THE UNIVERSITY OF MANITOBA
DEPARTMENT OF PHYSIOLOGY

TISSUE SPECIFIC EXPRESSION OF THE HUMAN GROWTH HORMONE-CHORIONIC SOMATOMAMMOTROPIN GENE FAMILY

BY

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TISSUE SPECIFIC EXPRESSION OF THE HUMAN GROWTH HORMONE-CHORIONIC SOMATOMMOTROPIN GENE FAMILY

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ARISTIDES LYTRAS

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

To Sophia and Sotiris

"Imagination is more important than knowledge ...."

Albert Einstein

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My being Dr. Cattini's student was a fulfilling experience. He provided an excellent educational evnironment for pure scientific thinking as well as many aspects of the scientific life, especially what could be called "focusing, targeting and communicating". His belief in "communication" has certainly influenced my approach in many aspects of science and life. I am now convinced that produciung a series of data or a concept is not more important than being able to "transfer" your ideas to your colleagues and to convince them about their value. I am grateful to Dr. Cattini for his confidence in my ability to work in science. He has encouraged my imagination and initiatives mroe than any other teacher I have ever had.

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ABSTRACT

The human growth hormone/chorionic somatomammotropin (hGH/CS) gene family is implicated in growth and developmental processes. Until recently, its expression was considered restricted to the pituitary (hGH-N) and placenta (hCS-A, hCS-B, hCS-L, hGH-V).

This thesis consists of three closely related studies on the tissue specific expression of hGH/CS genes and the cell-specific transcription machineries that are involved in their control.

Multiple criteria, such as the effects of monoclonal antibodies in the Nb2 bioassay, radioimmunoassay, immunoprecipitation, protein and RNA blotting analysis and RT-PCR, used in this thesis establish the extrapituitary expression of 22K hGH, in a cell line of lymphoid tumor origin.

Further, RT-PCR analysis coupled with diagnostic restriction digestion, establishes that the placentally expressed hGH/CS genes demonstrate differential relative mRNA profiles in distinct normal and abnormal trophoblasts.

Finally, gel mobility shift assays, mutational analysis, gene transfer experiments and reporter assays, establish the functional significance of two novel regulatory sequences, RF-1 and DF-1, found in proximity to the previously identified TEF-1 (Transcriptional Enhancer Factor-1) element in the 3' flanking sequences of the hCS-B gene. RF-1 and DF-1, may represent binding sites for TEF-2 and a member of the ets family of proto-oncogenes, respectively, and appear to participate in a complex, repression-derepression mechanism that modifies the function of TEF-1 and may confer placenta-specific enhancer activity. Gel mobility shift competition analysis suggests that the RF-1 and DF-1 sites participate in the formation of a

common complex or compete for common protein factors in a tissue specific manner. Further, they interact with the PSF-A and PSF-B repressor sequences in the 5' flanking regions of all placental GH genes suggesting their possible involvement in hGH/CS locus control.

These results emphasize the existence of diverse regulatory mechanisms that allow the expression of distinct hGH/CS genes in three highly differentiated systems, pituitary, immune system and placenta, and provides insight into the molecular mechanisms that may determine the existence of individual gene-sublocus organization within the hGH/CS locus.

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ABBREVIATIONS

Human growth hormone gene family

h	human
hGH-N	human growth hormone-N (normal) gene
hGH	human growth hormone-N mRNA and protein
hGH-V	human placental growth hormone variant gene and products
hCS-A,-B	Human chorionic somatomammotropin-A or -B genes
hCS	chorionic somatomammotropin-A or -B mRNA and protein
hCS-L	Human chorionic somatomammotropin-L gene and products

Hormones and growth factors

ACTH	adrenocorticotropin hormone
CSF	colony stimulating factor
CRH	corticotropin releasing hormone
EGF	epidermal growth factor
GnRH	gonadotropin releasing hormone
FGF	fibroblast growth factor
IGF-I,-II	insulin-like growth factor-I or -II
IL-	interleukin-

PDGF-A,B	platelet derived growth factor A or B
PG	prostaglandins
PRL	prolactin
SP1	Schwangerschafts protein 1 (PAPP-A, pregnancy-associated plasma protein-A)
TGF-α,ß	transforming growth factor-α or -ß

Cell types of reproductive tissues

vCT	villus cytotrophoblasts
vIT	villus intermediate trophoblasts
vST	villus syncytiotrophoblasts
CTsh	cytotropgoblastic shell
xvT	extra villus trophoblasts
prlT	proliferative trophoblasts
invT	invasive trophoblasts
evscT	endovascular trophoblasts
colT	columnar trophoblasts
chT	chorionic trophoblasts
dbIT	decidual (placental) bed intermediate trophoblasts
SC	stromal cells
dSC	decidual stromal cells
mesSC	mesenchymal stromal cells
aEC	amniotic epithelial cells

Transcription factors and DNA elements

OCT-	DNA element recognized by OCT-1 or OCT-2 POU domain transcription factors
GC box	CACC/GGTG core containing DNA element
SphI, SphII	DNA elements in the SV40 enhancer region, recognized by TEF-1
GT-IIC	DNA element identified in the SV40 enhancer, recognized by TEF-1
GT-I	DNA element identified in the SV40 enhancer, recognized by TEF-2
TEF-1 or 2	transcription enhancer factor-1 or 2, bind the
c-myc	proto-oncogene transcription factor
ets	family of proto-oncogene transcription factors
Sp1	GC box binding protein
AP-	activator protein-
GATA	Family of transcription factors
GAL4	yeast transcription factor
MAR	nuclear matrix associated regions of genomic DNA
NF-1	nuclear factor 1
IR	inverted repeat
DR	direct repeat

Materials and methods

M	molar
mM	millimolar
ml	millilitre
μl	microlitre

μCi	microCurie
PAGE	polyacrylamide gel electrophoresis
RT-PCR	reverse transcriptase polymerase chain reaction

INTRODUCTION

A. GENERAL PERSPECTIVES

Cancer has become a major research field for endocrinologists but although the hormonal dependence of certain malignant tumors is being used as a target for cancer therapy, it remains, in the majority of the cases, a terminal disease. It appears that even in the cases of hormonal dependence there is always an escape pathway from any hormonal control exercised on a malignant cell population. More fundamental mechanisms seem to deteriorate during malignant transformations and this results in the irreversible escape of these mechanisms from the normal hormonal regulatory control. It is this realization that has driven endocrinologists to apply methodological approaches used by cellular and molecular biologists in a search for new models for cell growth and its regulation. Terms, such as, "paracrine", "autocrine" and "intracrine" are used to describe the mode of action of "growth factors" which normally mediate several hormonal functions during regular developmental processes ranging from the development of the placenta and the mammary gland during pregnancy to the linear body growth during childhood and puberty. Embryonic development is one of the most fascinating aspects of animal life. This process of "outrageous" growth of undifferentiated stem cells is ultimately directed to cell differentiation and restricted proliferation. In a sense, it represents the antipode of the uninhibited growth of "de-differentiated" cancer cells.

Over the recent years studies on embryonic development and cancer have revealed a number of genes that control normal differentiation processes and cell growth. A

number of these genes have been found to be expressed irregularly (over- or underexpressed or bearing functionally important mutations) in several malignancies. It appears that an essential triumph has resulted from years of molecular biology research even in fields where studies were often considered to be undertaken just for the sake of the knowledge of these "magnificent" mechanisms that regulate gene expression and determine cell phenotypes and function.

In less than 40 years from the discovery of the structure of DNA *(1)* and 20 years from the breakthrough of molecular biology, the discovery of the restriction enzymes *(2,3)*, the knowledge that has been being acquired has a perfect practical purpose: the identification of gene abnormalities and repair of gene defects. This pushes medicine to a new level of invasiveness that goes beyond, and does not even require, the use of traditional surgical procedures. Instead, viruses and a variety of DNA vectors are used for carrying functional human genes into cells that have their own genes damaged and demonstrate an abnormal behaviour *(4)*. In this respect, knowledge of the mechanisms that control tissue-specific or temporary/hormone-stimulated gene expression will have substantial clinical interest in the near future.

Gene therapy is already under experimentation for diseases such as cystic fibrosis and muscular dystrophy *(5,6)* that are caused by mutations of genes or dysfunctional gene expression. The challenge is to direct an exogenously provided gene, "armed" with the appropriate regulatory elements, to the tissue of interest. Ideally, an exogenously provided gene should be able to respond to the needs of this tissue with precision referring to both time and level of expression. If we are about to succeed in such a task, full understanding of the mechanisms that determine gene expression is necessary.

These prospects result in a continuous interest in aspects of physiological

regulatory mechanisms and related aberrations with emphasis on the effects of such aberrations on the control of cell division, growth and differentiation.

B. TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION

I. Cell homeostasis and transcription factors

The cell structure appears tightly associated with the cell's biochemical balance and phenotype. Within the tissue of its origin or a target tissue after migration, a cell anchors, transiently or permanently, using a combination of macro- and micromolecular structures associated with a network, that consists of the cell's membrane, cytoskeleton and nuclear skeleton *(7,8)*. Information, for environmental changes, is transmitted to the cell nucleus through "tension" *(8,9)*, "shape changes" *(10-13)* and a variety of biochemical messages *(14)* in response to the structural and biochemical stimuli resulting from these environmental changes or the migration to a new environment. Cell nuclei are literally "vibrating" along with the rest of the cellular structures via a tissue tensegrity matrix system consisting of the nuclear matrix, the cytoskeleton and the extracellular matrix *(15)*.

A close "collaboration" of macro- and micromolecular pathways is apparently the basis of the "dynamic" structure of the cell nucleus and the genome. Nuclei are in a "ready state" to receive messages and respond with short and/or long term answers, demonstrating a control similar to the control that the brain exercises on the human body. It is the unique structure of a cell nucleus that allows this prompt and continuous response to cellular needs. The genome docks on a dynamic nuclear matrix that is able

to alter the "state" of chromatin, initiate events that lead to gene transcription, process the RNA transcripts and deliver them to the cytoplasm for translation *(16)*.

Transcription factors (used as general term that also includes proteins associated with chromatin and the nuclear protein matrix that participate in the activation or inhibition of gene expression) are messengers of cellular needs. This extremely versatile category of proteins, includes factors directed to the nucleus, in an active form, soon after their synthesis *(17)*, factors that are localized in the nucleus but they are inactive in the absence of their ligand that is transferred from the cytoplasm *(18)*. It also includes factors directed to the nucleus in an active form that can be specifically inactivated by nuclear phosphatases *(19)*, factors that are active in the nucleus but are inactivated by cytoplasmic factors when the nucleoplasm interacts with oocyte cytoplasm after germinal vesicle break down *(20)*, factors that are transferred to the nucleus from the cytoplasm following activation in response to a stimulus *(21-26)*, or even, possibly, hormones that are internalized after their binding to cell surface receptors and are directed to the nucleus *(27,28)*.

II. Association of the genomic DNA with chromatin, the "dynamic" state of chromatin and gene expression.

The packaging of genes in chromatin through the association of DNA with histones, forming the nucleosomes *(29-31)*, and further, through histone H1 and possibly H3 and H4 interactions, the solenoids of the 30 nm chromatin fibre *(32-40)*, represents an effective way of restricting gene expression to the genes for which a specific

mechanism results in at least a partial disassociation of the genomic DNA from nucleosomes. RNA polymerase II, once it has initiated transcription, can transcribe through nucleosomes *(41-43)*. A few models have been proposed to explain the mechanism of this phenomenon *(44 and references there in)*, and it appears that the only absolute chromatin modification requirement for the initiation of transcription is the displacement or disruption of the nucleosome from the promoter region (including the TATA box) of a gene that is going to be transcribed *(45-48)*. This represents a very important point of transcription regulation and most DNA regulatory sequences are likely to have an effect on the mode of interaction of a promoter region with the histones of its corresponding nucleosome *(49-52)*.

These effects are mediated through the interaction of the DNA regulatory regions with transcription factors that in turn can displace, disrupt or unfold a nucleosome directly *(47,48,53,54)* or, possibly, indirectly, by interacting with histones *(55)*. This interaction may in turn facilitate histone core modifications, such as loss of histones H2A/H2B, histone acetylation or histone ubiquitination, that have been shown to increase the accessibility of nucleosomal DNA to some transcription factors *(56,57, for a review 54)* or may prevent nucleosome refolding facilitating subsequent rounds of transcription *(58)*.

The action of a transcription factor through displacement of nucleosomes from a regulatory region or through facilitating the binding of other transcription factors that can displace nucleosomes, constitutes the "dynamic competition model" *(59, for reviews 54,60-67)*. Alternatively the interaction of transcription factors with putative nucleosome binding regions may occur during DNA replication where the higher order chromatin

structures are resolved and histones disassociated, at various degrees, from the DNA. This constitutes the pre-emptive competition model *(45,46,68,69, for reviews 54,60-67)*.

III. Role of transcription factors in animal development.

The most fundamental questions about the role of transcription factors in development refer to their role in growth and differentiation. Hormones and growth factors induce their effects on growth, differentiation and cellular functions by activating gene expression through second message signalling pathways and transcription factors. A considerable effort focuses on uncovering the role of transcription factors (through the activation of their target genes) in these signalling pathways and the control of cell proliferation and differentiation.

The identity of several transcription factors with nuclear oncogenes has created an additional exploratory pathway for approaching cellular events that lead to transformation. The current scientific effort focuses on increasing our knowledge of the "pathology" of the signalling pathways and transcription factors in cancer. How extensive is the transcription factor arsenal and how can we remove, replace or repair a transcription factor without influencing any other factor or pathway? Cascade events that result from tissue specific or temporary gene expression must be analysed thoroughly in a search for "control" or "threshold" points where intervention (restrictive or stimulatory) could be applied with minimal effects on overlapping pathways.

C. THE HUMAN GROWTH HORMONE FAMILY: GENE AND PROTEIN STRUCTURE, TISSUE SPECIFIC EXPRESSION, RECEPTORS AND POSSIBLE PROTEIN FUNCTIONS DURING GROWTH AND DIFFERENTIATION.

I. The study of gene families

Studies on gene families offer unique advantages for several aspects of medical research. Extensive similarities and characteristic differences between various members of gene families that derive from gene duplication events, may be used for the identification of protein segments or DNA sequences that are responsible for specific functions.

The growth hormone gene family represents an excellent model system for many aspects of medical research focusing on growth, differentiation and malignant transformation. The association of these proteins with growth and differentiation makes them an important research target for the identification of transcription factors that control developmental processes. Expression of members of this family in either the pituitary or the placenta is closely associated with important developmental changes suggesting that the transcription factors involved in the activation of these proteins may be responsible for inducing developmental changes as well. This notion was rewarded with success in the case of the identification of the pituitary specific factor GHF-1/Pit-1 that is required not only for the pituitary specific expression of GH, but for the development and differentiation of a mature pituitary gland as well *(70-74)*.

