of HSs as well as aid in the transcription of the β -globin genes in erythroid cells. It is interesting to note that HSs I and II of the hGH/CS gene LCR are localized in the immediate upstream sequences of the B-lymphocyte-specific CD79b gene, whereas the region containing the HSs III to V is located in the intronic sequences of the skeletal muscle specific SCN4A gene. Hence, future work will focus on revealing the transcriptional status of the CD79b and SCN4A gene in both pituitary and placental tissues. Furthermore, CD79b presumably forms an open chromatin structure in lymphocytes, and this could contribute to GH/CS genes expression in these cells. However, further work needs to be done to unravel the transcriptional status of the GH/CS genes both in lymphocytes and skeletal muscle cells and this may provide an interesting insight into the influences of neighboring chromatin structure on gene expression.

Furthermore, in this thesis, characterization of the downstream sequences of the hGH/CS gene loci revealed two constitutively and ubiquitously expressed genes, specifically BAF60b and TRIP-1. It was suggested that the presence of an open chromatin structure downstream of GH/CS genes may be responsible for the generation of placenta-specific 3'-HS, which in turn prevents the formation of a heterochromatin structure of the highly repetitive GH/CS gene sequences. However, further work needs to be done to characterize the intergenic sequences between the CS-B and BAF60b genes to characterize the 3'-HS as well as to verify the absence of any placenta-specific gene in these sequences.

The cell architecture including cytoskeletal and nuclear matrix structures are known to be involved in gene expression. Due to the dynamic nature of these structures, which respond to both internal and external stimuli, many investigators are now suggesting that even mechanical stimuli also influence the gene expression. Placental tissue is known to express many genes and some at very high levels. The mechanical stimuli brought about by

both contractions of the uterus and movements of the fetus could influence levels of expression of the CS genes in placental cells. Furthermore, nuclear localization patterns of the chromatin are also known to influence gene expression. The dynamic nature of the nuclear matrix and cytoskeletal structures in placental as well as pituitary cells may differentially localize the GH-N and CS/GH-V genes in their nuclei. This differential subnuclear localization may be responsible for differential expression of these genes in these tissues. Hence, further work may be aimed at the identification of the nuclear matrix regions of the hGH/CS gene loci in pituitary, placenta and non-pituitary/-placenta tissues. This information will be useful to understand the transcriptional control mechanisms involved in GH/CS gene expression. Interestingly, the 1.6G fragment comprising the HSS I and II, contains a highly conserved 60 bp fragment, which has homology with many human chromosomal sequences. This suggests that this sequence may have a common functional role *in vivo*. Analysis of these sequences might reveal their function.

As part of this thesis, a potent transcriptional activator in the form of a truncated TEF-1 was identified, and a role for this type of factor in gene expression was suggested. Further work needs to be done to characterize the nature of the interaction of this truncated form of TEF with other transcription factors or regulators both in HeLa and other cells of different tissue origin.

Finally, by combining results from various aspects of this thesis relating to hGH/CS gene expression, and with the anticipated results from the suggested future work, this will go a long way to address the original hypothesis that **the hGH/CS genes, for that matter any gene, are controlled by a complex network of transcriptional mechanisms which involve both proximal, far-upstream and far-downstream sequences as well as the neighboring chromatin structure and the nuclear matrix regions.**

APPENDIX 1

A.1. EFFECT OF PUTATIVE C-SEQUENCES ON THE EXPRESSION OF PLACENTA-SPECIFIC EXPRESSION OF THE HUMAN PLACENTAL GROWTH HORMONE GENES

A.1.1. Introduction

The four placental members of the GH/CS gene family are CS-L, CS-A, GH-V and CS-B. These genes are located as a single locus along with GH-N gene on chromosome 17 (Chen et al. 1989). The placental GH genes are expressed only in the syncytiotrophoblasts of placenta during pregnancy (Cooke and Liebhaber, 1995; McWilliams and Boime, 1980; Parks, 1989). Although CS-A, CS-B and GH-V genes are expressed in the placenta and released into the maternal circulation (Barrera-Saldana et al. 1982; Igout et al. 1988; Josimovich and MacLaren, 1962; Seeburg et al. 1977). the CS-L gene has been reported to be a pseudogene (Hirt et al. 1987) and capable of producing transcripts (Chen et al. 1989; Misra-Press et al. 1992) but no protein. However, there is both a placenta stage-dependent and an individual gene-dependent differential expression pattern among these four genes in the placenta. The CS mRNA is almost 4 times greater in the total RNA from term placenta than in total first trimester RNA (McWilliams et al. 1977). Furthermore, it was observed that 10% of the placental mRNA is from CS-A/CS-B relative to 0.01% and 0.001% for the CS-L and GH-V mRNA, respectively. This was revealed during screening and quantitation of individual clones in a cDNA library prepared from term placenta, hybridizing to gene specific probes (Chen et al. 1989). However, Cooke et al. (1988) observed that the population of GH-V mRNA in the placenta is approximately 0.06% of the total RNA. Regardless both these studies had suggested a lower level of GH-V and CS-L gene expression as compared with the CS-A and CS-B genes in the syncytiotrophoblast. Since these genes share greater than 90% sequence similarity in their structural and flanking DNA, the mechanism by which these genes are differentially regulated in the same cell during pregnancy is particularly intriguing. Although the GH/CS promoters were shown to

be active in GC cells after gene transfer, CS-A promoter (-496/+1) activity is consistently stronger than GH-V promoter (-496/+1) activity (Nickel et al. 1990b). Unfortunately the suggestion of a weaker GH-V promoter cannot be tested in placental cells, as the GH/CS promoters (-496/+1) are poorly active in placental cell lines after gene transfer (Fitzpatrick et al. 1990, and results from this thesis). Thus, there is a possibility of the presence and involvement of additional regulatory elements associated with GH/CS genes for expression in placental cells. These regulatory sequences may, for CS-L/GH-V genes, act as **repressors**, or in the case of the CS-A/CS-B genes, act as **enhancers**. As discussed in Chapter 3, our laboratory identified the PSF elements, present in the P-sequence regions of the placental genes, as repressors and showed that the PSFs were responsible for repression of placental members of the GH/CS gene locus in the pituitary (Nachtigal et al. 1993). Examination of the GH/CS gene flanking sequences also revealed **cytosine-rich** region, "C" sequences, of about 50 bp located between nucleotides (nts) position -680 and -620 of the GH-V, and of about 20 bp located between the nts -639 and -619 of the CS-L genes (Fig. A.1), but not CS-A and CS-B genes (Chen et al. 1989). Hence, it was hypothesized that these C-sequences contribute to the low level expression of GH-V and CS-L versus the CS-A and CS-B genes in the placenta.

A.2. Functional characterization of the C-sequences

A.2.1. Objective

The C-sequences in the 5'-flanking sequences of the CS-L and GH-V contain regulatory elements that are responsible for low level expression of these genes in the placenta. Hence, in the presence of the C-sequences, the activity of the GH/CS promoters would be repressed in the placental cells.

Fig. A. 1. Comparison of 5'-flanking sequences of GH/CS genes. The GH-N (-614/-496), the CS-A and CS-B (-625/-496), the CS-L (-638/-496) and GH-V (-685/-496) 5'-flanking sequences were compared. The sequences corresponding to Cytosine-rich sequences were double-underlined. (:) denotes similarity in sequence. (-) was introduced in the sequence to enhance the probability of homology.

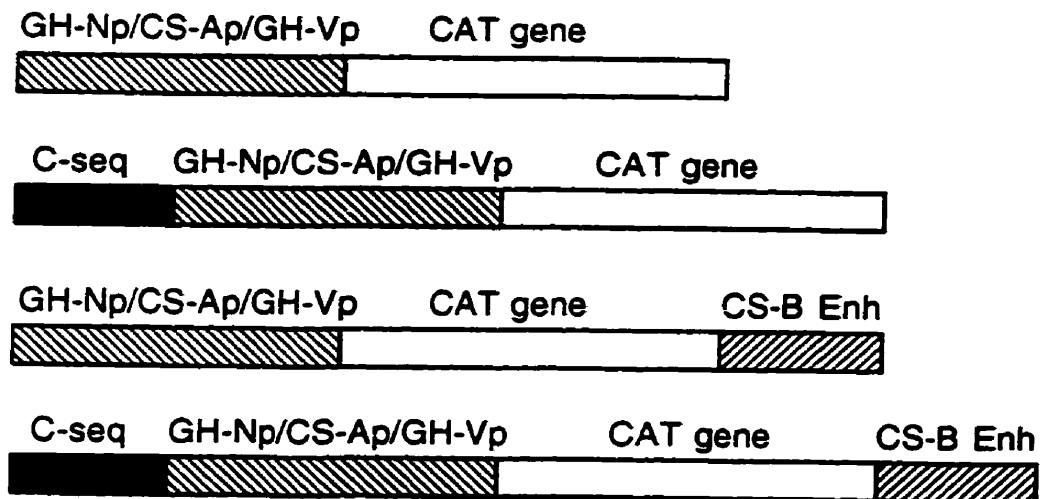
GH-V	<u>CCCCCCCCCC CCACACCCAC ACCCA--CCC CCACCCCCAC CCCCATCCCC</u>				
CS-L			:GC::	:--::	:CC::
CS-A					::
CS-B					::
GH-N					
GH-V	<u>ACGCCCCGCC CCCGCCCCCG CCCCAAGGCT CAGGTAAAGG GGAGAGCAAA</u>				
CS-L	-----	: T::	:--::	:G::	:T::
CS-A		: A::	:T::	:G::	:T::
CS-B		: A::	:T::	:G::	:T::
GH-N		: C	:AG::	:T::	:G::
GH-V	GTGTGGAGTT	TGTTCCCTCT	AGTGGTCAGT	ATTAGCACTG	CATCCAG---
CS-L	:G::	G::	:G::	:G::	:CTG
CS-A	:G::	G::	:T::	:G::	:CTG
CS-B	:G::	G::	:T::	:G::	:CTG
GH-N	:G::	G::	:T::	G::G:A::	:CTG
GH-V	-----GCGG	GCCTGTCTCA	GACGAGCCCA	GGAGGAGTCA	GCAAAAGTGG
CS-L	ACTCAG:T::	-----	-----	:TC::	:G::
CS-A	ACTCAG:T::	-----	-----	:TCA::	:G::
CS-B	ACTCAG:T::	-----	-----	:TCA::	:G::
GH-N	ACTCAG:T::	-----	-----A::	:TCC::	:G::

A.2.2. Experimental design

A 290 bp *Sma*I fragment, corresponding to -783/-496 region of the GH-V gene containing the putative C-sequences, was isolated and subcloned into the *Sma*I site of pUC19 in our laboratory. The putative C-sequence (300 bp) fragment was released by *Sma*I digestion and gel isolated. This fragment, in both orientations was introduced into

blunted *Hind*III site of either GH-Np.CAT, GH-Np.CAT.Enh, CS-Ap.CAT, CS-Ap.CAT.Enh, GH-Vp.CAT and GH-Vp.CAT.Enh vectors resulting in constructs C-seq.promoter.CAT or C-seq.promoter.CAT.Enh (Fig.4.2). These hybrid genes were transiently transfected into either human placental choriocarcinoma JEG-3 or rat pituitary tumor GC cells to determine the influence of these putative C-sequences on the GH-N, CS-A and GH-V promoter activity. Approximately 15 µg of each plasmid was used per 100 mm culture dish. The CAT activity was expressed as CAT units/mg of protein.

Fig. A.2. Hybrid CAT gene constructs containing the putative C-sequences. The CAT gene was placed downstream of the proximal promoter sequences (-496/+1) of the GH-N, CS-A and GH-V genes. C-sequences were introduced upstream to the GH/CS promoters. Constructs were also generated containing CS-B enhancer sequences.

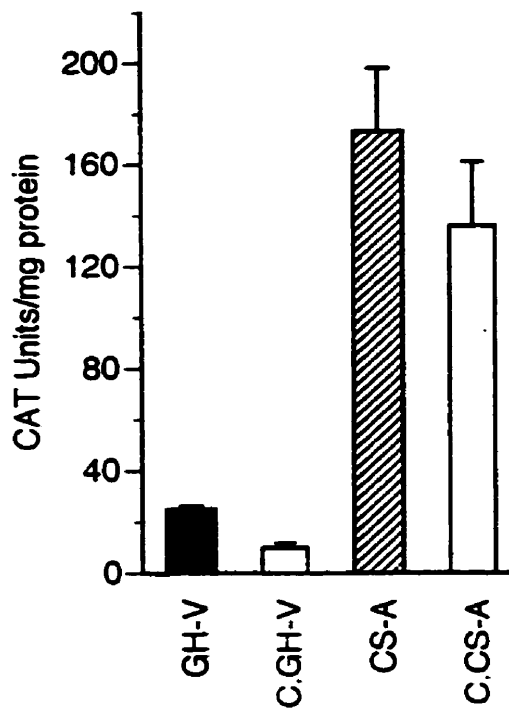


A.2.3. Results and Discussion

A.2.3.1. The putative C-sequences specifically repressed the GH-V promoter activity in the rat pituitary GC cells

Initially, the influence of the putative C-sequences on GH-V promoter was determined as the C-sequences were found upstream of this gene (Chen et al. 1989). The GC cells were transfected with constructs without enhancer sequences whereas the JEG-3 cells were transfected with all constructs. From the analysis of CAT gene expression, in GC cells we observed the GH-V promoter activity was repressed in the presence of putative C-sequences by more than 60% and was statistically significant (Fig. A.3; $n=3$, $p=0.0018$). Interestingly, the putative C-sequence did not have any significant effect on the CS-A promoter activity (Fig. A.3; $n=3$, $p>0.05$). However, the activity of CS-A promoter was consistently higher than that of GH-V promoter and this is in accordance with earlier observations made in our laboratory (Nickel et al. 1990). These preliminary results suggest that the putative C-sequences may harbour a repressor element. Furthermore, the putative C-sequences may also participate in restricting the pituitary expression of the GH-V gene, possibly in combination with PSF elements.

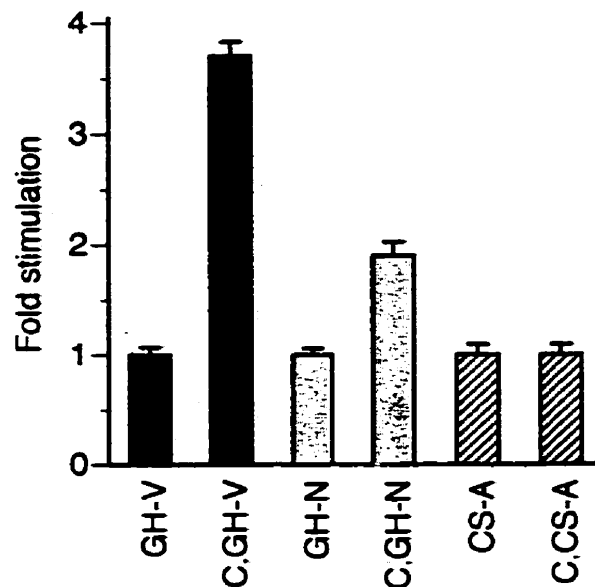
Fig. A.3. GH-V but not CS-A promoter activity is downregulated in the presence of putative C-sequences (C) in rat pituitary GC cells. Approximately 0.5×10^6 GC cells per 100 mm culture dish were transfected with 15 μ g of each GH-Vp.CAT (GH-V), C.GH-Vp.CAT (C.GH-V), CS-Ap.CAT (CS-A) or C.CS-Ap.CAT (C.CS-A). Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. The activity of GH-V promoter was significantly repressed in the presence of putative C-sequences ($n=3$, $p=0.0018$), whereas there was no significant difference in the CS-A promoter activity in the presence or the absence of putative C-sequences ($n=3$, $p>0.05$). The mean value in CAT units per milligram of protein is shown and was derived from at least three determinations. Error bars indicate standard error of the mean. (A DNA fragment containing nucleotides from -496 to +1 of the GH-V or CS-A gene was used as GH-Vp or CS-Ap, respectively).