II. The hGH gene locus, the hGH/CS genes and their tissue specific expression.

The growth hormone family consists of five highly homologous members and represents a model system for hormonal control over the functions of human organisms during all stages of the human life. The hGH/hCS genes [human pituitary growth hormone (hGH-N), human chorionic somatomammotropin (hCS-A and hCS-B), placental growth hormone variant (hGH-V) and hCS-like (hCS-L) gene] are located at a single locus extending over 66 kb on chromosome 17 (*Figure 1*) and have evolved by gene duplication as indicated by their considerable nucleotide ($\sim$94%) and amino acid ($>$80%) sequence similarity *(75-78)*. All five genes have a common structure with five exons and four introns. In spite of their great similarity, hGH/hCS genes are expressed in different tissues providing a model system for the study of tissue specific gene expression *(75,76,78)*. Pituitary hGH is responsible for controlling one of the most impressive phenomena of animal life, the longitudinal growth. The human chorionic somatomammotropin (hCS, identical product of two distinct genes, hCS-A and hCS-B *(79,80)* is expressed and secreted by the syncytiotrophoblast of the placenta *(81)*. Levels of hCS rise throughout pregnancy and correlate with placental size *(82)*. As a result, blood levels of hCS have been used as a biological index for the normal progression of pregnancy *(83-86)*.

Pituitary hGH secretion follows a relatively constant pattern under the control of growth hormone releasing hormone (GHRH) and somatostatin. Several small secretion surges during the course of the day and a major surge during slow wave sleep (stage 3 or 4), are the result of the ultradian rhythms of the hypothalamic secretion of

somatostatin and GHRH to the hypophysial portal blood. Resting daytime levels of hGH range between 1-2 ng/ml *(87)*.

The hGH secretory pattern is modified during pregnancy where eventually pituitary hGH secretion is diminished and the placental hGH-V protein is produced in increasingly high amounts *(88-91)*. However the most profound change for the secretion profile of the GH family is that the hCS-A and hCS-B genes, which are active only in

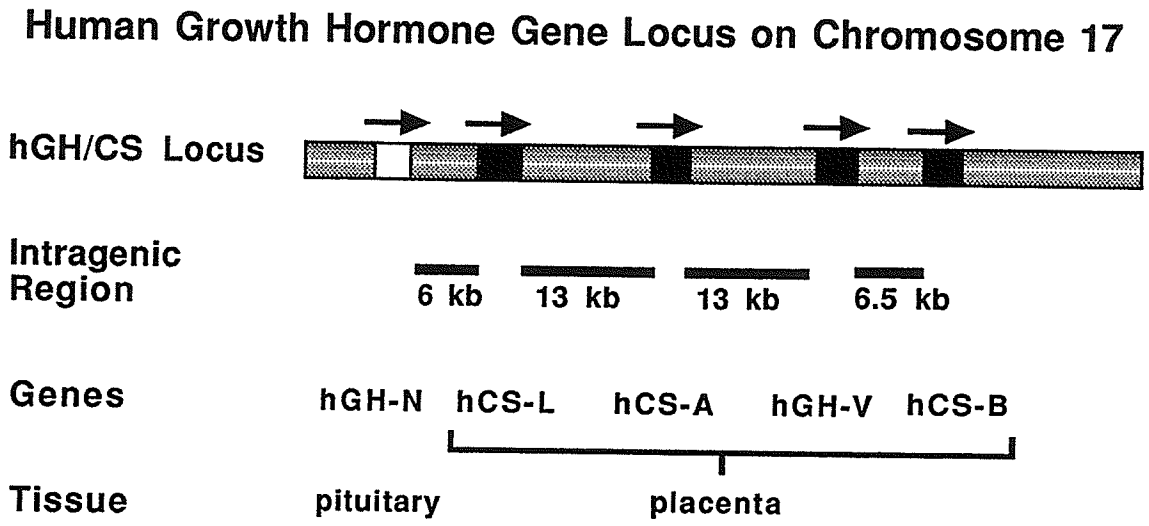

FIGURE 1: Schematic representation of the human growth hormone locus. The hGH/hCS genes [human pituitary growth hormone (hGH-N), human chorionic somatomammotropin (hCS-A and hCS-B), placental growth hormone variant (hGH-V) and hCS-like (hCS-L) gene] are located in the same transcriptional direction at a single locus extending over 66 kb on chromosome 17 and have evolved by gene duplication as indicated by their considerable nucleotide (~94%) identity. All five genes have a common structure with five exons and four introns *(Figure 2, p. 78)*. Distances between individual genes and the tissue of expression of each gene are indicated.

the placenta, are expressed and hCS (or hPL, for human placental lactogen) is released in great amounts into the maternal circulation *(92-96)*. Indeed, this protein is secreted at a rate higher than any other protein hormone in humans. Placental lactogen production reaches 1 g per day during late pregnancy and serum levels reach a peak of 5000-9000 ng/ml at the 30th week *(86,97)*.

III. Structure of hGH and hCS proteins.

a. Molecular forms of HGH and hGH-V proteins

The basic structure of GH proteins is a single amino acid chain with 191-198 amino acids, a single centrally located tryptophan, a large central disulphide loop and a short COOH-terminal disulphide loop *(98)*.

The HGH and hGH-V proteins show extensive similarities sharing 178 out of 191 amino acids. Differences between the two molecules are spread throughout their lengths and some of these amino acid and their corresponding nucleotide substitutions result in differential postranslational modifications or alternative RNA splicing.

The pituitary HGH appears as a duplet of 22 and 20K with the 20 kd representing up to 10% of the total *(77,99,100)*. The 20K variant is the product of an alternatively spliced mRNA lacking part of intron B and exon 3 *(101-103)*. It bears a 15 aminoacid deletion (amino acids 32-46) *(104)* relative to the full length HGH protein and has reduced affinity for the liver HGH receptor (HGH-R) and the HGH binding protein compared to the 22K *(105-107)*. However, it maintains IGF-I generating and lactogenic activity in rats and pigeons *in vivo (99,101,102,104,108-112)* likely due to lower clearance rate *(112)*. In addition, it has diminished insulin-like activity *(110,113-117)* but substantial chronic diabetogenic activity *(114,115,118,119)*.

In contrast to the negligible binding of the 20K to the liver hGH-R *(105)* and to the rat adipocyte GH-R *(116)*, it binds with high affinity to the hGH-R present in the IM-9 B-lymphocytes *(116,120)*. Interestingly, the IM-9 cells express predominantly

(79%) a hGH-R lacking exon 3 (due to alternative splicing) while in normal human liver the full length hGH-R is the only species expressed *(121)*. This raises the possibility that the hGH-Rd3 receptor isoform might be specific for the 20K variant. There is a distinct tissue distribution of the two hGH receptor isoforms during pregnancy. The predominant hGH-R is expressed in chorion and decidua (98 and 99% respectively) and the hGH-Rd3 is expressed in amnion and villi (both 100%) *(121)*. Puzzling, however, is that expression of pituitary hGH is considered minimal during the second half of pregnancy, being replaced by the placental hGH-V *(88)*. Due to the absence of a 20K hGH-V variant (see next paragraph) it appears that this preserved tissue distribution of the hGH-Rd3 receptor isoform may correspond to another specific ligand during pregnancy. Urbanek *et al (121)* suggested that hCS may be a ligand for this receptor. However, the predicted 174 amino acid hCS-L' protein may be another candidate, if the exon 3 truncated hCS-L' transcript, recently identified in placenta *(122,123, see "Molecular forms of hCS proteins" p 16)*, codes for a functional protein. An alternative, but rather remote, possibility is that the 20K pituitary hGH variant may be present during pregnancy but may have been undetected to date due to its reduced immunoreactivity to hGH antibodies and the limited availability of monoclonal antibodies able to distinguish hGH from hGH-V proteins.

The hGH-V molecule contains a potential N-glycosylation consensus sequence that results in a 25K glycosylated hGH-V in placenta *(87,100)*. As mentioned above, there is no 20K variant of hGH-V, due to nucleotide differences at the putative alternative splice site *(103)*. Further a different alternatively spliced (retaining intron D) product exists for hGH-V. The product of this RNA species, termed hGH-V2, is a 230 amino

acid protein with a MW of 26K, with a frameshift that results to a non hGH-like carboxy-terminal and no glycosylation site. Further it demonstrates a hydrophobic profile that suggests transmembrane localization *(124,125)*. Cooke *et al (124)* pointed at the quite extensive similarity of this exon 4 retaining GH RNA species with a bovine pituitary exon 4 retaining GH species *(126)* as an indication of a functionally significant evolutionary conserved hGH molecule. Further, in a more recent study *(123)* they reported that the exon 4-retaining hGH-V2 increases about 3-fold relative to hGH-V during pregnancy, reaching 15% of the total hGH-V/V2 mRNA at term.

A variety of other hGH forms resulting from alternative splicing, postranslational modifications, metabolic conversions, polymerization, association with immunoglobulins or laboratory artifacts have been reported [reviewed by Baumann *et al (100)*]. A particularly interesting form corresponds to a 5K, 43 amino acid hGH protein (aa 1-43), likely product of specific proteolytic cleavage. This molecule, representing about 1% of total hGH *(100)*, has no growth promoting effect, no hGH immunoreactivity but has insulin-potentiating effects in hypophysectomized mice or rats *(127-129)*. The generation by cleavage of this molecule would also result in the generation of the corresponding 44-191 hGH molecule with a predicted MW of 16,930[1] *(100)*. GH-related peptides of approximately this size have been reported *(127,131,132)* and a potent diabetogenic activity has been described for these products *(127,131,* also *133)*. In contrast, no growth promoting activity, no insulin-like activity and diminished lactogenic activity and hGH immunoreactivity (approximately 1%) have been described for the 44-191 molecule

[1] Two additional minor species, a 17.5 kd (151 aa hGH variant lacking aa 32-71 due to alternative splicing and removal of exon 3) and a truncated 48 aa (frameshifted after aa 31 due to alternative splicing at a site within exon 3, distinct from the site used to generate the 20 kd variant) have been described in pituitary tumors *(130)*.

(131). Interestingly hGH 44-191 does not appear to interact with bovine liver somatogenic receptors but interacts with bovine liver and rabbit mammary gland lactogenic receptors *(134)*. *Table 1* summarizes the receptor binding profiles of some of the hGH molecular forms.

Table 1. Receptor binding profiles of hGH molecular forms (ND: not determined)

Ligand	IM-9 hGH-R binding (79% hGH-Rd3)	human, rabbit or bovine liver somatogenic receptor binding (GH-R, 618-620aa)	bovine or rabbit liver lactogenic receptor binding (or lactogenic activity)	human placental chorion receptor binding	rat adipocyte receptor (or insulin-like activity)	Refs
hGH	+++	+++	+++	+++	+++	256, 44,56,57, 246,261, 257
hGH-20K	++	+/-	++	+	3% of hGH	44,56,57, 64,257
hGH_{1-43}	ND	-	ND	ND	++	64
hGH_{44-191}	ND	-	+	ND	-	70

Recently the development of a specific radioimmunoassay for the hGH 44-191 was reported (the 22K and the 20K hGH variant crossreacted very little in this assay, 0.1% and 0.5% respectively) and the mean value in normal men and women was 2.1 $\pm$ 0.1 ng/ml compared to a mean value of 0.4 $\pm$ 0.03 ng/ml for hGH *(135)*. An assessment of pregnant women revealed a mean value of 3.2 $\pm$ 0.2 ng/ml (mean $\pm$ SE) compared to 2.0 $\pm$ 0.2 ng/ml for hGH values produced by hGH RIA *(135)*. It is not known,

however, whether hGH-V can generate the 1-44 and 44-191 products and whether there was crossreactivity to the hGH-V in this assay, as hGH-V crossreactivity data were not reported *(135)*. Pituitary hGH secretion is considered minimal after mid-pregnancy *(88,90)*, and thus, assessing the levels of hGH 44-191 at different stages of pregnancy may provide some insight for the origin and possible functions of the hGH 44-191 in pregnancy.

b. Molecular forms of hCS proteins

The hCS-A or hCS-B gene product (hCS) is a 198 amino acid protein with 85% of its amino acids identical with those of hGH and only 13% identical to hPRL *(86)*. However, it appears that, in contrast to hGH and hGH-V, hCS is not subject to post-translational modifications *(86)*. Recently, the detection of alternatively spliced hCS-A and hCS-B RNAs, that maintained exon 4, was reported *(123)*. These species termed hCS-A2 and hCS-B2 followed a pattern of expression similar to their corresponding regularly spliced counterparts. The ratio of hCS-A to hCS-B increased during gestation from 1.5:1 at first trimester to 5:1 at term. Similarly the ratio of hCS-A2 to hCS-B2 increased from 2.6:1 to 12:1. The product of the hCS-A2 transcript is predicted to be a 230 amino acid protein with 80% similarity to the hGH-V2 protein *(123)*.

The hCS-L gene, initially considered to be silent, has recently received attention due to the demonstration of a developmentally regulated pattern of expression and the presence of a number of alternatively spliced mRNAs *(123)*. Two RNA species hCS-L and hCS-L' differing by 69 nucleotides represented 88% and 12% of the total hCS-L

transcripts and maintained a stable ratio throughout gestation. The hCS-L transcript was identical to the predicted species *(76)* utilizing an alternative donor site 19 nucleotides within intron B instead of the donor site used by the other hGH/CS genes (a G to A substitution in this site inactivates it in the hCS-L gene) and an acceptor site halfway within exon 3 resulting in the removal of 24 codons from this exon *(123)*. This processing resembles the removal of exon 3 sequences from the hGH-N transcript in pituitary that results in the 20K hGH variant *(101,102)*. Interestingly, hCS-A and hCS-B lack the exon 3 acceptor site while hGH-V maintains this site but exon 3-truncated transcripts are not produced *(123)*.

The hCS-L' transcript contains the same 19 bp 3' extension of exon 2 and utilized a unique cryptic splice donor site within intron B located 4 bp upstream of the normal splice site used by the other genes of the hGH family. Both, the predicted hCS-L and hCS-L' proteins maintain an open reading frame that results in mature 174 and 197 amino acid proteins and, with the exclusion of exon 3 deleted sequences have a 91% amino acid similarity to hCS *(123)*.

IV. The growth hormone receptor and the cytokine receptor superfamily.

Distinct and common properties of the protein products of the members of GH and PRL gene families have facilitated the assessment of the function of different protein domains and their interactions with the corresponding receptors of the cytokine receptor superfamily. The cytokine receptor superfamily *(136)* includes members such as the IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, GM-CSF, PRL, GH and erythropoeitin receptors as well as the glycoprotein gp130 that is associated with the IL-6 receptor. They are all single transmembrane domain receptors with highly conserved cysteine residues in their extracellular domain and variable intracellular domains. Further a conserved extracellular WSXWS (Trp-Ser-X-Trp-Ser) motif is present very close to the transmembrane domain of these receptors. Interestingly this motif is absent from the GH-R *(136)*.

GH and PRL receptors are widely distributed in mammalian organisms *(137,138-141)*. GH-R are present in liver, adipose tissue, lymphoid cells, intestine, heart, kidney, lung, pancreas, brain, cartilage, skeletal muscle, corpus luteum, placenta and testis. PRL-R are found in mammary gland, liver, ovary (including corpus luteum), uterus, placenta, thymous, lymphoid cells, erythrocytes, neutrophils, intestine, kidney, adrenal, pancreas (ß-cells), brain, eye, prostate, epididymis, seminal vesicle and testis *(137,138-141)*. A long (the human, rabbit, rat, mouse, sheep and cow GH receptors have been cloned: 614-626 aa) and a short (extracellular binding protein, GH-BP, 255-273 amino acids, alternative splicing mechanism) form of GH-R have been identified *(142,143)*. Similarly, a long and a short form, of the PRL-R have been identified [long, 591-598 amino acids, the human, rat and rabbit PRL-R have been cloned *(144-146)*, and short, 284-291 amino

acids, rat and mouse PRL-Rs have been cloned *(147,148)*. In addition, an intermediate form, resulting from a mutation (594 bp deletion) that produces a receptor with truncated cytoplasmic domain, has been identified in the rat Nb2 lymphoma cells *(149)*.

A degree of crossreactivity is observed between GH and PRL receptors with more characteristic the lymphoproliferative effects of hGH on Nb2 lymphocytes that are mediated through the lactogenic Nb2 PRL-R *(150-152)*.

There are several reports *(153-163)* on the specific molecular and ion requirements for efficient hGH, hCS, hGH-V and hPRL binding to a variety of GH and PRL receptors. As might be expected, both a redundancy in receptor binding as well as extreme paradigms of specificity were revealed. Human GH-BP binding by hPRL is 100,000-fold weaker than hGH binding and PRL-BP binding by hGH is 130-fold weaker than hPRL binding.

In the presence of Zn^{++}, however, hGH binds hPRL-BP with 80-fold higher affinity than hPRL. This effect is due to a 8,000-fold increase in the affinity of hGH for hPRL-BP in the presence of 50 μM $ZnCl_2$ and is explained by the participation of Zn^{++} in the hGH/PRL-BP complex through interactions with the His^{18}, His^{21} and Glu^{174} of hGH and His^{188} of the hPRL-BP. Interestingly, hCS demonstrates the same Zn^{++} requirement, as hGH, for binding to the hPRL-BP and can bind with almost equal affinity to hGH (K_d: 0.046 nM versus 0.033 nM respectively), to hPRL-BP and Nb2 PRL-R but has a 2300-fold lower affinity for hGH-BP than hGH. *Table 2* summarizes some of these results and *Table 3* summarizes the results of a similar analysis by Ray *et al (163)* for the receptor binding profiles of hGH versus hGH-V.

Table 2: Receptor binding characteristics of hGH, hCS and hPRL. (ND: not determined)

Ligand	hGH-BP binding (K_d, nM)	hPRL-BP binding (291aa, K_d, nM)	human liver (L, 620aa) fetal liver (FL) or fetal muscle (FM) receptor	hGH, PRL or CS plac. chorion receptor	Refs
hGH	0.34 nM 1.6 nM ($+Zn^{++}$) 0.42 nM ($-Zn^{++}$)	0.033 nM ($+Zn^{++}$) 270 nM ($-Zn^{++}$)	K_d^{FL}:1.6 nM and 8.6 nM (hCS^{IC50}:x5.9 $hPRL^{IC50}$:x7.1)	++++	256 105 120 154 161 157
$hGH_{glu174 \rightarrow ala}$	0.075 nM	46 nM	ND	ND	156
hCS	770 nM	0.046 nM ($+Zn^{++}$) >10 nM ($-Zn^{++}$)	K_d^{FL}: 2.2 nM and 24.0 nM (hGH^{IC50}:x9.1 $hPRL^{IC50}$:x8.3) K_d^{FM} 5.6 nM (hGH^{IC50}: ∞, $hPRL^{IC50}$: ∞)	+	154 155 157
$hCS_{Glu174 \rightarrow Ala}$	252 nM	46 nM	ND	ND	155
hPRL	>40,000 nM	2.6 nM ($+Zn_{++}$) 2.1 nM ($-Zn_{++}$)	K_d^{FL}: 2.7 nM (hGH^{IC50}:x.025 hCS^{IC50}:x.2)	+++	154 157 159

Table 3: Receptor binding profiles of hGH versus hGH-V. (ND: not determined)

Ligand	rat Nb2 receptor binding (PRL-R, 393 aa/ IC_{50}, ng/ml)	rabbit liver somatogenic receptor binding (GH-R, 620aa/ IC_{50}, ng/ml)	rat liver lactogenic receptor binding (PRL-R 291aa/ IC^{50},ng/ml)	IC^{s}_{50}:IC^{l}_{50} (ratio)
hGH	3.1	11.2	4.1	3.03 (1.0)
hGH-V[2]	94.0	9.4	29.9	0.43 (7.37)
$hGH/ex3_{hGH-V}$	ND	27.0	16.1	2.16 (1.43)
$hGH-V/ex3_{hGH}$	ND	9.1	9.6	1.26 (2.60)
$hGH/ex3_{rPRL}$	ND	-	+	

V. Signalling pathways for the cytokine-superfamily receptors

The receptor mediated pathways by which growth hormone proteins exert their effects finally began to be elucidated with the recognition that the GH-R, as well as the other members of cytokine receptor superfamily, is associated with a family of protein kinases (Jak1, Jak2, Jak3 and tyk2) *(reviewed in 164)* which are bound to a membrane proximal region of the receptor and are rapidly tyrosine phosphorylated after GH ligand binding. More interestingly, these protein kinases are in turn associated with the

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2 hGH and hGH-V differ only in three aminoacids in exon 3 corresponding sequences: hGH-N→hGH-V: Pro^{38}→Leu, Glu^{65}→Val, Glu^{66}→Lys. Exon 3-swap hybrid molecules were included in this analysis and revealed that part of the difference in receptor binding could be attributed to 3 amino acid differences of hGH versus hGH-V in this region.

activation of a family of transcription factors, that includes the p91 and p113 proteins, which were identified as part of the interferon-stimulated ISGF3 transcription complex *(24)* which are in turn substrates for the Jak kinases *(164)*. It appears that cytokine receptors, in general, are associated with Jak kinases which explains the similarity in the effects of various cytokines *(164)*.

It has been suggested that this signalling system may also mediate the insulin-like effects of GH *(165)*. Jak-2 was suggested to mediate, directly or indirectly, the GH-induced tyrosine phosphorylation of SHC, a SH-2 (Src homology type 2) domain-containing protein *(166,167)*. SH-2 containing proteins are also part of the insulin receptor signalling pathway *(168)*. If proven true, this hypothesis may explain the basis of the insulin-mimicking effects of GH. It is particularly interesting that the insulin-like effects of hGH are maintained by the hGH_{1-43} subfragment. In contrast, 20-fold stronger anti-insulin action is obtained by the 44-191 segment compared to the full length protein *(133)*. This raises the possibility that hGH (or hGF-V if a 44-191 generated), may represent prohormones for glucose homeostasis, subject to protein cleavage. If this were true, examination of the integrity of this mechanism during pregnancy might provide some insight for pathological conditions such as gestational diabetes. In this context inappropriate processing of hGH/hGH-V might result in an imbalance between their insulin-like (as expressed by hGH_{1-43}) and anti-insulin (as expressed by hGH_{44-191}) activities. The recent measurement of the hGH_{44-191} segment in pregnant women that revealed a mean value of 3.2 ng/ml compared to 2.0 ng/ml for hGH values produced by hGH RIA *(135)*, supports the possibility that hGH/hGH-V processing occurs during pregnancy. This, in combination with the detection of, moderately but significantly,

lower hCS levels at 9-10 weeks, as well as at 20 weeks of pregnancies complicated by insulin dependent diabetes, when compared to normal pregnancies *(85)*, supports the possibility that the balance between the different hGH/CS molecules may be an important determinant of glucose metabolism and homeostasis in gestation.

VI. Biological functions of members of the human growth family.

a. Effects on body homeastasis and linear growth

The functions attributed to the members of the human growth family are very diverse. However, dissection of the function of individual members has been difficult due to the high similarity and redundancy of function between these proteins. Recently monoclonal antibodies *(88)* and oligonucleotide specific probes *(76,169)* as well as RT-PCR *(123)* approaches have been used to assess the levels of expression of individual members.

Well described effects of hGH include stimulation of the epiphysial cartilage growth, growth of connective tissue, bone (periosteum), muscle, skin and viscera (heart, lungs, liver, kidneys, adrenals, intestines and pancreas), increase in nitrogen uptake (increase in uptake of amino acids by muscle, increase in protein, RNA and DNA synthesis), decrease in protein catabolism, increased lipolysis and lipogenesis (transient insulin-like action), decreased glucose utilization and increased glyconeogenesis, anti-insulin effects (GH increases glucose levels and ketogenesis in diabetics, although *intra-venus* GH causes transient decrease in plasma glucose demonstrating an acute insulin-like

action), increased intestine absorption and urinary excretion of calcium (positive balance of calcium), increased renal tubular reabsorption and serum levels of phosphorus (positive balance), decreased sodium and potassium excretion, increased chondroitin sulphate and collagen synthesis, increased collagen degradation, the increased urinary hydroxyproline, increased glomerular filtration and the decrease of blood urea concentration and increased conjugating capacity for several substances *(87,98)*.

Functions of hPRL (and possibly hCS and hGH) include mammotropic, lactogenic, luteotropic or crop-sac stimulating (rodents and doves) and behavioral effects *(98)*.

Further ovine PL stimulates IGF-I production *(170)*, epiphysial growth and weight gain *(171)*, ornithine decarboxylase activity in liver *(172)* and aminoacid transport (*173.*). HCS also binds to the Nb2 lymphocyte PRL receptors and stimulates the growth of these cells *(174)*

b. Effects on cell proliferation, and differentiation

Members of the hGH/hCS family have been also implicated in a variety of biological effects ranging from cell proliferation to tissue specific differentiation processes *(86,133,175-186)*. Cell differentiation, organ development and functions of the immune system are also, at various degrees, dependent on the presence of growth hormone or prolactin *(187-193)*. Further, certain aberrant situations were also reported to be accompanied by abnormal expression of hGH or hCS *(194-196)*.

The "dual effector theory" proposed by Zezulak and Green *(197)*, gains

recognition as a model for these hGH functions. Human GH is hypothesized to be a "priming" factor for immature cell populations. According to this hypothesis, stem cells following exposure to GH become able to respond to growth stimuli, such as growth factors, that would be otherwise unable to promote proliferation. First demonstrated by the GH induced differentiation of preadipocytes to adipocytes which were subsequently able to respond to IGF-I stimulation with proliferation (an effect described as "clonal expansion", *197*), the "dual effector theory" has been also proposed for the action of GH on chondrocytes *(176,177)* and lymphocytes *(187)*. A variety of precursor cell types such as fibroblasts *(184)*, cytotoxic lymphocytes *(198)* and hematopoeitic cells *(186)* as well as pancreatic islet cells *(180-182)*, have been shown to respond to GH with differentiation and/or proliferation. In many cases local production of IGF-I in response to GH treatment has been reported *(180-182,184)* supporting the validity of the "dual effector theory" in these systems, as well.

D. THE SITES OF GH FAMILY EXPRESSION: GROWTH, DEVELOPMENT AND DIFFERENTIATION OF THE ANTERIOR PITUITARY, THE PLACENTA AND THE IMMUNE SYSTEM.

I. The anterior pituitary

The anterior pituitary derives from Rathke's pouch formed from a placodal ectoderm region on the roof of the primitive mouth *(70,199,200)*. Five different cell types appear one after the other: corticotrophs (POMC), thyrotrophs (ß-TSH),

gonadotrophs (ß-FSH or ß-LH), somatotrophs (GH) and lactotrophs (PRL) *(70)*. Both somatotrophs and lactotrophs appear to derive from a common GH expressing somatotroph stem cell *(201)*. Surrounding mesodermal and neural tissues may influence the proliferation rate *(70)* or the differentiation *(202)* of these pituitary cell types.

Interestingly, three of these cell types (thyrotrophs, somatotrophs and lactotrophs) were extinct in the mouse dwarf locus [Snell dwarf, (dw)] mutants and this was shown to be the result of mutations in the POU domain of the Pit-1/GHF-1 transcription factor *(73)*. This result linked Pit-1/GHF-1, the transcription factor required for the expression of GH and PRL in pituitary *(203-207)*, with the differentiation of the specific cell types that produce these hormones and the development of the pituitary gland. In addition direct effects of Pit-1/GHF-1 in the proliferation of pituitary cells have been demonstrated *(74)*.

Although Pit-1 exerts developmental effects at the level of thyrotroph precursors[3], it is not able to activate GH or PRL gene expression in these cells. Similarly, although present and able to transactivate both GH and PRL promoters *in vitro (209)*, Pit-1 is unable to activate the GH promoter in lactotrophs and the PRL promoter in somatotrophs *in vivo*.

There are many possible explanations for this phenomenon, one being the existence of multiple Pit-1 forms *(210-214)* that can exert some but not all of the effects, or even different effects than those that can be attributed to Pit-1. It might have been

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[3] The fact that lactotroph differentiation may be stimulated *in vitro* by POMC after birth (*208.*), suggests paracrine effects between different pituitary cell types. This raises the possibility that the effect of a defective or absent Pit-1 on thyrotrophs might be indirect and due to the fact that thyrotroph proliferation, differentiation or survival may be dependent on the presence of intact somatotrophs and lactotrophs *(202)*.

very difficult to distinguish these forms by means of *in situ* hybridization and immunocytochemistry with polyclonal antibodies in initial studies *(70,206,207)*. Multiple forms of Pit-1 *(210-213)*, as well as Pit-1 binding sites which are distinct from the initially identified bipartite elements [able to bind an alternatively spliced Pit-1 variant that lacks the POU-specific domain with the same affinity as the regularly spliced protein *(212)*], have been described. The diversity in Pit-1 functions is also supported by the presence of human hypopituitarism, with severe GH and PRL deficiency and mild central hypothyroidism, in the absence of pituitary hypoplasia, as determined by magnetic resonance imaging (MRI). This paradoxical observation was explained by a point mutation of Pit-1 that reduced transactivation but not DNA binding function *(214)*. This raises the possibility that Pit-1 may exert additional effects, independent from its DNA-binding/transactivating function, such as a direct effect on DNA replication similar to that proposed for the Oct-1 mediated activation of viral DNA replication *(215)*.