A.2.3.2. The putative C-sequences specifically stimulated GH-V promoter activity in the placental cells

In contrast to GC cells, the putative C-sequences enhanced (ranging from 3.5 to 10 fold) GH-V promoter activity in placental JEG-3 cells (Fig. A.4; $n=5$, $p=0.0064$). Furthermore, a similar but more modest (~1.9 fold) stimulatory effect was seen with GH-N promoter activity (Fig. A.4; $n=3$, $p=0.0044$). However, the putative C-sequences did not significantly influence CS-A promoter activity (Fig. A.4; $n=5$, $p=0.5797$).

Fig. A.4. GH-V and GH-N but not CS-A promoter activity is upregulated in the presence of putative C-sequences (C) in human choriocarcinoma JEG-3 cells. Approximately 1.0×10^6 JEG-3 cells per 100 mm culture dish were transfected with 15 μ g of each GH-Vp.CAT (GH-V), C.GH-Vp.CAT (C.GH-V), GH-Np.CAT (GH-N), C.GH-Np.CAT (C.GH-N), CS-Ap.CAT (CS-A) or C.CS-Ap.CAT (C.CS-A). Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. Effect of C-sequences are expressed as fold stimulation over the respective promoter activities in the absence of C-sequences. The activities of GH-V promoter ($n=3$, $p=0.0064$) and GH-N promoter ($n=3$, $p=0.0044$) were significantly enhanced in the presence of putative C-sequences, whereas there was no significant difference in the CS-A promoter activity in the presence or the absence of putative C-sequences ($n=3$, $p=0.57$). Error bars indicate standard error of the mean. (A DNA fragment containing nucleotides from -496 to +1 of the GH-N, GH-V or CS-A gene was used as GH-Np, GH-Vp or CS-Ap, respectively).

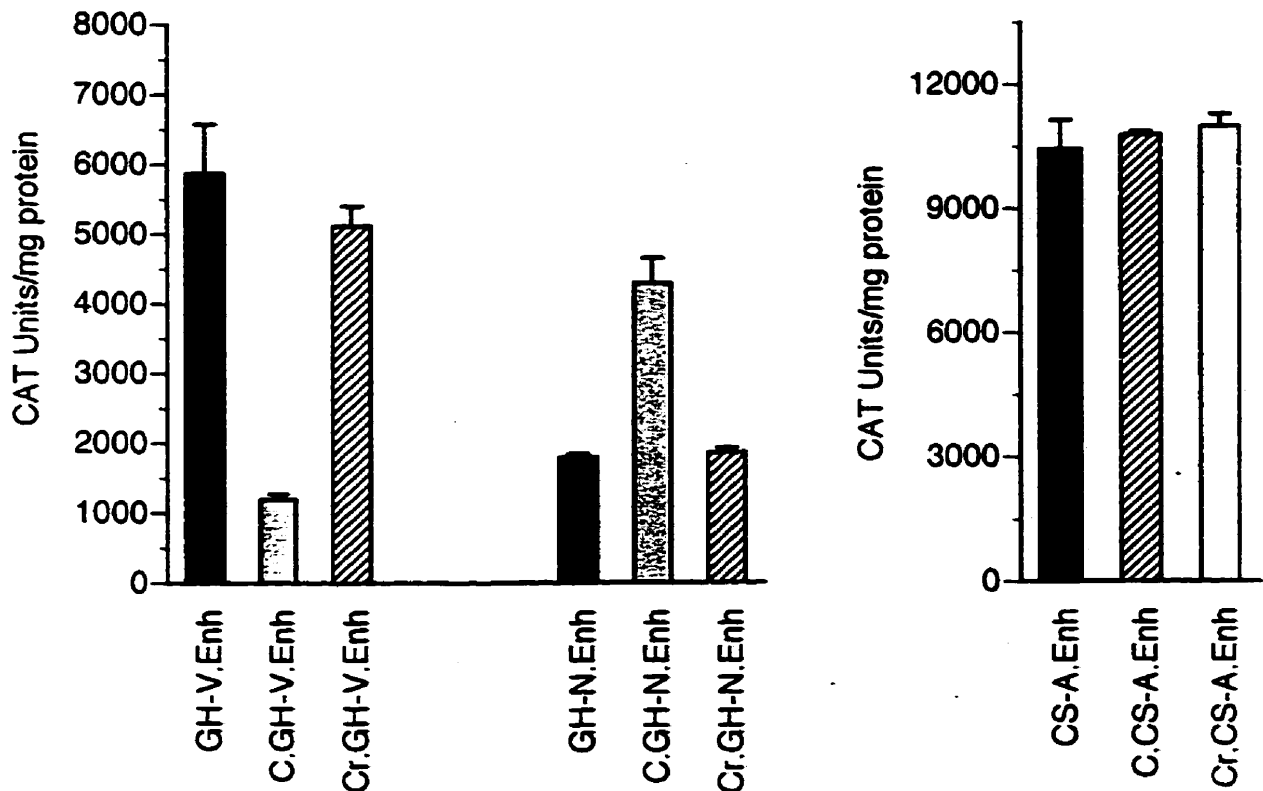


A.2.3.3. Differential regulation of GH-V/CS-A promoters by the putative C-sequences in the presence of CS-B enhancer region in placental cells

Additional transient transfection assays in JEG-3 cells with GH-V and CS-A promoter constructs bearing CS-B enhancer elements were performed. These assays revealed a differential regulatory effect of the putative C-sequences. In their original orientation, the putative C-sequences had a significant repressor effect on CAT gene expression which was now under the influence of the CS-B enhancer and GH-V promoter (Fig. A.5; $n=6$, $p<0.0001$). The GH-V gene differs from CS genes by the lack of an enhancer in its 3'-flanking sequences. However, this repressor activity in the presence of CS enhancer could reflect an important transcriptional regulatory event as the CS-A enhancer is located upstream of the putative C-sequence region of the GH-V gene in the context of the GH/CS locus on chromosome 17.

The putative C-sequences did not have any effect on CS-A.CAT.Enh activity (Fig. A.5; $n=6$, $p=0.65$). However, GH-N.CAT.Enh was 2.4 times more active in the presence of the putative C-sequences (Fig. A.5; $n=6$, $p<0.0001$). These differential effects of the putative C-sequences on the GH/CS promoters were unexpected and this prompted an investigation of the factors binding to the putative C-sequences. These results also raised the possibility of an interaction between the factors binding to the elements in the GH/CS promoters and the putative C-sequences, which in turn might be responsible for the differential activities of individual GH/CS gene promoters. In terms of their activity in rat pituitary GC cells, CS-Ap is more active than GH-Np, as well as GH-Vp (Nachtigal et al. 1993; Nickel et al. 1990). Until recently it was believed that GHF-1/Pit-1 was not expressed in placenta (Bodner and Karin, 1987), however, recent studies from three separate groups have detected GHF-1/Pit-1 in human placenta, choriocarcinoma JEG-3 cells and rat placenta (Bamberger et al. 1995; Lee et al. 1996; Schanke et al. 1997). This suggests a role for GHF-1/Pit-1 in GH/CS and/or rat placental lactogen gene expression in the placenta. Therefore, it might be possible that GHF-1/Pit-1 recognizes the corresponding elements in the GH/CS promoter sequences in placenta and placental cells.

Fig. A.5. Differential regulation of CS-B enhancer-associated GH/CS promoter activity in the presence of the putative C-sequences (C) in human choriocarcinoma JEG-3 cells. Approximately 1.0×10^6 JEG-3 cells per 100 mm culture dish were transfected with 15 μ g of each GH-Vp.CAT.Enh (GH-V.Enh), C.GH-Vp.CAT.Enh (C.GH-V.Enh), GH-Np.CAT.Enh (GH-N.Enh), C.GH-Np.CAT.Enh (C.GH-N.Enh), CS-Ap.CAT.Enh (CS-A.Enh), or C.CS-Ap.CAT.Enh (C.CS-A.Enh). Effect of C-sequences in reverse orientation was also tested in these experiments. Forty eight hours after transfections, cell extracts were prepared and assayed for CAT gene expression. The influence of the presence of putative C-sequences on the CS-B enhancer-associated GH-V and GH-N promoter activities was highly significant ($n=6$, $p<0.0001$). The mean value in CAT units per milligram of protein is shown and was derived from at least six determinations. Error bars indicate standard error of the mean. (C-sequence fragment in reverse orientation was indicated as "Cr" in the figure. A DNA fragment containing nucleotides from -496 to +1 of the GH-N, GH-V or CS-A gene was used as GH-Np, GH-Vp or CS-Ap, respectively).



Nuclease protection analysis of the the GH/CS promoters with rat pituitary GC cell nuclear extracts revealed differential GHF-1/Pit-1 binding to these promoters (Lemaigre et al. 1989; Nickel et al. 1991). The GH-N promoter typically has two, proximal and distal, GHF-1/Pit-1 elements, whereas CS-A has only the proximal and the GH-V contains only the distal GHF-1/Pit-1 site (Lefevre et al. 1987; Lemaigre et al. 1989; Nickel et al. 1991). This could be one of the reasons for the differential activities observed with CS-A, GH-N and GH-V promoters in GC cells as well as in this study in the presence of the putative C-sequences and the CS-B enhancer in JEG-3 cells.

Furthermore, the differences in the location of GHF-1/Pit-1 binding sites on these promoters may vary the distances between the putative C-sequence regulatory elements and GHF-1/Pit-1 sites, and this may impart different interactions among these factors, which would be reflected in terms of the differential influence of the putative C-sequences on the GH/CS promoter activities. Indeed, this could be the reason an effect of the putative C-sequences was observed on both GH-V and GH-N promoters (Figs. A.3, A.4, A.5) as the distal GHF-1/Pit-1 binding element in these promoters was 'closer' to the putative C-sequences. In pituitary GC cells, the GH-V promoter was repressed (Fig. A.3) due to a possible interaction between the GHF-1/Pit-1 and the factors recognizing the elements in the putative C-sequences. However, the lack of an effect of the putative C-sequences on CS-A promoter activity (Fig. A.3) could be due to the inability of GHF-1/Pit-1 to interact with the putative C-sequence factors. The CS-A promoter has only the proximal GHF-1/Pit-1 site and it is possible that the changes in the distance between the putative C-sequence elements and the proximal GHF-1/Pit-1 site does not permit the interaction. A similar situation might also exist in placental cells. The effect (the repressor activity on GH-V promoter or the enhancing effect on GH-N promoter) was almost abolished when the putative C-sequences were placed in reverse-orientation (Fig. A.5, n=6). However, the GH/CS promoters in this experiment are under the influence of the CS-B enhancer. Again and consistently the putative C-sequences did not affect CS-A promoter activity (Fig. A.5, n=6). These results also suggest the presence of the regulatory elements at the 3'-end of the

putative C-sequences. Furthermore, this also indicates the distance between the putative C-sequence regulatory element(s) and GHF-1/Pit-1 element may be an important determining factor, as the putative C-sequences did not influence CS-A promoter activity. Hence, a possible mechanism governing this differential regulation could be due to distance-dependent interactions between the factors recognizing the putative C-sequences and the factors recognizing the promoter elements, possibly GHF-1/Pit-1. Distance-dependent interactions among regulatory elements and their role in promoter activity are well known. One of the examples of this type of regulation includes the effect of an interaction between basal, cAMP and thyroid hormone response elements in rat growth hormone promoter activity (Tansey et al. 1993). In addition, GHF-1/Pit-1 was shown to exhibit a unique promoter spacing requirement for activation and synergism (Li et al. 1993; Smith et al. 1995). However, the differential effects observed in these experiments in pituitary and placental cells could also be attributed to the nature of GHF-1/Pit-1 in these cells. Recently it was suggested that the activity of GHF-1/Pit-1 is determined by a regulated balance between a co-repressor complex and a co-activator complex (Xu et al. 1998).

Thus, it is important to localize and identify the regulatory elements in the 290 bp putative C-sequence fragment to understand the distance-dependent interactions between the factors recognizing the putative C-sequences and the factors recognizing the promoter elements.

A.3. Structural characterization of the putative C-sequences

A.3.1. Objective

To identify the regulatory elements in the putative C-sequences and their possible binding factors in order to decipher the regulatory mechanisms associated with putative C-sequences.

A.3.2. Experimental design

The *Sma*I and *Eco*RI fragment (290 bp) containing the putative C-sequences was released from a pUC19 plasmid and was gel purified. To generate a putative C-sequence probe, the *Eco*RI site was Klenow filled with [α - 32 P]dATP and was used in both mobility shift and DNase I protection assays. Nuclear extracts from JEG-3, GC and HeLa cells were used in gel shift and footprint reactions.

A.3.3. Results and Discussion

A.3.3.1. The putative C-sequence fragment is not the 5'-flanking region of the GH-V gene and does not contain the Cytosine-rich sequences

In gel shifts only a low mobility complex was observed with GC, JEG-3 and HeLa nuclear extracts due to the large size of the fragment (Fig. A.6), and this did not allow us to identify the number of complexes and possible number of binding elements present in the putative C-sequences. Therefore, the DNase I protection assay was chosen to map the protein-DNA binding sites on the putative C-sequence fragment. The probe was incubated with 10 μ g each of JEG-3, GC and HeLa nuclear protein extracts and treated with DNase I. The probe was also subjected to the Maxam-Gilbert (1977) method of chemical sequencing to identify the sequences, if any, protected by these nuclear extracts. This assay revealed the presence of a protected region in the putative C-sequences and the footprint was observed with all nuclear extracts used in the assay (Fig. A.7). This suggested an ubiquitous factor and the region protected corresponded to an AP-1 site. Surprisingly, the chemical sequencing reactions (G+A, C+T, C) performed with the labelled putative C-sequence fragment did not reveal the presence of the repeat units of CCCCCA sequence. At this stage further sequencing analysis of this fragment, which was previously isolated as GH-V C-sequence was performed. A homology search of the sequences obtained identified this fragment as the sequence present -497 bp upstream of the CS-A gene transcription initiation site and in the same location as that of C-sequences of the GH-V gene. However,

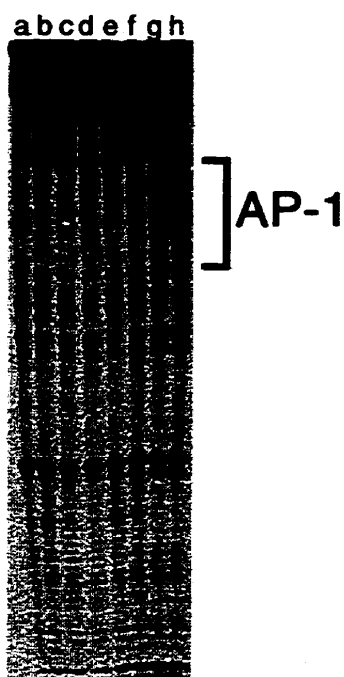
these CS-A upstream sequences are devoid of the C-rich sequences. This sequence has a very high homology with upstream sequences present in the equivalent location of all GH/CS genes (Fig. A.9).

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Fig. A.6. The putative C-sequences are recognized by nuclear proteins from various cells. Gel mobility shift assays were performed using a C-sequence probe (10,000 cpm/1 ng) without (a) and with nuclear extracts from rat pituitary GC (b), human placental JEG-3 (c) and human cervical HeLa (d) cells non-pituitary and non-placental nuclear extracts. All types of nuclear extracts resulted in a very low mobility DNA-protein complexes. The DNA-protein complexes are indicated by arrow heads.