A second possible explanation is offered by reports in the literature focusing on the presence of multiple cis-elements in the regulatory regions of the GH and PRL genes and synergistic interactions of Pit-1 with other transcription factors *(216-231)*. In this case the contradiction between the absence of GH or PRL expression in lactotrophs and somatotrophs respectively, as well as in thyrotrophs, and the importance of Pit-1 for the differentiation of these endocrine cells, would point at the modulation of Pit-1 action by additional factors that may have either a positive or a negative regulatory effect. Negative regulatory regions have been described in the human growth hormone promoter *(232)*. Two silencer elements 300 and 500 bp upstream of the rat GH mRNA start site function to decrease transcriptional activity in non pituitary cells but had no effects in pituitary

cells *(233)*. However, the significance of these negative regulatory regions in Pit-1 function in thyrotrophs versus lactotrophs or somatotrophs has not been addressed. Nevertheless, the distal hPRL enhancer that contains four Pit-1 binding sites as well as additional DNA elements [*(234-237)*; a similar structure is conserved in the distal rPRL enhancer *(238-250)*], has been shown to contribute to lactotroph versus thyrotroph specific expression of the hPRL gene in transgenic animals *(251,252)*. In addition, in spite of the fact that initiation of Pit-1 expression in the pituitary follows the initiation of TSHß *(70)*, either Pit-1 or a related protein has been suggested to be a trans-acting factor that mediates cAMP regulatory effects on thyrotropin-ß gene expression suggesting that Pit-1 may also exert transcriptional effects in thyrotrophs through DNA-binding *(253)*. Thus, it is possible that TSHß gene activity may be modified upon activation of Pit-1 expression. It has not been determined, however, whether the transcriptional function of factors with a thyrotroph-specific temporal pattern, such as the thyrotroph embryonic factor (TEF) that is able to bind and activate the TSHß promoter *in vitro* may be modified by Pit-1 action. TEF is expressed specifically in the thyrotrophs after embryonic day 14, coinciding with TSHß expression, however, is expressed in a variety of tissues after birth *(70,254)*. It is conceivable that Pit-1 interaction with the TSHß promoter might confer tissue and cell specificity to TEF action in the mature animals.

An important link between the developing CNS and the pituitary is GHRH. GHRH has been suggested to promote pre-somatotroph proliferation and initiates Pit-1 expression in pituitary through cAMP mediated pathways. Transgenic mice expressing the GHRH gene are large *(255)* but more impressively have enormous pituitary glands *(256)*. The promoter region of Pit-1 contains two cAMP responsive elements (CREB

binding sites) as well as binding sites for Pit-1 itself *(257)*. This explains the autoregulation of Pit-1, once basal transcription levels have been established through the GHRH action on its receptors (GHRH receptors are associated with adenylase cyclase-stimulating G-proteins). However, additional DNA elements and corresponding transcription factors have been proposed to contribute to the establishment of the pituitary specific expression of Pit-1 before it can reach levels sufficient for autoregulation *(258)*. Interestingly, a pituitary specific receptor, shown to be a member of the seven-transmembrane-helix/G-protein coupled receptor family, was cloned and was able to mediate GHRH effects *(259)* and sequence comparison revealed it was identical to the GHRH receptor that had been cloned a little earlier *(260)*. This receptor gene is silent in the pituitaries of Snell (dw) dwarf mice that lack functional Pit-1, suggesting that Pit-1 is, directly or indirectly, responsible for its activation *(259)*. This is consistent with the appearance of the GHRH receptor after the appearance of GHRH in the nerve endings of the median eminence, that results in the stimulation of the Pit-1 gene through cAMP activation *(259)*. Clearly, for GHRH to exert an effect on Pit-1 transcription, either Pit-1 gene activation has to have initiated earlier via non-GHRH mechanism, so as to have induced the expression of GHRH receptor, or GHRH receptor gene activation has to have initiated via a non Pit-1 mediated mechanism. Since GHRH receptor is absent in the Pit-1 defective Snell dwarf mice, it is likely that early Pit-1 activation is GHRH independent and responsible for GHRH receptor induction. Alternatively, a remote possibility is that GHRH might exert some of its effects by crossreacting with another receptor, member of the seven-transmembrane-helix/G-protein coupled receptor family, before the expression of its own receptor.

II. The immune system

a. The development of the immune cells, thymus and lymph node

The development and maturation of the immune system is very complex and dependent on the support of microenvironmental factors. Migration and stem cell maturation are often linked, with migration to a new environment being the trigger for proliferation and differentiation *(261,262)*.

Precursors of blood cells likely originate from mesenchymal tissue that surrounds the aorta of the embryo. These stem cells migrate to the yolk sac where they form islets. Subsequently, formation of organs within the yolk sac brings the blood cell islets into different microenvironments that determine the differentiation processes that will dictate the maturation of these stem cells. Stem cells migrate from the yolk sac to the liver of mammalian organisms beginning at the 8th week of pregnancy *(262,263)*.

The liver is the primary location for the initial development of erythroid, myeloid and cells of the B-lineage until the second trimester *(262,264 and references there in)*. Subsequently, stem cells migrate to the bone marrow that becomes the major hematopoietic organ producing blood cells throughout life *(265,266)*. Pre-B cells in the bone marrow undergo immunoglobulin (Ig) μ heavy chain gene rearrangement and subsequently Ig light chain rearrangement *(266,267)*. This allows the expression and the presence of surface IgM molecules that characterize the immature B-cell. Further differentiation results in mature, resting, B-cells that express, also, surface IgD molecules *(267)*. After antigen or mitogen stimulation B-cells can either differentiate to plasma cells

(the terminally differentiated antibody producing B-cells) or proliferate and then return to a resting state characterized by small postmitotic B-memory cells *(262,268)*. These cells, that have now lost their surface IgD molecules *(268)*, can be rapidly induced to differentiate after a new exposure to the same antigen. Mature plasma cells are primarily found in lymphoid tissues such as the medullary cords of lymph nodes, splenic red pulp regions, lamina propria of intestine, as well as, airways and sinusoids of the bone marrow *(262)*.

Similar migratory events take also place for cells of the T-lineage. T-stem cells migrate from the bone marrow to the thymus where they proliferate within the subcapsular cortex and migrate towards the central (deep) cortex. During this endocortical migration they progressively lose their ability for division while at the same time they acquire T-cell specific markers, such as the T-cell receptor antigen. About 90% of the stem cells undergo apoptotic death during this process, but successfully developed mature T-cells finally gather in the medulla, from where they are released to the blood stream. T-cells released in the blood stream aggregate in peripheral lymphoid organs such as the spleen, the Payer's patches and the inner cortex of lymph nodes. Subsequently, within 24 hours, they reenter the circulation *(261)*.

Following antigenic stimulation the primary follicles of the lymphoid tissue develop into "germinal centres" which represent groups of activated lymphoblasts, macrophages and plasma cells *(268)*. A high proliferative rate and extensive cell death, balance eventually lead to the production and maintenance of a relatively stable memory B-cell population in these regions *(268,269)*. Antigenic stimulation, however, induces further cell proliferation *(262,268)*.

b. Neuroendocrine-immune communication

The existence of an extensive lymphocyte arsenal of hormone or neurotransmitter receptors suggests a broad communication between the immune and the neuroendocrine systems [thoroughly reviewed by Berczi *(187)*]. CRH *(270)*, ACTH and endorphins *(271)*, CG, TSH, FSH and LH *(272,273 and references there in)*, VIP, substance P, neurophysins, oxytocin as well as GH- and PRL-like activities *(272-289)* have been detected in immune cells. Of particular interest is the function of the CRH-ACTH-glucocorticoid axis in the immune system. CRH has been reported to stimulate leucocytes to produce POMC-related peptides (ß-endorphin, ACTH and α-MSH), to secrete interleukin-1 and -2, to proliferate (with or without lectin co-stimulation) and to increase IL-2 receptor expression *(290 and references there in)*. In turn, interleukins and other cytokines have a stimulatory effect on pituitary ACTH secretion *(291-295)*. Stimulation of glucocorticoid secretion by ACTH has a systemic inhibitory effect on almost all functions of the monocyte-macrophage cell series as well as certain B- and T-cell functions *(187)*. In contrast, an augmentation of local inflammatory responses *in vivo* that may counterbalance the immuno-suppressive (through glucocorticoids) systemic effects has been described for CRH *(290.)*. The effects of glucocorticoids on lymphoid tissues are also antagonized by thymic hormones, IL-1 and IL-2 as well as by interferons *(296-301)*.

c. *Possible functions of GH proteins in the immune system*

The hypothesis that GH proteins (as well as PRL proteins) have immune functions has been based on *in vivo* observations of immunodeficiency and/or thymus involution in old, hypophysectomized or dwarf animals, or GH-deficient children, that can be reversed by GH or PRL treatment. Further, a variety of *in vitro* GH or PRL effects on immune functions have been observed and hyperprolactinemia has been associated with the presence of active autoimmune diseases *(187-193 and references there in, 302-309).* Berzci *(187)* suggests that a "dual effector theory" *(310,197)* explains the basis of the restoration of immunodeficiency by GH or PRL. He proposes that, initially, GH or PRL and, at later stages, antigens are the "priming" factors that render lymphocyte populations ready to respond to mitogenic stimuli. According to this view, the function of GH/PRL would be more important at earlier differentiation stages during lymphocyte development, at the level of the primary lymphoid organs. Precursor cells in the embryonic liver and the primary organs (bone marrow and thymus) cannot proceed with antigen recognition as no antigen receptor molecules are present on the surface of these cells. Thus, in the absence of the machinery for an antigen mediated pathway, precursor cells must depend on other "priming" or "competence" factors *(187 and references there in).* The impaired DNA synthesis and the involution of both thymus and the bone marrow after hypophysectomy, and the restoration of these effects by GH/PRL administration is consistent with the dependence of precursor lymphoid cells on GH/PRL. GH or PRL may exert their effects directly *(311)* or by inducing the local production of growth factors such as CSF (colony stimulating factor), IGF-I, IL-1, IL-2 or thymic hormones

such as thymulin *(187,312-317)*. Documented GH/PRL effects that may reflect functions on immature populations include a transient decrease in the percentage of B- and T-cells in vivo *(318)*, a stimulating effect on B-cells *in vitro (319)*, the enhancement of granulopoiesis *(320)*, and a decrease *(303,318)* or increase *(98,321,322)* of PHA induced lymphoproliferation. However, no universal conditions have been determined for consistently reproducible results. Human placental lactogen (hCS) was also reported to restore immunodeficiencies in hypophysectomized or dwarf rodents *(187)*.

In addition to the possible "priming" effects on stem cell populations, diverse functions of GH proteins also in the secondary organs of the immune system have been suggested. The germinal centres of the secondary lymphoid organs may be targets for GH/PRL protein action. GH and/or PRL may directly increase the survival rate of germinal centre non-memory cell populations, which are sensitive to apoptotic death *(269)*, or the maintenance of a pool of memory cells in the absence of antigenic stimulation. Alternatively they may influence the final maturation of memory clonal cells, indirectly, by exerting their effects on antigen presenting macrophages. Effects of GH/PRL on peripheral cell populations include proliferation of peripheral blood lymphocytes *(303)*, promotion of cytotoxic lymphocyte generation acting preferentially on the CTL precursor before cell division *(198)*, the augmentation of superoxide anion secretion by mononuclear macrophages and neutrophils *(323-325)*, the production of antibodies *(307,319)* and the increase in the production of IL-1α and IL-2 by mononuclear lymphocytes *in vitro (312)*.

More recently the synthesis of PRL and GH proteins by lymphocytes has been investigated *(272-289)* and a few reports, raise the possibility that these molecules may

exert autocrine functions *(285,289)*.

A complete GHRH/GH/IGF-I-like axis appears active in lymphoid cells. Expression of GHRH-like molecules and GHRH receptors, GH-like activities and GH receptors, IGF-I-like activities and IGF-I receptors, have been reported *(326-332,272,273)* suggesting a functional importance of this pathway in immune cells. These experimental data, however, have not demonstrate conclusively the identity of the GH-activities produced by lymphocytes, as convincing protein and RNA analyses and sequencing data were not produced.

In spite of the number of reports on GH and PRL expression and function in lymphoid cells it appears that the appropriate systems for the study and conclusive demonstration of possible functions, have not been developed yet. Studies elucidating the immune, autocrine or paracrine, functions of PRL or GH axis may have to include mixed culture systems studying complex processes, such as generation of antibodies or mixed lymphocyte reactions. The existence of GH and/or PRL producing lymphoid cell lines would provide an environment with a minimal GH and/or PRL presence that can be modified with antibodies or agents that can modulate the expression of these genes. Studying the effects of these modulations on immune functions of GH/PRL producing lymphocytes or co-cultured blood normal lymphocytes or clonal populations may provide insight on the role of GH or PRL in immune cell-cell communication and intracellular pathways. However a thorough analysis of the production of GH and/or PRL in all types of blood cells would have to precede the initiation of such experiments.

III. The Placenta

Due to the intense research that led to the identification and cloning of Pit-1 and the study of its function, extensive knowledge, of the mechanisms directing the development of the pituitary gland, is available. In contrast, knowledge of the transcription factors and mechanisms that determine placental cell phenotypes, is limited. Two major cell types, cytotrophoblasts and syncytiotrophoblasts, can be recognized in placenta *(336)*. Further distinction of placental cell types, particularly in the cytotrophoblastic layer, is dependent on the identification of cell specific markers, as the two major placental products, hCS and hCG, are both primarily expressed in syncytiotrophoblasts *(81)*. The syncytiotrophoblastic layer of the chorionic villi is often considered as a uniform entity although intermediate forms of syncytiotrophoblasts have been described *(337-344)*. This implies that, at least temporally but possibly also throughout placental life, there is some cellular diversity in the syncytiotrophoblastic layer, as well. However, due to the extensive mRNA and protein similarities, *in situ* hybridization and immunocytochemistry can not distinguish between the different members of the hGH/CS family and reports on hCS expression by these techniques have rarely provided data on the cell specific expression of individual members of the hGH/CS family. Liebhaber *et al (125)* and Scippo *et al (345)*, have identified the syncytiotrophoblast as the site of both hCS and hGH-V expression.

a. The development of the placenta.

A description of the development of the human placenta, from the time of implantation to formation of terminal villi, focusing on the major events that determine the placental structure and a description of identifiable placental cell subtypes, follows. This is necessary for the comprehension of the placental structure and its implications in cell-cell communication and hormone secretion to the maternal or fetal circulation.

(i) Implantation

Originating from the outer layer of the 6 day old blastocyst, trophoblasts penetrate the columnar epithelial cells of the maternal endometrium completing the implantation by the end of the first week. This is followed by the regeneration of the covering endometrium by day 10 which concludes the "interstitial" implantation *(346,336).*

(ii) Trophoblast differentiation, formation of syncytium, trophoblast migration and formation of the cytotrophoblastic shell and primary stem villi

These trophoblasts differentiate into two layers, an inner "cytotrophoblastic" mononucleated cell layer (proliferative Langhans's cytotrophoblasts) and an outer "syncytiotrophoblastic" multinucleated cell layer which establishes a sponge-like formation (day 8) that communicates with maternal sinusoids. These sinusoids, likely derived from endometrial capillaries, form the precursors (lacunae) of the intervillous spaces to be filled with maternal blood and secretions from degraded endometrial glands

(346,336).