Fig. A.7. Presence of AP-1 element in the upstream flanking sequences of CS-A gene. A DNA fragment corresponding to nucleotides -625/-496 upstream of the CS-A transcription initiation site was radiolabelled and nuclease protection assay was performed in the presence of 10 μ g each of JEG (lanes: a and d), GC (lanes: b and e) and HeLa (lanes: c and f) nuclear proteins. The nuclease protection assays were performed in the presence of either 1 μ g (lanes a, b and c) or 2 μ g (lanes d, e and f) of poly [dIdC]. Lanes g and h represent the DNase I pattern generated from the -625/-496 fragment in the absence of nuclear proteins.



Previously, the C-sequence and P-sequence regions were isolated in our laboratory from a 5.0 kb *EcoRI* fragment which was misidentified as the upstream sequences of the GH-V gene (Nachtigal et al. 1993). The present study reveals that the P-sequences are also from the upstream sequences of the CS-A gene, and not of the GH-V gene as was reported earlier (Nachtigal et al. 1993). Although this information does not alter the sequence of PSF-B element, but it does change the PSF-A sequence by one nucleotide (Fig. A.8). Regardless, fragments containing either CS-A or GH-V-specific PSF-A sequences possessed repressor activity (Nachtigal et al. 1993). However, this revelation brought about a complete change to the objective related to the CCCC-A-rich sequence, as this motif is not present in the sequence investigated.

Fig. A.8. One nucleotide difference in PSF-A sequence of P-region located upstream of the CS-A and GH-V genes. (:) denotes similarity sequence.

PSF-A in CS-A	TTCAATGTTGGTTGCCAACACCACTGCCAACCCTTCT
PSF-A in GH-V	::::::::::::::::::::A:::::::::::::

A.3.3.2. Presence of an AP-1 element in the upstream sequences of the GH/CS genes

Further examination of this sequence upstream of the CS-A gene revealed that the footprint region has the consensus sequence of a AP-1 site (Fig. A.7). This provides a possible explanation for the footprint which could be observed with all nuclear extracts tested, because AP-1 is an ubiquitous factor. This AP-1 site (TGACTCA) is present in all GH/CS genes including the GH-N gene in the same (upstream) region, with a nucleotide change in the crucial core sequence (TGtCTCA) in the region upstream of the GH-V gene (Fig. A.9).

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Fig. A.9. AP-1 core sequence located in the upstream sequences of the GH/CS genes. An AP-1 element is present at nucleotide positions -531/-525 (with respect to transcription initiation site of individual gene) of GH/CS genes. AP-1 sequence is underlined and the one nucleotide variation in the AP-1-like sequence of the GH-V gene is indicated by lower case.

GH-N	TGCATCAGCT <u>TGACTCAGGT</u> GGG
CS-L	TGCATCAGCT <u>TGACTCAGGT</u> GGG
CS-A	TGCATCAGCT <u>TGACTCAGGT</u> GGG
GH-V	TGCATCAGCT <u>TGtCTCAGGT</u> GGG
CS-B	TGCATCAGCT <u>TGACTCAGGT</u> GGG

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The presence of an AP-1 site upstream of the GH/CS genes has not been reported previously. Many laboratories investigating the GH/CS promoters, worked with the -496/+1 proximal sequences which can be isolated easily as a *EcoRI* (-496) and *BamHI* (+1) fragment. Furthermore, this -496/+1 promoter region itself was sufficient to confer the pituitary-specific activity of GH-N (Cattini et al. 1986), and also the equivalent regions of the CS-A and GH-V genes were shown to be active in rat pituitary GC cells (Cattini and Eberhardt, 1987; Nachtigal et al. 1993; Nickel et al. 1990).

This discovery of an AP-1 site upstream of the GH/CS genes might also contribute to our understanding of transcriptional regulatory mechanisms involved in the expression of these genes. One possible explanation for low levels of expression of the GH-V gene in placenta could be due to the presence of a modified AP-1 element, and a resulting effect on AP-1 binding. Further studies were performed with the AP-1 sequence of CS-A to determine the role of AP-1.

A.4. ROLE OF THE TRANSCRIPTION FACTOR AP-1 IN GH/CS GENE EXPRESSION

A.4.1. General introduction

AP-1 was initially identified as a transcription factor binding to the phorbol ester, 12-O-teradecanoylphorbol-13-acetate (TPA) responsive elements (TREs) in several promoters (Angel et al. 1987; Lee et al. 1987). AP-1 responds to protein kinase C, which is in turn is stimulated by the phorbol esters (TPA), thus linking the signals from plasma membrane to the transcriptional machinery (Angel et al. 1987; Lee et al. 1987). Furthermore, AP-1 was shown to be a homodimer of proto-oncogene *jun* (Angel et al. 1988; Bohmann et al. 1987) or heterodimer of *fos* and *jun* proteins (Sassone-Corsi et al. 1988a,b). This dimerization occurs through the basic-leucine zipper sequence present in the *fos* and *jun* proteins (Sassone-Corsi et al. 1988b). *Fos* and *jun* are proto-oncogenes and are the earliest genes to respond to many mitogens (Lamph et al. 1988). Autoregulation of *jun* gene transcription occurs in response to TPA, and reflects the presence of an AP-1 site in the *jun* promoter region (Angel et al. 1988). This positive regulatory loop is likely to be responsible for prolonging the transient signals generated by activation of protein kinase C (Angel et al. 1988).

It was noticed that AP-1 can also be activated by c-AMP, protein kinase A and, furthermore, AP-1 can bind to c-AMP responsive elements (CREs) (Beard et al. 1991; de Groot and Sassone-Corsi, 1992; Hoeffler et al. 1989; Masquillier and Sassone-Corsi, 1992; Sassone-Corsi et al. 1990). The CRE consists of a palindromic sequence (TGACGTCA) and is recognized by CRE binding protein (CREB; Yamamoto et al. 1988; Gonzalez et al. 1989). The CRE sequence is similar to that of TRE (TGA(C/G)TCA; Sassone-Corsi et al. 1990). However, transcription directed by the CRE is stimulated by cAMP and not TPA, although the basal expression mediated by this element was augmented by endogenous protein kinase-C activity (Hoeffler et al. 1989). In contrast, the TRE mediates transcriptional responses to both cAMP and TPA, and the two agents together give

synergistic responses (Hoeffler et al. 1989). The similarity between TRE and CRE elements may involve an interplay in transcriptional regulation and 'cross-talk' between components of the two major signal transduction pathways. This type of cross-talk was also observed with the transcription factor AP-2, which mediates induction by protein kinase C and cAMP signal-transduction pathways (Imagawa et al. 1987).

A.4.2. The role of AP-1 sequences in GH/CS gene expression

The GH/CS constructs, with or without the AP-1 sequences, were tested for their inducibility in the presence of phorbol ester (PMA, which induces the PKC and AP-1), forskolin (which increases adenylyl cyclase levels) and 8-Bromo cAMP (a stable analogue of cAMP) in placenta JEG-3 cells. Twenty four hours after gene transfer, the cells were supplemented with medium containing either 10 μ M forskolin, 100 nM PMA or 1.0 mM 8-Bromo cAMP. The cells were exposed to these agents for another twenty four hours and cell extracts were prepared for CAT activity.

A.4.3. Results and Discussion

A.4.3.1. No influence of phorbol esters on GH/CS promoter activity in placental cells

Interestingly, CAT assays revealed that only forskolin and 8-Bromo cAMP had an effect on CS-A and GH-V promoter activity. The CS-A promoter activity was increased by 4.5-8 fold by both forskolin and 8-Bromo cAMP (Fig. A.10A; $n=3$, $p<0.05$), whereas the GH-V promoter activity was increased by only 2-3 fold by both agents either in the presence of or the absence of AP-1 sequences (Fig. A.10B; $n=3$, $p<0.05$, **note- the two fold activity of the AP-1.GH-V.CAT in the presence of forskolin was not quite statistically significant, $p=0.09$**). However, the presence of AP-1 sequence had an enhancing effect only on the GH-V promoter as stated earlier, but not on the CS-A promoter activity (Fig. A.4). Furthermore, PMA did not influence the basal promoter activity in the presence or

absence of AP-1 sequence (Fig. 4.10; n=6).