"Migratory" trophoblasts that originate from the chorionic base form "columns" that develop into the primary stem chorionic villi *(346)*. The stem chorionic villi separate initially incompletely and finally completely (anchoring villi) the lacunae (day 14). Migratory cytotrophoblasts from the tips of the anchoring villi will invade the syncytiotrophoblast layer fuse with neighbouring invading trophoblasts and form a cytotrophoblastic shell around the 15 day conceptus *(346,336)*. The cytotrophoblast shell separates the primitive syncytium into the inner ("definitive") and the outer syncytium which separates the conceptus from the decidua *(346,347)*.

(iii) Decidua and decidualization

The decidua, until the fourth month, consists of three distinct regions: (a) decidua basalis, which underlies the conceptus and represents the maternal component of the placenta (b) decidua capsularis, which overlies the conceptus and projects into the uterine cavity and (c) decidua parentalis, which represents the rest of the uterine mucosa. The decidua capsularis eventually degenerates allowing the associated chorion laeve to fuse with the decidua parentalis as the uterine cavity disappears *(346,347,336)*.

The "decidualization" under the influence of progesterone of endometrial periarteriolar stromal cells that enlarge, accumulate glycogen and lipids and are exposed to the vascular and glandular modifications of the endometrium, provides trophic support and restricts uncontrolled invasion of maternal tissues by the migratory trophoblasts which are soon to invade the decidualized endometrium (decidua basalis). The migratory

cytotrophoblast invasion occurs preferentially along decidualized columns that surround the blood vessels in the decidua basalis *(346,336)*.

(iv) Trophoblastic invasion and vascular adaptation of decidua and myometrium:

Initially "interstitial" ("stromal") trophoblasts invade in groups the decidua basalis and degrade maternal arterioles and arteries, allowing blood flow to the intervillous spaces. Blood vessel degradation extends gradually to the level of the spiral arterioles and their venules allowing direct arterial blood flow at 37-38 days of gestation. "Perivascular" interstitial trophoblasts closely surround the superficial distal (nearer the intervillous space) branches of the spiral artery and appear almost intramural *(346)*.

The "interstitial" trophoblasts eventually (8-18 weeks) also invade the myometrium although perivascular distribution is absent in this tissue. The dispersed "interstitial" trophoblasts in the myometrium are believed to contribute to the preparation of the myometrial segments of the spiral arteries for the subsequent migration of "endovascular" migratory trophoblasts. Edema and disorganization of the myometrial segments of these arteries, likely, occur following local steroid production by the "interstitial" trophoblasts. At term, most of the "interstitial" trophoblasts have either disappeared or fused to form the "placental bed syncytial giant cells" (346).

The "endovascular" migratory cytotrophoblasts originate initially from the cytotrophoblastic shell and later from the anchoring villi. The decidual segments of the main spiral arteries, up to the junction with the myometrium, is invaded earlier, until the 10th week, by the "endovascular" trophoblasts that become embedded in the wall of these arteries disrupting their muscular and elastic elements. At about 14-16 weeks, a new

invasion phase extends to the myometrial segments of the spiral arteries reaching the distal portions of the radial artery *(346)*.

These events lead to the formation of functional "uteroplacental" arteries that are able to provide constantly the increased blood flow necessary for the fetus but do not respond to systemic blood pressure or autonomous system regulators *(346-350,336)*. Control of the blood flow to the placenta is obtained at the level of the radial arteries which can respond to such stimuli as their neuromuscular component is intact *(346)*[4].

(v) Formation of mature villi and fetal-maternal exchange surface

The "primary" villi develop mesenchymal cores forming the secondary villi which in turn will be branched ("branch" villi) and extend to the entire surface of the chorionic shell *(346,348,336)*. Mesenchymal cell differentiation into blood capillaries in the "secondary" villi will result in the formation of an arteriocapillary venous network which

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[4] Disruption of this normal transformation process results in abnormal conditions such as *preeclampsia*. In this situation only the decidual portion of the spiral arteries are subject to the invasion by endovascular trophoblasts which results in a myometrial portion of these arteries that contain an intact muscular component. This leads to both reduced blood flow to the intervillous space as well as, spiral arteries sensitive, at the myometrial level, to the systemic and autonomous system regulatory control of blood pressure (may need to add: atherosis of myometrial portion, acute necrotizing arteriopathy, p.35). It is characteristic that at about 50% of the cases of uncomplicated intrauterine growth retardation there is failure of trophoblastic invasion and restriction of these physiological changes similar to preeclampsia.

In extreme cases, failures for vascular adaptation result in late first trimesters spontaneous recurrent abortions (when the decidual portions of the spiral arteries fail to adapt) or second trimester abortions (when both decidual and myometrial portions bare insufficient or no changes). In certain cases, absence of endovascular or sparse interstitial trophoblasts is observed implying a defect in the normal migration process*; in others insufficient or no vascular changes occur despite the presence of interstitial trophoblasts implying a defect in trophoblast-uterine interaction *(346 and references there in)*.

*It has been reported that in preeclamptic women cytotrophoblasts which invade the uterus retain cell adhesion molecules that are normally expressed only in villous stem cells and cytotrophoblasts in the proximal region of the cell columns. In addition they fail to upregulate the expression of an integrin receptor that in normal pregnancy is expressed only by placental bed cytotrophoblasts. This, presumably, switches the adhesion molecule phenotype to a profile that is more compatible with only limited uterine invasion *(351)*.

characterizes the "tertiary" villi (day 20). This network will soon communicate with the embryonic heart through vessels derived from the mesenchyme of the chorion and the connecting stalk *(346)*.

The mature villous structure includes three different types of villi: (a) the "stem" villi (containing centrally located fetal arteries and veins with media, highly branched, their branches being smaller stem villi or intermediate villi and with a rich connective tissue stroma) (b) "intermediate" villi (containing a reticular stroma and arterioles and venules lacking media) that can be distinguish as "mature" (thin, containing many terminal villi) or "immature" (thick, with few terminal villi) (c) "terminal" villi which represent the final branches with fetal capillaries and a minimal fetomaternal diffusion distance, where the fetomaternal exchanges occur *(346,348,336)*.

Until week 12 four layers will separate the fetal and maternal circulations *(346)*: (a) syncytiotrophoblasts (b) cytotrophoblasts (Langhans's layer) (c) connective tissue cores of the villi, containing also stellate mesenchymal cell and Hofbauer cells (fetal macrophages, with large nuclei and vacuolated cytoplasm) (d) endothelium of fetal capillaries *(346,348,336)*. With the progression of pregnancy the syncytiotrophoblastic layer becomes thinner and the cells of the Langhans's cytotrophoblastic layer are reduced in both size and number almost disappearing by term *(336)*. This results in an increased occupation of the villi's inner by the fetal capillaries that dilate, form sinuses or fuse with the outer syncytiotrophoblastic layer providing a vasculosyncytial membrane important for fetal-maternal oxygen and CO_2 exchange. At the same time the villi's stroma condenses and Hofbauer cells are compressed by stromal tissue and vessels *(346,336)*.

Syncytial knots, formed by the sequestration of nuclei, increase in size the last

12 weeks of pregnancy and connect with knots from adjacent villi forming intervillous syncytial masses that project into intervillous space and are often released to the maternal venous system and may embolize the lung capillaries *(336)*. By the end of pregnancy a fibrinoid material forms on the villous surface *(346,336)*.

(vi) Mechanism of formation of syncytial structures from cytotrophoblasts.

Several differences exist between cells of the Langhans's layer and the syncytiotrophoblasts. Langhans' cells have a less dense cytoplasm, with larger mitochondria, less developed endoplasmic reticulum (ER), fewer inclusions and a number of ribosomes, unattached to membranes *(337)*.

A number of cells, present either between Langhans' cells or in the basement membrane show characteristics that resemble syncytial cells, having smaller and more dispersed Golgi system, many granular inclusions, more granular ER and denser cytoplasm. In some cases a disruption of the plasma membrane can be observed on the syncytial site of these "intermediate" type of cells [also termed by some investigators as "X" cells *(338)*], and small isolated stretches of membranes with desmosomes are found in the syncytium *(337)*. "Intermediate" types of cells have been also described as round or polyhedral, mononuclear, with larger hyperchromatic nuclei, and abundant, less eosinophilic than in syncytiotrophoblasts, cytoplasm *(342,343)*. Based on their description, placental localization and hormonal profile, "intermediate" cytotrophoblasts, may either represent a transient type of cytotrophoblasts which differentiate into the terminally differentiated syncytiotrophoblasts that form the syncytium *[336-338, first*

described in the first trimester placenta by Terzakis (344)], or a type associated with trophoblastic columns and the trophoblastic shell involved in implantation, decidual invasion, establishment of uteroplacental circulation or formation of placental bed giant cells *(342,343)*. The latter form presumably corresponds to the migratory interstitial trophoblasts described above in sections *ii* and *iv*, p.37 and p. 39, respectively *(346)*.

Cell "fusion" was suggested early as the possible mechanism for the final transition to syncytiotrophoblasts. At first cytotrophoblasts are subject to differentiation events that render them similar to syncytiotrophoblasts. Incorporation of these differentiated trophoblasts into syncytial structures is accompanied by the maintenance of only small membrane fragments, that often bear desmosomes and eventually disappear from the syncytial cytoplasm *(338,344,352)*

The sequence of these events was conclusively shown, by time lapse cinematography and microinjection of fluorescently labelled α-actinin 24-120 h after isolated mononuclear trophoblasts from human term placentas were plated in culture *(353)*. Two sizes and two types of cytotrophoblasts were observed by transmission electron microscopy of freshly isolated Percoll-gradient purified cells: 10 and 20-30 μm cytotrophoblasts appeared either **immature**, with little rough ER, few lipid droplets and microvilli and some glycogen or **mature**, with numerous microvilli, many coated pits, abundant smooth membrane vesicles, well developed Golgi, many lipid droplets, branched segments of rough ER and scattered multivesicular bodies. Occasional cells formed intermediate junctions and rarely had internalized desmosomes *(353)*.

A number of growth factors has been implicated in the proliferation and progression of cytotrophoblasts to more differentiated forms. IGF-II and PDGF may

induce trophoblast proliferation through induction of c-myc expression *(338,354-356)*. Morphological differentiation to syncytiotrophoblasts is enhanced when the culture surface is coated with extracellular matrix proteins such as laminin, fibronectin or collagen or when EGF or cAMP agonists are added to the culture medium *(357-362)*.

b. The placenta as an endocrine organ - Endocrine profile of differentiating, terminally differentiated and neoplastic trophoblasts

Fetal health could be threatened by maternal infections or the immune response against fetal and placental antigens and the excess of growth factors needed during embryonic development could be harmful to the maternal homeostasis if the placenta did not act as barrier between the embryo and the mother. This anatomical and biochemical placental barrier *(348,336)*, likely limits maternal-fetal hormonal exchange and may prevent fetal signals from modulating the hypothalamic and pituitary function of the mother. To override this problem, trophoblasts, cytotrophoblasts and syncytiotrophoblasts appear to assume some pituitary functions, functions of other endocrine organs, as well as hypothalamus in the context of interactions between different placental cell populations that secrete hypothalamic or pituitary factors[5] *(349,336,363)*. Further, cytotrophoblasts and syncytiotrophoblasts may respond differentially to maternal or embryonic signals and establish distinct hormonal profiles in the maternal or embryonic circulation.

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[5] The endocrine function of the trophoblasts has received increased attention the last 15 years. Studies on the endocrinology of pregnancy have shown that the placenta overrides the pituitary and hypothalamus in the production of many of the "classical" pituitary hormones and hypothalamic releasing factors. GnRH, somatostatin, CRH, TRH, oxytocin, hCG, hCS, ACTH, dynorphins, PGE, PGF, MPF, 6-keto-PGF, TxB2, inhibin, relaxin, TGFa, FGF, IGF-I etc are produced by the placenta or other intrauterine maternal or embryonic tissues (fetus, amnion, chorion and decidua) *(363)*.

i. Expression of human chorionic gonadotropin (hCG), chorionic somatomammotropin (hCS) and growth hormone variant (hGH-V) by normal and abnormal trophoblasts.

α. Expression of hCG, hCS and hGH-V by cytotrophoblasts and syncytiotrophoblasts

HCG and hGH/CS proteins are major placental products and represent markers of the differentiation status of trophoblasts *(364,365)* and valuable clinical diagnostic and prognostic parameters for the evaluation of the normal progression of a pregnancy *(86,366)*. The actual site of synthesis of these hormones has been the subject of extensive investigation. A study of the localization of GH/CS proteins in human placenta demonstrated localization of both hCS and hGH-V in chorionic villi *(124,125)*. In situ hybridization of 7-12 week placentas with hCGα and hCS probes showed that both signals were present in the *syncytial layer (81,365)*. HCGα signal (deriving from only one functional gene that also codes for the α subunit of LH, FSH and TSH *(364,367,368)* was also present in the *cytotrophoblast layer (81,365)*. HCGß signal (deriving from at least two functional genes, hCGß3 and hCGß5 *(369,370)*, was present also in the *syncytial layer* and in *few cytotrophoblasts (365,371)*. This suggested that hCG expression is activated during the cytotrophoblast to syncytiotrophoblast transition, after the exit of the cytotrophoblasts from the cell cycle *(81)* but even before the final fusion to syncytiotrophoblasts. In contrast, hCS expression rather requires syncytial formation *(365,372)*. *Table 4* summarizes the sites of expression of hCG and hCS/GH-V.

Interestingly, hCG mRNA is significantly decreased with the progression of

pregnancy (6-fold for hCGα and to barely detectable levels for hCGß *(365)*, ratio hCGα:hCGß from 1.7:1 at the first trimester to 12:1 at term *(373)*. This correlates with

Table 4. Sites of expression of hCG and hCS and hGH-V

HORMONE	CELL TYPE	References
hCG	Intermediate trophoblasts (multinucleated) Syncytiotrophoblasts	81,364,371
hCGα	Intermediate trophoblasts (mononucleated and multinucleated) Syncytiotrophoblasts	81,364
hCGß	Intermediate trophoblasts (multinucleated) Syncytiotrophoblasts	364,371
hCS/GH-V	Villus syncytiotrophoblasts Extravillus trophoblasts Intermediate trophoblasts	81,103,124 125,338 364,365 371,372

the gradual marked decrease in the number of cytotrophoblasts that represent a very small portion of placental cells at term *(365,346,336)*. The same pattern is followed during prolonged culture of cytotrophoblasts that form "villus-like" structures but lose their ability to produce hCG after several days although the cells are still viable *(374)*.

These data suggest that hCGα and hCGß expression is dependent on the presence of cytotrophoblasts and that a "multinucleated intermediate", not distinguishable by the already applied techniques from the true syncytial cells, may be responsible for their production in the syncytial layer *(365)*. This would imply that the cytotrophoblastic layer steadily supplies the syncytiotrophoblastic layer with this transient "intermediate" form but as soon as the cytotrophoblasts are depleted, hCG expression rapidly regresses. In

serum free culture, in the absence of extracellular matrix proteins, however, mononuclear cytotrophoblasts that cannot spread, aggregate or fuse, have been shown to secrete large amounts of hCG *(357)* implying that "mononuclear intermediates" have already acquired differentiation characteristics allowing them hCG production. In either of these cases, hCGα expression appears to start earlier in the "committed" Langhans's cytotrophoblasts while hCGß expression initiates later, following further differentiation events that lead to the formation of an "intermediate" (either mono- or poly-nucleated) form. Intermediate cell population peaks at the 8-10 weeks and starts declining after this point *(365)*.

An alternative theory for the accumulation of mRNA for hCGα and hCGß during differentiation focuses on the regulation of gene expression at the post-transcriptional level following, possibly, cellular microenvironmental changes. HCGα mRNA half life raises from 40 minutes to 3 hours during differentiation of trophoblasts *(375)*. Similarly, in human choriocarcinoma JEG-3 cells treated with 1μM methotrexate which inhibited DNA replication, there was a 15 to 20-fold increase in hCGα steady state mRNA level while hCGß mRNA was constant. Nuclear run on experiments showed no difference in transcriptional activity between the two subunits *(375)*. These methotrexate-treated JEG-3 cells have lost their proliferating potential and possess a complex reticulated cytoplasm resembling cytotrophoblasts at an "intermediate" state on the verge of differentiation *(371)*.

β. Expression of hCG, hCS and hGH-V in choriocarcinomas and hydatidiform moles

Choriocarcinomas consist of not fully differentiated trophoblasts. A typical histological characteristic is that although there are clusters of mononucleated trophoblast cells, enlarged mononucleated trophoblast cells and remnants of polynucleated syncytiotrophoblasts, no villi formation is observed in sections of these tumors *(365)*. **HCG** signal could be observed in *syncytial cells* and in few large cytotrophoblasts with both cytotrophoblast and syncytiotrophoblast characteristics (*"intermediate" cytotrophoblasts"*), while no **hCS** signal could be detected in early studies *(365)*.

Hydatidiform moles (HMs) still maintain the placenta villi phenotype although there is swelling of the villus stroma, hyperplasia of the cytotrophoblasts and absence of fetal vessels *(365,376)*. Two different forms of HM exist *(376)*. The **complete** HMs derive from "empty", non nucleated, oocytes that have been fertilized either by a sperm with double genetic material or two different sperms *(377,378)*. Thus, the resulting 46XX or 46XY caryotype, with diploid chromosomal content, is exclusively of paternal origin and no fetal development is observed in these cases *(376,377,379-384)*. In contrast, the **partial** HMs derive, usually, from the fertilization of normal nucleated eggs either by a sperm with double genetic material or two different sperms *(376,380,385)*. Thus, in these cases, the resulting caryotype, with a triploid 69,XXX or 69,XXY chromosomal content, is of paternal and maternal origin with the excess chromosomal content deriving usually from the paternal side *(376,380,382,384)*. Fetal development is observed in partial HMs, fetal vessels are usually present, trophoblastic hyperplasia is less profound

and abnormal tissue is interspersed within normally developed placental tissue *(376,380)*. The diagnosis of the type of a HM by the caryotypic analysis, however, may be not absolute, as a number of triploid complete HMs and a number of diploid partial HMs have been reported *(380)*. DNA fingerprinting, however, of a HM that was macroscopically and microscopically characterized as partial, revealed the simultaneous presence of a complete HM and an abortion *(382)*. In this case caryotypic analysis of either the embryonic or the HM material would have attributed a diploid caryotype to a HM that might be falsely diagnozed as "partial" due to the concurrent abortion.

Both hCG and hCS signals have been detected by *in situ* hybridization in these tumors *(365)* with their secretion profile being characterized by very low hCS and higher than normal hCG levels *(376,383)*.

ii. Hormonal production in the placenta and embryonic membranes

An intriguing aspect of placenta endocrinology is the multipotency of trophoblasts for the production of hormones and protein factors that are normally produced by a variety of endocrine organs, including the pituitary and the hypothalamus *(336)*. Trophoblasts either display an "infinite" ability for synthesis of transcription factors specific for several other tissues, or use mechanisms that override the requirements and restrictions that rule tissue-specific gene expression in other organs. It is possible that trophoblasts maintain the potential for expression of a variety of genes but lack the ability to express other genes (like the HLA MHC antigens) due to the absence of development of restriction mechanisms that would blunt expression or activating mechanisms that

would allow expression of certain genes, respectively. This would be consistant with the hormonal gene expression being, in most of the cases, under negative, rather than positive, regulatory control while for other genes (like the HLA antigens) the reverse may be the case. An alternative possibility is that this "multipotency" is just the reflection of the existence of many different subtypes of trophoblasts that we have not been able to distinguish until now.

Several hormones and factors identified in placental or decidual tissues and the cell types of synthesis are presented in *Table 5*.

Table 5. Placental or decidual hormones, releasing factors, growth factors and oncogenes and the cell types of their synthesis.

HORMONE/ RELEASING FACTOR	CELL TYPE	References	GROWTH FACTOR/ GENE	CELL TYPE	Refs
GnRH	vCT, vST (early pregnancy)	338,363 386-388	IGF-I	vCT, vST	355 375
CRH	vIT, vST, xvT, dSC dbIT, evscT (>15 week) aEC, invT	363,389 390	IGF II	vCT, prlT, colT, TI, vSC (EP), mesSC, CTSh	355,354 375
ACTH	aEC	391	TGF-ß	vCT, CTSh (EP)	355,375
Somatostatin	vCT	392	TGF-α	endometrium	375
IL-2	vST	375	EGF	not expressed	375
IL-6	vST	375	FGF	prol-vCT,vST	401
Renin	chT	338	PDGF-B (c-sis)	vCT, prolT-CTSh (α' trimester), InvT	338,355 356,375
Inhibin	vCT,dC	388,393-397	c-myc	vCT	355,375 383,402
Activin	vCT	388,395-397	IGF-IR	vCT	375
Relaxin	IT,chCT	341,398,399	IGF-IIR	vCT	338
Prostaglandins	chT (PGE), IT (produce PGDH), aEC (produce PGH_2 synthase)	338,398,400	PDGF-BR (c-fms)	vCT	338,356
Estrogen	vST[6]	338-340	EGFR (c-erb-B)	vST	360,375
Progesterone	vST, chT	338,340	SP1	vST, colT, xvT	338

[6] *Villus syncytiotrophoblasts* secrete progesterone, by converting cholesterol, as well as estrogens by aromatizing fetus-derived androgens *(338,339)*.

c. *Possible functions of GH proteins in pregnancy.*

i. *Distribution of hGH proteins and hGH receptor isoforms and general metabolic effects of hGH/hCS during placental and embryonic development.*

It is conceivable, that the growth hormone "analogues" produced by the placenta might promote similar direct effects for the embryo as in infancy and childhood: growth and differentiation. However, the fact that hGH/CS proteins may be unable to cross the placental barrier *(83,403)* compromises their potential for direct hGH/CS effects on the embryo. In contrast, hCS has been implicated in fetal growth and other functions during pregnancy through general metabolic effects on the maternal circulation *(86,98,179,403-407)*, suggesting indirect effects on fetal metabolism. The anti-insulin effects of hCS that result in lipolysis, proteolysis and increased blood glucose maintain amino acids and energy substrates readily available for the fetus *(86)*. Fasting and low levels of glucose induce hCS secretion *(404,86,407)* supporting its role in food and energy homeostasis. Further, it would be consistent with the function of hGH in other systems if hCS or hGH-V had similar direct effects on the differentiation and proliferation of placental trophoblasts. An acceleration of differentiation of trophoblasts, which possess hCG/LH receptors, has been described for hCG *(408)*, but no reports have appeared on similar effects of hCS or hGH-V proteins. In fact, during trophoblast differentiation in culture, hCS expression initiates only when the fusion of cytotrophoblasts to syncytiotrophoblasts is essentially complete which coincides with the peak of hCG expression *(372)*. By analogy to the effects of hGH in immune cells and other systems *(175-177,180-*

193,197,309,311-317), however, placental hGH/CS may modulate the hormone, growth factor (such as IGF-I or IGF-II), cytokine (such as interleukins IL-1 and IL-2), or nuclear factor (such as c-*myc*, which is present in undifferentiated cytotrophoblasts but not in syncytiotrophoblasts; *355,375,383*) synthesis by trophoblasts. In spite of the fact that normal pregnancies have progressed in the absence of normal hCS levels *(409-413,77)*, Parks *(77)* has pointed that in none of these cases complete absence of hGH/CS proteins has been detected. Particularly interesting, in light of the demonstration of hGH-V and hCS-L transcripts in human placenta *(123)* was a pregnancy where hGH and and a hCS-like protein (likely to be hCS-L) were detected in placenta *(409)*. This was due to a gene rearrangement that deleted both alleles of the hCS-A, hCS-B and hGH-V genes and brought the hGH-N and hCS-L genes in proximity to the hCS-B enhancer *(413)*.

Interestingly and in contrast to the reported inability of hGH/CS proteins to cross the placenta barrier, distinct receptors specific for hCS or ovine placental lactogen exist in fetal liver *(153,154,414)*, and a variety of effects have been shown for hCS or placental lactogens of other species in fetal tissues in culture *(86,153,170,172,173,180,415-417)*. Similar effects were not always observed for hGH in spite of its ability to cause similar effects on postnatal tissues *(86,415,416,173)*. If indeed, hCS is present at minimal levels in the fetal circulation *(154,403)*, any possible effects of hCS on fetal tissues would have to be generated by a very small fraction of the maternal plasma hCS concentration that would suggest a great difference in responsiveness to hCS between the maternal and fetal tissues. The absence of GH effects on the fetus is supported by transgenic mice overexpressing GH that show no size augmentation until after the 3rd week postnatally *(418)*.

Two major questions, rather closely associated, remain unanswered: (a) whether there are distinct hGH, hGH-V and hCS receptors in the maternal or fetal tissues and (b) the reason behind the release of **gram** quantities of placental lactogen in the maternal circulation, daily.

Recently the identification of the alternatively spliced-exon 3 truncated GH-R *(121)* suggested the presence of a distinct form of GH-R in trophoblastic populations. Interestingly the hGH-Rd3 was the only form of the receptor found in chorionic villi and the amnion while the full length hGH-R represented more than 98% of the receptor population in chorion and decidua. Provocative results were produced by the analysis of normal tissues and tumor cell lines: choriocarcinoma (JEG-3) cells in contrast to the chorionic villi appeared to express predominantly (95%) the full length form *(121)*. The reverse pattern was also observed in normal liver (hGH-R:100%) versus hepatoma Hep3B cells (hGH-Rd3:100%). A very interesting observation was that the IM9 lymphoid cell line that responds to hGH with proliferation expresses primarily the hGH-Rd3 form (79%). The hGH-Rd3 form appears more similar to the PRL receptors due to the absence of exon 3, a common feature for the PRL receptors *(121)*. In spite of that similarity, however, IM9 cells do not appear sensitive to PRL treatment *(277)*. Urbanek *et al (121)* suggested that hCS could be a ligand for hGH-Rd3 and subsequently confirmed this possibility *(419)*. However, hGH, hGH-V and hPRL were also shown to bind to hGH-Rd3 *(419)*.

The presence of hGH-V2 suggested to be a transmembrane protein adds another component that could be involved in cell-cell communication during processes such as trophoblast differentiation or migration. The function of hGH-V2 has not been elucidated

and its tissue localization, in the chorionic villi, appears coincident to the localization of hGH-V and hCS although no cell-specific colocalization has been demonstrated *(103,124,125)*.

ii. Placental lactogens and possible functions in animal models

The development, in Friesen's laboratory, of the radioreceptor assay (RRA) for prolactin and other lactogenic hormones by Shiu *et al (420,421)* and the Nb-2 bioassay by Tanaka *et al (152)* was followed by extensive research for the identification of "placental lactogens" in mammals. This led to the identification of a highly conserved family of proteins with similarities to either GH or PRL *(422-435, for reviews 436-440)*. Ogren and Talamantes *(438)* categorized these proteins into two groups: (a) a single-chain 20-25K polypeptide group, with 2 or 3 intrachain disulphide bonds at positions similar to the positions of disulphide bonds in GH (two bonds) and PRL (three bonds) molecules (hCS, monkey CS, mPL-II, rPL-II, hrPL-II, oPL, baboon CS are some of the proteins of this group) and (b) a single-chain 30-34K polypeptide group, including bPL, mPL-I, hamster PL-I and rPL-I, with reduced structural similarity to GH/PRL. Characteristic for this group, is the example of mPL-I that lacks the large disulphide loop of the GH/PRL family *(438)*.

Research in rats and mice has revealed a diverse family of placental lactogens and PRL-like proteins that follow distinct cell-specific and/or temporal patterns of expression during gestation (*Table 6*, includes rat and mouse PLs and PRL-like proteins, their similarities to PRL at the amino acid level, the cell types of their synthesis and their

temporal pattern of expression; data were drawn from Duckworth *et al (437)*, Talamantes *et al (439)* and Soares *et al (440)*.

Functions of PL/PRL-like proteins, during the rodent pregnancy, may include, regulation of somatomedin activity *(441)*, maternal metabolism, mammary gland production of IGF-IBP (mainly mPL-I, also mPL-II) and α-lactalbumin (mainly mPL-II, also mPL-I), corpus luteum steroidogenesis (PRL), inhibition of ovarian follicular development and promotion of luteal development and function (PL/PRL-like proteins in pregnancy), while hepatic, uterine, lymphatic, extraembryonic and fetal (liver, muscle) tissues have been shown to possess PL or PRL receptors and respond to PRL-like proteins with growth and differentiation or modulation of their metabolic and secretion profiles *(440 and references there in)*. Interestingly, some PRL-like proteins have been detected in the fetal circulation *(438,427,428)* suggesting a mechanism that overrides possible placental-barrier restrictions. Finally, rPLP-A appears to be subject to transportation to the nucleus of trophoblasts cells *(429,431)*. This phenomenon, that has been also described for PRL in hepatic and lymphoid cells *(27,28,442,443)*, raises the possibility that rPLP-A may exert nuclear functions as well.

iii. Immune regulation at the placental-maternal interface.

For the maternal immune system, the fetal-placental unit represents a "graft" rather than a self organ. This suggests that there must be a mechanism that inactivates the maternal immune system and allows the normal progression of pregnancy. As a result, several immune functions have to be modulated during pregnancy to permit the

development of the placenta.