Fig. A.10A. The presence of AP-1 element did not influence forskolin- and 8-Bromo-cAMP-dependent stimulatory activity as well as did not confer phorbol ester (PMA) stimulation of GH-V and CS-A promoters in human choriocarcinoma JEG-3 cells. Approximately 1.0×10^6 JEG-3 cells per 100 mm culture dish were transfected with 15 μ g of each CS-Ap.CAT (CS-A) or AP-1.CS-Ap.CAT (AP-1.CS-A). Twenty four hours after transfections, cells were fed with medium containing 10 μ M forskolin (For), 1.0 mM 8-Bromo-cAMP (8-BrcAMP) and 100 nM PMA and the cells were exposed to these reagents for 24 h. After this incubation time, cell extracts were prepared and assayed for CAT gene expression. The results were presented as fold induction of CS-A promoter (with or without AP-1 sequences) activity by forskolin, 8-Bromo-cAMP and PMA over the respective promoter activities in the absence of above reagents. (A DNA fragment containing nucleotides from -496 to +1 of the CS-A gene was used as CS-Ap). **Note:** The designation "C-sequences" (C) is replaced by "AP-1" in the constructs as the C-sequence fragment is an AP-1 containing upstream flanking DNA of the CS-A gene.

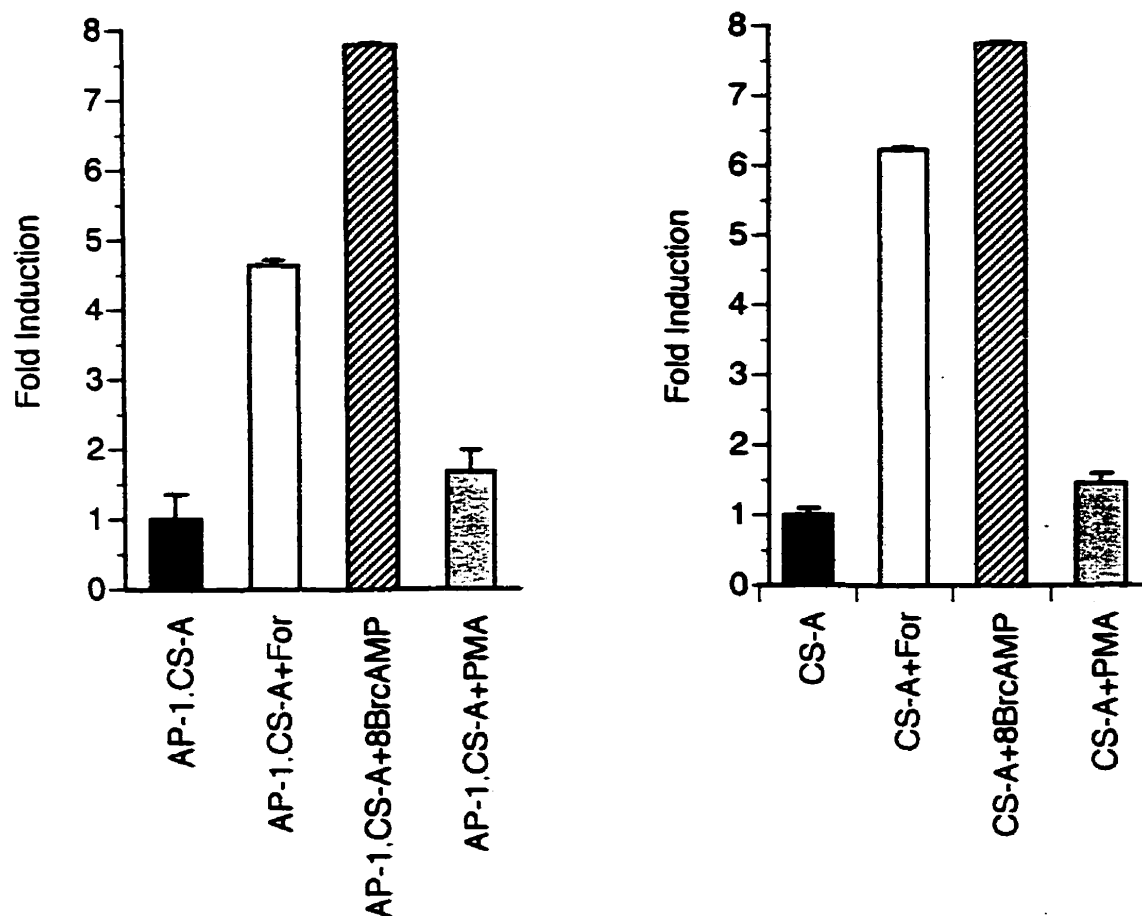
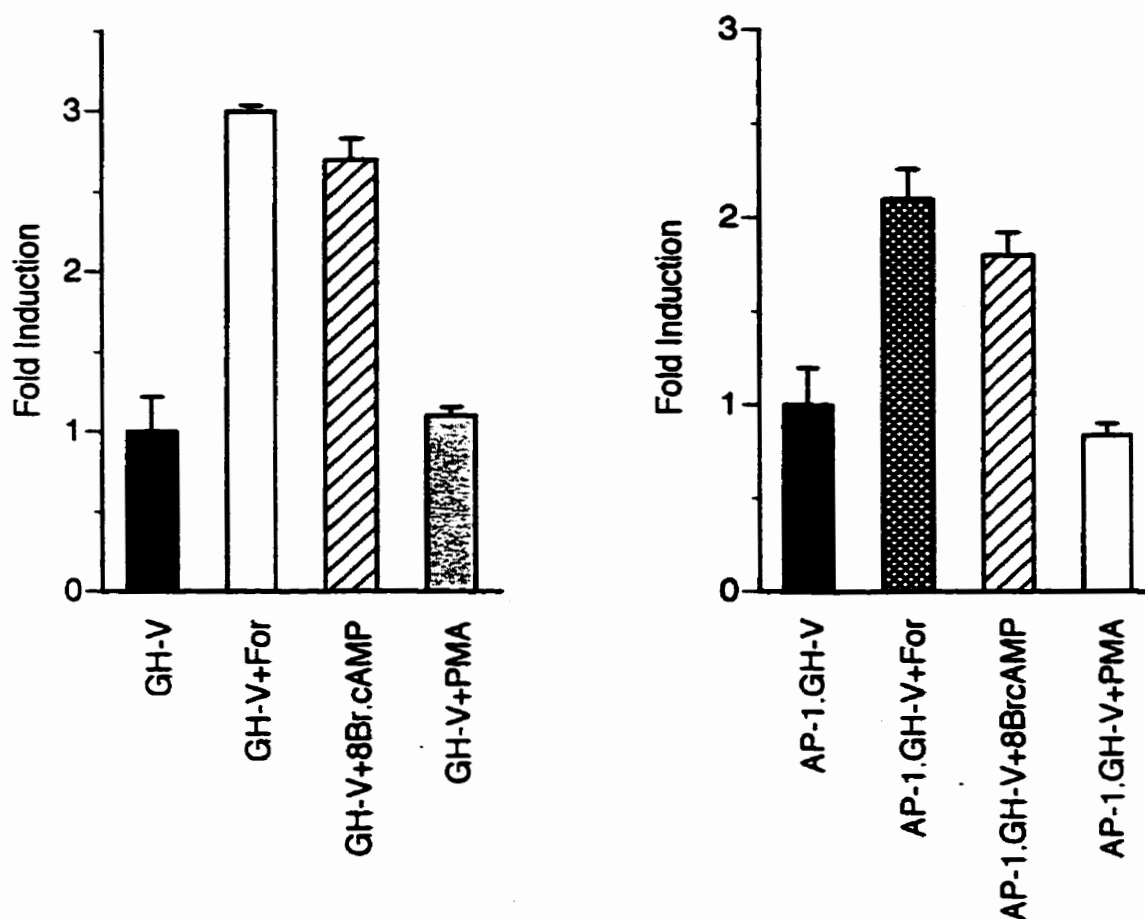