Although the basis of the acceptance of the placenta as a self organ exists due to the expression of the non-polymorphic HLA-G by extravillous trophoblasts in direct contact with the decidual tissue and the non expression of HLA-A, -B, -C, -D, and -DR by syncytiotrophoblasts *(444-447)*, additional mechanisms appear to be required for blunting a possible immune response. A sialomucin coating over trophoblasts and the generation of suppressor cells and suppressor-blocking factors or antibodies may also contribute to the prevention of an immune response *(444,446)*[7].

Of particular interest, are certain types of immune cells that may contribute to the successful progression of pregnancy. **Macrophages**, which process and present antigens, secrete monokines that can be either immunostimulatory (IL-1) or immunosuppressive (PGE_2) depending on local interactions *(444)*. **Suppressor cells** inhibit the activation of other effector cells specifically through MHC antigens (alloantigen specific suppressor T lymphocytes) or non-specifically (natural suppressor cells, non-bearing surface markers of mature B- or T- lymphocytes or macrophages) blocking non-T cell immune responses *(444,446)*. **Natural suppressor** cells are localized primarily in the decidual placental bed where they may control locally the activity of the large numbers of decidual **natural**

[7] Four different categories of potentially blocking antibodies are generated during pregnancy (a) allo-antibodies against parental alloantigens (b)inti-idiotype antibodies against the antigen combining site of a primary antibody that can block mixed lymphocyte reactions (MLR) or non cytotoxic IgG antipaternal antibodies (c) cytotoxic antibodies that can inhibit T-cell proliferation in a MLR and (d) anti-TLX (trophoblast-lymphocyte cross-reacting antigen system) antibodies against non-HLA antigens shared on trophoblasts and lymphocytes. No conclusive evidence, however, has been produced for the role of these antibodies in the limitation of a mother versus placenta immune response and their contribution to a successful pregnancy *(444)*.

Table 6. PRL-like proteins of the rodent pregnancy, cell-specific and temporal patterns of expression.

PRL-LIKE PROTEIN	CELL TYPE	INITIATION OF EXPRESSION	TERMI-NATION
rPRL	pituitary lactotroph	**day 0** (*two daily surges*) **day 20** (*pituitary secretion increases*)	**day 11** term (**day 21**)
rPL-I (38%)	trophoblast giant cells (TGC)	**day 6**	**day 12**
rPL-Iv	TGC, spongio-trophoblasts (SPGT)	**day 12-14**	term
rPL-II (47%)	TGC labyrinth TGC	**day 11** (*TGC*), **day 15-16** (*labyrinth TGC*)	**day 13-14** (*TGC*)
rPLP-A (39%)	SPGT, TGC	**day 12-14**	term
rPLP-B (*LTH?*) (39%)	decidual cells (dC) SPGT	**day 6** (*dC*) **day 12-14** (*SPGT*)	**day 13-14** term
rPLP-C (34%)	SPGT, TGC	**day 12-14**	term
mPL-I (33%)	TGC	**day 6-8**	**day 13-14**
mPL-II (39%)	TGC	**day 8**	term (**day 19**)
mPROLIFERIN (mPLF, 32%)	TGC	**day 8**	**day 18**
mPLF-RP (**R**elated **P**rotein) (30%)	SPGT	**day 10**	term

killer (NK) cells *(448)*[8]. Natural suppressor cell presence in the maternal-placental interface appears to be controlled by both hormonal and trophoblast factors *(449)* while NK cells appear to be attracted by embryonic and trophoblast signals *(450,451)* andprimarily serve as a barrier against the unrestricted invasion of maternal tissues by trophoblasts *(444)*. To emphasize on the importance of suppressor cell populations, in a mouse system, antibodies against natural suppressor cells resulted in the termination of the pregnancy or decreased litter size *(452)*.

An interesting aspect of trophoblastic phenotype is their reduced susceptibility to either specific or non specific cell lysis *(453-455)*. In addition to the absence of HLA antigens (that makes them resistant to cytotoxic lymphocytes), the inability to stimulate in a mixed lymphocyte reaction (MLR) suggests that no other antigens are stimulatory for T-cells *(454,456)*. Further, an increase in membrane fluidity, induced by progesterone *(457)* and a reported relative deficiency in surface structures [membrane structures possibly regulated by τ-interferon (τ-IFN) or related to MHC *(458)*] necessary for NK recognition and binding, may further reduce cell lysis susceptibility *(444,459,460)*. However, lymphokine activated killer cells (LAK cells, likely an activated form of NK cells) are able to lyse trophoblasts *(461)* which underlines the importance of the control of the NK activity at the decidual level. NK activity is inhibited by

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[8] Natural killer cells are large granular lymphocytes with no cell surface markers characteristic of mature B- or T- cells or macrophages. They are active against tumor cells, viral infections and they control the hematopoietic stem cells. They bear cell specific markers such as asialo-GM1, CD57, CD16 (Fc receptor) and CD56 and represent up to 50% of the decidual cells apparently attracted to the uterus by embryonic and trophoblast signals. Lymphokines such as GM-CSF secreted by the NK cells may have some growth factor action on the placenta. Systemic NK activity is decreased from early to mid pregnancy but is again increased before parturition. Decidual NK cells are progressively inactivated by PG_{E2} produced by decidual cells and macrophages in response to progesterone. Similarly hCG and LH have been shown to have suppressive effects on NK activity. Interestingly although the lytic capacity of NK cells is reduced in pregnancy their binding capacity to effector cells remains intact *(444 and references there in)*.

progesterone which inhibits also cytotoxic lymphocyte activity and prostaglandin F2a (PGF2a) synthesis *(462,463)*[9]

A number of substances, including hCG, hCS, progesterone, α-fetoprotein, human early factor, τ-IFN, PGE_2 (produced by small lymphocytes, macrophages and large cells in decidua), decidual and trophoblast suppressing factors[10] may have immunosuppressing activity *(444 and references there in)*. Contractor and Davies *(475)* reported inhibitory effects on phytohemagglutinin-induced lymphocyte transformation for both hCS and hCG and suggested that suppression of cell-mediated immunity in such a mode may contribute to the immune tolerance of the fetal-placental unit.

The association of growth hormone and prolactin with the immune system has been raised in many occasions. Lymphoid cells express receptors for PRL and/or GH *(139,308,312,476-479)* and both proteins can induce proliferation of the Nb2

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[9] On peripheral blood lymphocytes, high doses of progesterone [100-fold greater than pregnancy serum levels but equivalent to local placental tissue and maternal placental interface *(444,462,464,465)*] suppresses mixed lymphocyte reactions, mitogen activation and cytotoxic T-cell generation *(466,467)*. These effects appear direct effects on lymphocytes. A 34K protein produced at levels proportional to the levels of progesterone receptor present in lymphocytes appears to induce immunosuppressive phenotype *(462,463)*. Binding of progesterone-treated lymphocytes to alloantigens is not affected but their lytic capacity is reduced. Exogenous arachidonic acid will block and indomethacin increase suppressive activity *(468)*. Interestingly peripheral blood lymphocytes, normally responsive to progesterone during pregnancy are 100-fold less responsive in threatened spontaneous abortion, preterm labour or men *(468,469,470)*. Further, a remarkable salvation of fetal resortion after administration of a progesterone receptor blocker was obtained by administering the 34K protein from progesterone-primed pregnancy lymphocytes *(462)*. Although the presence of progesterone receptors in lymphoid cells has been proposed, progesterone effects are considered to be exerted through the glucocorticoid receptor *(187 and references there in)*. This may explain why "progesterone" receptor levels in lymphocytes are not upregulated by estrogen in contrast to most other tissues, but they are regulated by a combination of specific MHC antigen expression and alloantigenic stimulation *(462)*. This pattern resembles the pattern of regulation of the glucocorticoid sensitivity of thymocytes which is also influenced by MHC antigens, as well as by thymic hormones *(187)* supporting the idea that progesterone effects may be, indeed, exerted through the glucocorticoid receptor.

[10] A decidual suppressing activity is produced by natural suppressor cells and is distinct from TGF-ß2; this activity, however can be inhibited by anti-TGF-ß2 monoclonal antibodies *(471,472)*. This activity blocks T cell response to IL-2 *(449,473)*. A similar suppressing activity can be isolated from trophoblasts and it is able to inhibit IL-2 production by lymphocytes and macrophages and down regulate the IL-2 receptor expression in lymphocytes *(474)*.

lymphocytes by binding to the PRL-R present on the surface of these cells *(150-152)*. Additionally, hGH can induce proliferation of the IM-9 lymphocytes by binding to its own receptor *(308)* and regulate antibody and cytokine (IL-1 and IL-2) production in immune cells *(312)*. Although hCS can stimulate Nb2 proliferation *(152,174)* through prolactin receptors as well, similar function has not been established for the IM-9 lymphocytes. Interestingly, it has been shown that increased amounts of hGH result in internalization of the hGH-R of the IM-9 lymphocytes *(308,477)*.

Whether the functions of the hCS during pregnancy could include substituting for, or inactivating the "priming" effects described for hGH and/or hPRL *(187)* due to either, internalization of the hGH-R, or, a "squelching effect" *(155)* is not known and direct experimental data addressing these possibilities have not been generated.

iv. Maintenance of pregnancy and induction of labor

No direct connection has been established between the hGH/CS proteins and the maintenance of pregnancy, the maturation of the myometrial contractile potential and the induction of labour *(348)*. However, the reported effects of hGH on immune cells *(312)* raise the possibility that hGH/CS proteins may regulate the synthesis and secretion of cytokines (such as IL-1 and IL-2) in decidual lymphocyte, decidual and/or placental trophoblastic populations. This may be the basis for a possible interaction of hGH/CS proteins with a cytokine-prostaglandin-CRH-ACTH-glucocorticoid axis that has been implicated in the induction of labour *(389 and references there in)*[11].

[11] Induction of cytokine secretion by the immune system in response to infection can modify hypothalamic secretions (e.g. IL-1, IL-2 and TNFα induce hypothalamic release of CRH or secretion of

A possible scheme for parturition, suggested by Riley and Challis *(389)*, includes, as the first step, the maturation of the fetal HPA axis. This results in an increase of fetal glucocorticoid secretion that in turn increases CRH synthesis and secretion from placental and fetal membranes[12]. The increase in maternal and fetal plasma and amniotic fluid CRH concentration further enhances the maturation of fetal HPA axis and possibly stimulates the maternal HPA axis. This results in a CRH-ACTH-glucocorticoid positive feedback cascade that through CRH stimulates cytokine and prostaglandin production which in turn further increases CRH secretion. This positive feedback mechanism primes the myometrial contractility through the action of prostaglandins and potentiates the myometrial contractility in response to oxytocin through the action of CRH. CRH

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various anterior pituitary hormones *(291-295)*. Placental trophoblasts, placental mononuclear phagocytes and decidua cells release interleukin-1 and interleukin-1 stimulates CRH secretion from placental cells in culture. Cytokines, including interleukins and tumor necrosis factor, are present in the amniotic fluid of 50% of pregnant women with premature delivery. This raises the possibility that cytokine-induced prostaglandin release by decidual lymphocytes, macrophages and/or trophoblasts may contribute to the premature labour *(389 and references there in,480-482)*.

Prostaglandins increase in maternal and fetal plasma in normal late pregnancy and have been implicated in the stimulation and maintenance of myometrial contractility at term *(480-482)*. Interestingly, CRH, ACTH and prostaglandin synthase are all localized in amnion and syncytiotrophoblasts *(483)*. In primary cultures, CRH stimulates placental ACTH secretion *(484,485)* which in turn stimulates PGE_2 and PGF_{2a} release from amnion, chorion, decidua and placental cells *(486,487)*. As part of a feedback loop, PGE2 and PGF2a stimulate, in turn, placental CRH and ACTH release *(484)*.

Maternal serum CRH is increased in complications such as hypertension, fetal asphyxia or growth retardation and premature labour or rapture of membranes, supporting the role of a CRH-ACTH-prostaglandin positive feedback mechanism in the induction of labour. Correlations between increased CRH and documented infections with increased amniotic fluid cytokine levels, however, have not been established *(389 and references there in,488-490)*. Nevertheless, a possible absence of correlation between maternal CRH and increased amniotic fluid cytokine, would not be necessarily surprising. The mechanism for the CRH-induced increase of cytokine production could based on local paracrine or even autocrine effects of CRH produced by placental or decidual lymphocyte populations. In decidua, lymphocytes may represent up to 50% of the cell population. CRH has been reported to stimulate leucocytes to produce POMC-related peptides (ACTH, ß-endorphin and α-MSH), to secrete interleukin-1 and -2, to proliferate (with or without lectin co-stimulation) and to increase IL-2 receptor expression. Further an augmentation of local inflammatory responses in vivo that may counterbalance the immunosuppressive (through glucocorticoids) systemic effects has been described *(290 and references there in)*.

[12] CRH is synthesized primarily by villus syncytiotrophoblasts and intermediate trophoblasts. Maternal CRH levels are very low until mid second trimester, but then increase steadily, and at higher rate after the 35th week. The production of CRH, also, by fetal membranes raises the possibility for paracrine effects on the closely associated myometrium *(389)*.

potentiates the ionotropic effects of oxytocin although it has no ionotropic effect of its own and oxytocin further increases the secretion of CRH and ACTH *(389)*.

A possible involvement of hGH/CS in such a pathway would be based on stimulation of prostaglandin and CRH production by hGH/CS-induced cytokine secretion. This would have a positive effect on the cytokine-prostaglandin-CRH-ACTH-glucocorticoid axis supporting its activity until the maturation of the fetal hypothalamic-pituitary-adrenal (HPA) axis and the establishment of the positive feedback cascade loop. A reduction of hCS levels in response to intra- and extra-amniotic injection of PGF_{2a} has been reported *(491)* raising the possibility that a putative hGH/CS-cytokine-CRH-prostaglandin interaction would be regulated by a negative feedback loop.

Progesterone and CG[13] counterbalance the function of the cytokine-prostaglandin-CRH-ACTH-glucocorticoid axis, both having a major role in the maintenance of pregnancy through relaxation of the myometrium and control of immune function at the placental-maternal interface possibly by inhibiting NK activity, cytotoxic lymphocyte activity and PGF_{2a} release *(340,341,347,462-470,444)*. Ultimately, however, the positive stimuli prevail leading to the induction of labour.

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[13] CG secretion is regulated by GnRH, and human GnRH levels follow a pattern similar to hCG but with attenuated peak. GnRH activity has been localized by immunofluoresence, predominantly in cytotrophoblasts and also in syncytiotrophoblasts in very early pregnancy. GnRH increases αCG, estrogen, progesterone and prostaglandin levels in placental cultures, but has no effect on CS release. GnRH activity is regulated by *chorionic peptidase-1* that degrades GnRH by proteolytic cleavage, decreasing the levels of chorionic GnRH activity. It inactivates also TRH, angiotensin II and the reduced form of oxytocin and is upregulated by cAMP, estrogen, prostaglandins and CRH *(363 and references there in)*.