Fig. A.10B. The presence of AP-1 element did not influence forskolin- and 8-Bromo-cAMP-dependent stimulatory activity as well as did not confer phorbol ester (PMA) stimulation of GH-V and CS-A promoters in human choriocarcinoma JEG-3 cells. Approximately 1.0×10^6 JEG-3 cells per 100 mm culture dish were transfected with 15 μ g of each GH-Vp.CAT (GH-V) or AP-1.GH-Vp.CAT (AP-1.GH-V). Twenty four hours after transfections, cells were fed with medium containing 10 μ M forskolin (For), 1.0 mM 8-Bromo-cAMP (8-BrcAMP) and 100 nM PMA and the cells were exposed to these reagents for 24 h. After this incubation time, cell extracts were prepared and assayed for CAT gene expression. The results were presented as fold induction of GH-V promoter (with or without AP-1 sequences) activity by forskolin, 8-Bromo-cAMP and PMA over the respective promoter activities in the absence of above reagents. (A DNA fragment containing nucleotides from -496 to +1 of the GH-V gene was used as GH-Vp). **Note:** The designation "C-sequences" (C) is replaced by "AP-1" in the constructs as the C-sequence fragment is an AP-1 containing upstream flanking DNA of the CS-A gene.



In vitro structural studies confirmed binding of a factor to the AP-1 element, which could be AP-1, however, the failure to see a PMA response after transient transfections would not be consistent with induction of AP-1 and AP-1 binding. Thus the presence of a consensus sequence is not sufficient to assign a regulatory event, at least in this instance, even though it was shown an AP-1 element alone can confer TPA inducible activity on either genes possessing these elements or heterologous promoters (Angel et al. 1987; Lee et al. 1987). However, there are several lines of evidence that the presence of an AP-1 element alone may not be sufficient for phorbol ester-induced promoter activity. Although the collagenase gene has an AP-1 element in its promoter region, the AP-1 sequence alone is insufficient for phorbol inducibility (Auble et al. 1991). Phorbol responsiveness could be achieved when other sequence elements within the collagenase promoter were present (Auble et al. 1991). There are also examples where the AP-1 site is essential for basal expression of a gene but not necessarily for TPA-inducibility (Buttice et al. 1991). Furthermore, it was also shown that, in some cases, TPA induced gene expression is dependent on combinatorial interactions between positive and negative regulatory elements in a promoter (Harris et al. 1998). A DNA binding protein known as MDBP can counteract the stimulation of transcription by AP-1/CREB like proteins when the binding sites of these proteins are present contiguously (Asiedu et al. 1994). Furthermore, glucocorticoids antagonize the AP-1 induced gene activation without affecting AP-1 binding activity (Jonat et al. 1990; Konig et al. 1992) and this antagonizing mechanism explains the anticancer and antiinflammatory functions of glucocorticoids (Jonat et al. 1990). Tseng and Verma (1995) showed that AP-1 elements in the intronic regions of the ornithine decarboxylase gene are unresponsive to TPA. It was suggested that the flanking sequences of the AP-1 site in the introns possess negative regulatory elements and may be crucial to determine whether the AP-1 site is accessible to the TPA-induced transcriptional factor(s) (Tseng and Verma, 1995).

Similarly, it was shown that the purified AP-2 protein could bind hGH promoter sequences at nucleotide positions -270/-280 and -150/-160 by footprint assay (Imagawa et al. 1987), but later it was concluded that it was a non-functional binding event (Dana and

Karin, 1989)

The presence of one nucleotide difference in the consensus AP-1 binding site, TGA(C/G)TCA, to TGACTCc, could cause the AP-1 site to be unresponsive to phorbol esters, and this could be due to subtle differences in affinity for AP-1 proteins (Timmers et al. 1990). Although different TRE elements bind a common factor, they have the ability to bind to different subsets of a family of related transcription factors, because the bZip protein dimers (fos and jun) differ in their ability to tolerate variations from the canonical TRE sequence (Smith et al. 1993). In GH-V, the upstream equivalent AP-1 binding region contains a nucleotide change in the core AP-1 consensus sequence (Fig. A.9). Whether this sequence binds AP-1 is yet to be determined. Also the mechanism by which upstream CS-A sequences containing the AP-1 were able to stimulate GH-V promoter activity in placental cells is yet to be elucidated. The lack of any PMA response suggests that AP-1 alone might not be involved in this mechanism.

A.4.3.2. The role of cAMP in GH gene expression

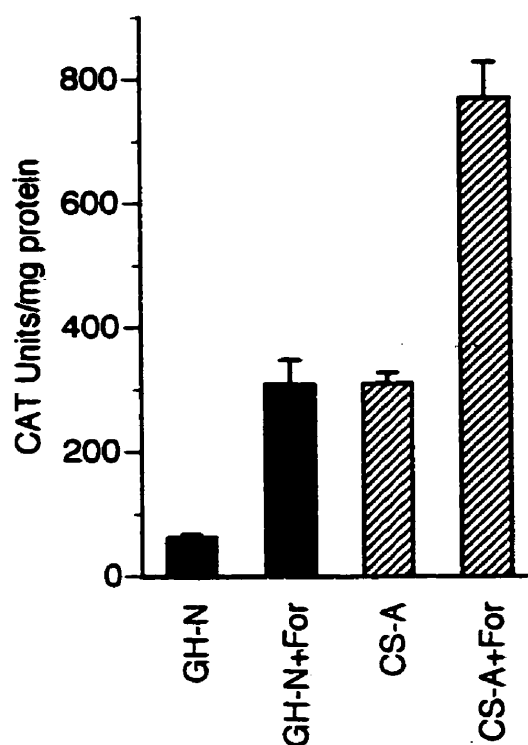
Results from the above experiments suggest that both forskolin and 8-Bromo-cAMP have a stimulatory effect on the CS/GH promoters. Many genes are modulated by cAMP at the transcriptional level and these transcriptional effects are mediated by the cAMP response element binding protein (CREB) (Yamamoto et al 1988; Gonzalez et al 1989). After activation of cAMP-dependent protein kinase A (PKA), the catalytic subunits of PKA phosphorylates numerous cytoplasmic and nuclear substrates. Among these substrates, the phosphorylation and activation of CREB is very important (Gonzalez and Montminy, 1989). Cyclic AMP is known to stimulate both GH-N secretion and GH-N gene expression, to play an important role in somatotroph differentiation, and to stimulate somatotroph proliferation (Struthers et al. 1991; Bertherat, 1997). Generally, in all species of vertebrate animals, the control of GH-N gene expression in anterior pituitary somatotrophs requires cAMP. Growth hormone releasing hormone (GHRH) positively regulates the level of GH-N mRNA by stimulation of GH-N gene transcription (Barinaga et

al. 1983) and also GH-N secretion. GHRH binds to a G-protein-coupled seven membrane receptor (GHRHR) and activates adenylyl cyclase through the G- α s subunit, which catalyses cAMP production (reviewed by Mayo et al. 1995). Somatostatin, on the other hand, inhibits GH-N release, and binds to a G-protein-coupled receptor (G- α i), but inhibits adenylyl cyclase and protein kinase-A (reviewed by Eberhardt et al. 1996).

As stated earlier, the major cAMP-responsive transcription factors are CREB/ATF, AP-1 and AP-2 (Beard et al. 1991; de Groot and Sassone-Corsi, 1992; Gonzalez et al 1989; Hoeffler et al. 1989; Imagawa et al. 1987; Masquillier and Sassone-Corsi, 1992; Sassone-Corsi et al. 1990; Yamamoto et al 1988). Among these factors, the importance of CREB in GH-N gene expression was established through a transgenic mouse model. Overexpression of an inactive, non-phosphorylatable CREB mutant form in a transgenic mouse resulted in severe somatotroph hypoplasia and dwarfism (Struthers et al. 1991). Furthermore, the GHF-1/Pit-1 gene was stimulated by cAMP at the transcriptional level (McCormick et al. 1990). GHF-1/Pit-1 not only regulates expression of the GH-N gene in somatotrophs, it positively controls GHRH receptor gene expression (Lin et al. 1992). It is also known that cAMP positively regulates the GH-N gene through a non-canonical cAMP-responsive region (Copp and Samuels, 1989; Shepard et al. 1994). Although GHF-1/Pit-1 binding sites are important, additional elements seem to be required for full cAMP stimulation of the GH-N promoter (Copp and Samuels, 1989; Shepard et al. 1994). However, while strong cAMP-responsiveness was observed with both hGH-N and rGH-N promoters, interestingly, none to a very weak response was noticed with highly similar hCS promoters in pituitary cells (Cattini et al. 1988; Shepard et al. 1994). A possible explanation for this discrepancy may be in the minor variations in promoter sequences between the GH-N and CS genes. According to Shepard et al. (1994), two CGTCA motifs in hGH-N promoter sequences (nts -187/-183, distal and -99/-95, proximal) were suggested to be core cAMP-responsive elements. Although expression of the rat GH-N gene was also upregulated by cAMP (Barinaga et al. 1983; 1985), the promoter sequences do not have the CGTCA motifs. The CS promoter possesses only the proximal CGTCA motif, whereas the distal (CaTCA) motif is varied by one nucleotide and this was thought

to account for the CS-A promoter unresponsiveness to cAMP (Shepard et al. 1994). Several genes were shown to possess this cAMP-responsive CGTCA motif (Shepard et al. 1994 and references therein). However, the CS-A promoter is more active than the GH-N promoter in pituitary GC cells (Nachtigal et al. 1993) and, in preliminary studies, upon activation with forskolin, the GH-N promoter responded better than the CS-A promoter in pituitary GC cells (Fig. A.11; $n=3$; $p<0.05$). Although, the CS-A promoter was activated by forskolin (two fold), the values for the CAT gene expression under the influence of the GH-N promoter activated by forskolin and that of the CS-A promoter without forskolin were similar (Fig. A.11; $n=3$).

Fig. A. 11. Differential stimulation of GH-N and CS-A promoter activity by forskolin in rat pituitary GC cells. Approximately 0.5×10^6 GC cells per 100 mm culture dish were transfected with 15 μ g of each GH-Np.CAT (GH-N) or CS-Ap.CAT (CS-A). Twenty four hours after transfections, the cells were fed with medium containing 10 μ M forskolin (For) and exposed for 24 h. After this incubation time, cell extracts were prepared and assayed for CAT gene expression. Although forskolin stimulated both GH-N and CS-A promoters ($n=3$, $p<0.05$), there was no significance difference between the GH-N promoter activity in the presence of forskolin and the CS-A promoter activity in the absence of forskolin. The mean value in CAT units per milligram of protein is shown and was derived from at least three determinations. Error bars indicate standard error of the mean. (A DNA fragment containing nucleotides from -496 to +1 of the GH-N or CS-A gene was used as GH-Np or CS-Ap, respectively).



Furthermore, it was suggested that downstream sequences in the CS-A gene (the primary transcript and/or 3' - flanking sequences) may be important for cAMP and phorbol ester responsiveness in pituitary cells (Cattini et al. 1988). In a study, it was noted that the CS-A promoter responds better than GH-N promoter to thyroid hormone *in vitro* in GC cells (Cattini and Eberhardt, 1987). *In vivo*, the CS genes are not expressed in the pituitary and the CS-A promoter contains only one GHF-1/Pit-1 site (Lemaigre et al. 1989; Nickel et al. 1991). At present it is not known why the CS-A promoter is consistently more active than GH-N promoter in pituitary cells as observed in transient transfections.

In the current study, both the CS-A and GH-V promoters were activated by cAMP modulators (forskolin and 8-Bromo-cAMP) in the presence or absence of AP-1 element in choriocarcinoma JEG-3 cells (Fig. A.10). These results contrast with the results of earlier studies (Cattini et al. 1988; Shepard et al. 1994). However, these earlier studies were performed in pituitary cells, and possibly the lack of response of the CS promoter may account for one of the pituitary restricting mechanisms of the human placental growth hormone genes. Although there were reports about the essential role of cAMP (Handwerger et al. 1973; Harman et al. 1987; Wu et al. 1988; Golos et al. 1992), and protein kinase-C (Harman et al. 1986; Wu and Handwerger, 1992) on primate placental growth hormone release and expression in placental cells, the regulatory elements responsible for these effects were not elucidated. Recently, Oury et al. (1997) identified both cAMP and phorbol ester response elements upstream of the CS-A and CS-B genes. This element was not similar to the AP-1 element identified in this study. The AP-1 element was present in the region of -532/-525 in all GH/CS genes with a point mutation in the AP-1 element associated with GH-V (with respect to their transcription initiation site, +1), whereas the CRE and TRE elements were localized to -1210/-1059 (152 bp sequence) sequences of the CS-A gene (Oury et al. 1997). However, these elements endorsed both cAMP and PMA response to the CS promoters in primary trophoblasts, JEG-3 and non-placental HeLa cells, suggesting that the mechanisms involved are not cell-specific. It was also shown that two Sp1/Sp3 sites as well as additional elements directly adjacent to these sites contribute to trophoblast-specific cAMP-responsiveness of the monkey GH-V

proximal promoter (Schanke et al. 1998). Furthermore, cAMP stimulation resulted in a variable response for hCS secretion in first and third trimester trophoblasts (Kato and Braunstein, 1989; 1990). In this context, it is interesting to observe that GHRH, which is important for pituitary regulation of GH-N gene expression and GH-N secretion, was also identified in human (Losa et al. 1990, Berry et al. 1992), rat (Meigan et al. 1988; Nogues et al. 1997; Perez-Riba et al. 1997) and sheep (Lacroix et al. 1996) placenta. The presence of placental GHRH suggests its possible role in the regulation of placental GH gene expression in the placenta in similar way to GHRH regulation of GH-N gene expression in the pituitary. However, the function of GHRH in placentas of rat and sheep is not understood clearly.

The present study suggests the function of the AP-1 site might be redundant with regard to regulation of GH/CS gene expression, as this element was not responsive to either cAMP or phorbol ester in placental cells. Existing data, as discussed earlier, also support the non-specific nature of other TRE and CREs present upstream of the CS-A gene (Oury et al. 1997) and the variable responsiveness of the endogenous CS genes to cAMP in placental cells (Kato and Braunstein, 1989; 1990). In this context, it would be interesting to study other regulatory mechanisms involved in the placenta-specific expression of the GH-V/CS genes. It is possible that the tissue-specific expression of these genes might have been achieved by the combination of several of the above responses and the presence of enhancer mechanisms.

A.5. POSSIBLE ROLE OF C- AND UPSTREAM SEQUENCES OF THE GH-V GENE

Although this study was started with the objective of investigating the role of C-sequences, unfortunately, the fragment thought to contain the C-sequences was found to be another region of the GH/CS locus. However, it is still possible that the presence of the CACCC sequences in the 5'-flanking sequences of the GH-V gene may have a definite role in the lowered expression of GH-V gene in the placenta. Alternatively, these sequences

may be harbouring positive regulatory elements as GH-V is the only placental GH gene which does not have an “enhancer” sequence. There are at least four CACCC and CCAC sequences clustered within a 35 bp region of the upstream sequences of the GH-V gene at nucleotide position -674/-630. Several CACCC binding factors, including Sp1 (Li et al. 1998), Erythroid Kruppel-like factor (EKLF) (Miller and Bieker, 1993), BKLF/TEF-2 (Crossley et al. 1996) Lung Kruppel-like factor (Anderson et al. 1995) and other uncharacterized factors (Sabath et al. 1996) were identified. Binding of EKLF to the CACCC element was shown to be important for regulating β -globin gene expression (Antoniou and Grosveld, 1990; Hartzog and Myers, 1993; Reddy and Shen, 1993). Studies to unravel the role of C-sequences still need to be done.

CHAPTER 7

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