E. BACKGROUND, RATIONALE AND OBJECTIVES OF THE RESEARCH PRESENTED IN THIS THESIS

I. PRL and GH expression in cells of the immune system

Recent research in Friesen's and Cattini's laboratories has examined several aspects of the expression and function of PRL and GH proteins in placental cells *(169,174,422-424,426,433,434,436,437,492-497)*. In addition to hGH and hCS, hGH-V had been shown by both groups *(174,492)* to be stimulatory in the Nb2 assay demonstrating the lymphoproliferative and lactogenic properties of this molecule. Further, the work of DiMattia *et al (493)* identified an upstream promoter in the PRL gene located 6 Kb upstream from the transcription initiation site responsible for the synthesis of PRL mRNA in pituitary. This promoter contained the initiation site for the PRL mRNA produced ectopically in the lymphoid IM-9-P3 cell line *(277)* and, as subsequently shown, in decidua *(494)* and myometrium *(498)*.

The coincidence of the PRL promoters directing lymphocyte, decidual and myometrial expression of hPRL *(493,494,498)* raises questions about (a) the function of PRL in the context of a pregnancy where paracrine functions for factors produced by decidual lymphocytes had been suggested *(see "Immune regulation at the placental-maternal interface" footnote #8, p. 58)* and (b) the specific cell type of origin of decidual PRL. Immunohistochemical studies have localized hPRL to the secretory endometrium *(499,500)* and decidual stromal cells *(398,501)*. Syncytiotrophoblasts and choriocarcinoma cells have also been reported to express the hPRL gene *(286,501)*. No *in situ* hybridization data have been produced to confirm the specific cell type of PRL

production and the possibility that decidual lymphocytes may also express hPRL has not been thoroughly examined.

The decidual production of PRL and the apparent importance of immunoregulation in this tissue intensified the interest in the possible functions of PRL proteins in the immune system. For a long time the production and possible roles of PRL-like or GH-like molecules in the immune system were subjects of debate. Reports had appeared on their influence in the development, maturation and function of the immune system *(for reviews see references 187,191-193)*. PRL and GH receptors had been identified in lymphocytes *(276,478)* and a variety of effects from increased c-*myc* expression *(309)* and direct lymphoproliferation *(303-305,308-311,285,289,399,150-152)* to increased antibody *(307)* cytokine *(312)* and IGF-I production *(316,317)* had been attributed to PRL and/or GH.

The secretion of PRL-like molecules by rodent or human lymphocytes had been reported in a number of studies *(274-285)*. It was not clear, however, whether the presence of PRL-like molecules had resulted from activation of the authentic PRL or a PRL-related gene in immune cells, or whether PRL was sequestered from serum *in vivo* or during culture. In the IM-9-P3 human lymphoma cell line, synthesis of hPRL identical to pituitary hPRL was demonstrated *(277)* while in the L2 murine T-lymphocyte line sequestration of biologically active bovine PRL was shown *(306)*. In addition to the detection of hPRL mRNA in the IM-9-P3 cell line *(277)* PRL-like mRNAs had been detected in lymphocytes in a few additional studies *(274,282)*.

At about this time, a particularly interesting report on a hPRL-like autocrine activity in lymphoma cells, appeared. A 29K hPRL-like peptide molecule was reported

to be secreted by a Burkitt lymphoma cell line (Ramos) under serum-free (sf) condition *(502)*. Conditioned medium from these cells had a proliferative effect on Nb2 rat lymphoma cells, known to respond with growth to lactogenic hormones (PRL, hGH and hCS) which interact with the PRL (lactogenic) receptor present on their surface *(279)*. Additionally an autocrine effect on sfRamos cells was observed and a polyclonal antibody against hPRL inhibited the autonomous growth of these cells *(502)*.

To re-examine the **hypothesis** that these serum free-grown lymphoid cells produced a hPRL-like lactogenic activity, I initiated a series of experiments with the **objective** of confirming and characterizing this lactogenic activity and subsequently cloning the respective gene. This would provide the molecular tools (specific cDNAs and genomic DNA clones and antibodies) for studying the distribution, biological effects of its product(s) and the factors that determine its tissue specific gene expression. The latter would also provide, in combination with the knowledge generated from studies on the decidua/lymphocyte specific expression of hPRL, a comparative system for the study of decidua/lymphocyte specific transcription factors.

II. Trophoblast expression of individual members of the hGH family.

Chorionic somatomammotropin (hCS) is expressed and secreted by the syncytiotrophoblast of the placenta *(81)*. Levels of hCS rise throughout pregnancy and correlate with placental size *(403)*. As a result, blood levels of hCS have been used as a biological index for the normal progression of pregnancy *(83-86)*. Through cloning and the use of monoclonal antibodies it has been possible in spite of their considerable

nucleotide (~94%) and amino acid (>80%) sequence similarities *(75,76)* to confirm that in addition to high levels of hCS (hCS-A and -B), hGH-V and hCS-L RNAs are present at low levels in the placenta while the pituitary hGH-N gene is not expressed *(76,88)*. Due to the sequence similarities between these genes, however, earlier studies to assess hCS RNA expression during pregnancy using radiolabelled hGH or hCS cDNAs as probes, would have also detected hGH-V as well as hCS transcripts since both cDNAs would crossreact with transcriptional products of the hGH-N, hCS-A, hCS-B as well as hGH-V and hCS-L genes. Similarly, polyclonal antibodies raised against hCS and used to assess hCS secretion might crossreact with hGH-V *(174)* and, possibly, products of the hCS-L gene. As a result, no routine methodology was available to detect individual members of the hGH/hCS family. More recently, reverse transcriptase-polymerase chain reaction (RT-PCR) was employed to assess the expression of hGH/CS genes during pregnancy using specific sets of primers for each family member *(123)*. Multiple hGH-V and hCS-L RNAs are generated in placenta with specific patterns of temporal distribution *(123)* implying a functional significance for these two genes.

Deregulation of the normal development of the human placenta leads to pathological conditions such as hydatidiform mole and choriocarcinoma with characteristic structural and biochemical changes *(503,378)*. In both of these pathological situations there is an increase in the levels of human chorionic gonadotropin and a reported decrease in the levels of hCS *(365,385,503-505)*. Due to the crossreactivity between probes used to assess hCS with other members of the family, possible changes in hGH-V and/or hCS-L levels would have been masked.

I questioned whether the decrease in hCS levels is accompanied by a pattern of

hGH/hCS gene expression similar to that of the normal placenta or by certain members of the family being expressed at higher or lower levels. If differential levels of expression for these genes were observed, it would support the **hypothesis** that distinct mechanisms are responsible for the regulation of individual hGH/CS genes in trophoblasts. I initiated a series of studies with the **objective** of determining the relative levels of expression of hGH/CS mRNAs in human placental tumor cells, using samples of hydatidiform moles, as well as, choriocarcinoma cell lines, such as BeWo, JAR and JEG-3. The BeWo, JAR and JEG-3 cell lines were reported to produce minimal amounts of hCS when they were established *(338)* and have been used as a convenient model for investigating hCS-A and -B gene regulation *(497,505-509)*.

III. Transcriptional control of the hCS-B gene expression.

Initiation of transcription from promoters with very low basal activity is frequently under the control of tissue-specific enhancer elements distinct from promoter sequences. This mode of regulation provides a very tight control-mechanism for gene expression that blocks inappropriate activation but is able to provide very high levels of temporary or constitutive tissue-specific expression. In contrast to many tissue-specific enhancers, 3'-flanking sequences are implicated in the efficient expression of the hCS genes, hCS-A and hCS-B, in trophoblasts *(507,497)*. A 1022 bp 3' flanking sequence of the human chorionic somatomammotropin (hCS-B) gene was shown to direct placental specific expression of this gene. A single DNA-protein interaction involving a 22 bp element and a protein factor termed TEF-1 was demonstrated in this region by DNAase I protection experiments. Work in our laboratory showed, however, that a synthetic 22

bp TEF-1 element was neither as potent nor as specific an enhancer as was the full length fragment (*509, also Nickel & Cattini, unpublished results*).

This led to the **hypothesis** that additional elements within the 1022 bp 3' flanking hCS-B enhancer fragment confer tight control over the tissue specific activity of this enhancer region. The **objective** of these studies was a detailed characterization of the 3' enhancer region and the identification of the DNA elements and transcription factors that interact with these DNA elements and determine placenta-specific expression.

MATERIALS AND METHODS

MATERIALS

Human GH (18-10-1985, standardized against NIH hGH 2019G, 2.2 U/mg) and human PRL (29-7-1987, standardized against NIH oPRL PS14, 31 U/mg) were purified in Friesen's laboratory (Department of Physiology, University of Manitoba) by monoclonal antibody affinity chromatography. All monoclonal and polyclonal antibodies were also developed in Friesen's laboratory by standard procedures *(510,511)* with the exception of anti-hGH #1 antibody (Dako, Corp., Santa Barbara, CA).

Chemicals and reagents were obtained from Sigma, St. Louis, MO; GIBCO-BRL, Burlington, Ontario; ICN/FLOW, Cleveland, OH; Pharmacia, Baie d'Urfé, Quebec; Mallickrodt, Paris, Kentucky; Fisher, Fair Laun, NJ; BMB, Indianapolis, IN; Bio-Rad, Mississauga, Ontario; Aldrich Chem. Comp., Inc., Milwaukee, WI and DuPONT, Boston, MA.

Sterile filters 0.22 and 0.45 μm were obtained from Nucleopore, Pleasanto, CA, and Baxter, Corp. Canlab Div., Toronto, Ontario; culture flasks and dishes from Corning, Corning, NY, and Falcon Plastics, Los Angeles, CA; and dialysis tubing MWCO 3500 and 6000-8000 from Spectrum Med.Ind. Inc., Los Angeles, CA.

CELL CULTURE

SfRamos cells, cultured under serum-free conditions for more than one year *(502)*, were grown as suspension culture in RPMI 1640 with the addition of 5μg/ml

human transferrin and 3 x 10^{-8} M selenium dioxide at 37^0C in a humidified atmosphere of 95% air and 5% carbon dioxide. Cell number was kept between 6 x 10^4 and 5 x 10^5/ml by replacing 65-75% of the culture medium with fresh medium every 2-3 days. Ramos cells were also obtained directly from ATCC (American Type Culture Collection, Rockville, MD) and were grown in RPMI 1640 / 10% FBS (referred to as Ramos ATCC).

Choriocarcinoma cells, JEG-3, JAR and BeWo were obtained from the ATCC and grown in monolayer in RPMI 1640 or Ham's F12 (BeWo) both supplemented with 8% fetal bovine serum (FBS) in a humidified atmosphere of 95% air and 5% carbon dioxide. Cells were grown on 100 mm culture dishes and harvested when they were about 80 % confluent.

Human cervical (HeLa) and rat pituitary (GC) tumor cells were also obtained from ATCC and were grown in monolayer in DMEM/8% FBS.

MEDIA CONCENTRATION

Several preparations of sfRamos conditioned media were obtained as follows: two to three day old culture media were centrifuged at 1000 x g and the supernatant was dialysed against 0.03 M NH_4HCO_3, 0.5 M Tris-HCl pH 7.6 or against H_2O, at 4^0C. Dialysed media were lyophilized and resuspended in phosphate buffered saline (PBS) or H_2O to yield 150-1000 times concentrated conditioned media. This material was used in the Nb2 assay for determination of lactogenic activity.

For immunoprecipitation and Western blot analysis, lyophilized medium was resuspended in buffer (0.05 M Tris-HCl pH 7.6, 0.15 M NaCl, 2 mM EDTA, 1% NP-

40, 0.5% aprotinin) *(512,513)* to yield 600 times concentrated conditioned medium.

Nb2 ASSAY

Nb2 cells, a T-cell lymphoma line derived from a lymph node of Nb rats *(150,151)*, exhibit a mitogenic response to hormones that interact with the PRL (lactogenic) receptor present on their surface. PRL of different species and hGH are able to bind to the PRL receptor and stimulate the proliferation of Nb2 cells, in a dose responsive way. Nb2 cells have been extensively used in a sensitive and precise bioassay for the presence of lactogenic hormones *(152)*.

The Nb2 11 and 11C clones of the rat lymphoma line *(152)* were used to estimate the lactogenic activity of sfRamos concentrated conditioned media. Nb2 cells were grown in FISHERS media with 10% horse serum (HS) and 10% FBS with the addition of 10^{-4} M ß-mercaptoethanol, 0.075% $NaHCO_3$, 50 U/ml penicillin and 50 μg/ml streptomycin *(152)*. Nb2 assay was performed as previously described *(152,514)* with 50 to 200 μl samples added to 2 ml Nb2 cell suspension. In certain assays, GMS-S supplement (100x = 0.67 μg/ml sodium selenite, 11 mg/ml sodium pyruvate, 1 mg/ml insulin, 0.55 mg/ml transferrin, 0.2 mg/ml ethanolamine; GIBCO,BRL) and 2mg/ml BSA were added to the media (in these cases the Nb2 assay was performed in FISHERS media with 10% HS, 15 mM HEPES, supplemented with GMS-S, 10^{-4}M ß-mercaptoethanol, 50 U/ml penicillin, 50 μg/ml streptomycin and 2mg/ml BSA). Human GH purified in Friesen's laboratory (18-10-85) was used for standards. Monoclonal antibodies 2A1, 3B1, 3D5 (anti-hGH), 9C3 (anti-hPRL) and polyclonal antibodies BR-3-4 (anti-hGH) and AR-38-7 (anti-hPRL) were used at a final dilution of 1:4000. At this dilution the anti-hGH or

anti-hPRL antibodies completely blocked the effect of homologous standards (20 ng/ml hGH or hPRL, respectively) but did not inhibit the effect of heterologous standards (20 ng/ml hPRL or hGH, respectively). No effect on the basal proliferation of Nb2 cells in the absence of hGH, hPRL or concentrated conditioned media is observed in the presence of the antibodies at the dilutions used. Antibody anti-hGH #1 was used at a final dilution of 1:2000. Cell numbers were determined using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL).

RADIOIMMUNOASSAY

The double antibody radioimmunoassay method *(515)* was used for determining hGH levels. BH-12 anti-hGH polyclonal antibody was developed in Friesen's laboratory and was used at a $1:3x10^5$ final dilution. Human GH, also purified in Friesen's laboratory (18-10-85), was used for standards. Sheep anti-rabbit serum and normal rabbit serum (NRS) were added at a final dilution of 1:160 and 1:700, respectively. Briefly, iodination of hGH was performed according to the IODO-GEN method (PIERCE, Rockford, Illinois, U.S.A.) and resulted in ^{125}I-hGH with a specific activity of 64 μCi/μg. Samples or standards (100 μl) were incubated overnight (18-24 hours) at 4 C with BH-12 Ab 1: $3x10^5$ final dilution, ^{125}I-hGH (27500 counts per minute (cpm)/tube in a buffer of 1% BSA/PBS pH 7.4 and a final volume of 500 μl. Addition of 100 μl of 1:23 dilution of sheep anti rabbit serum and 100 μl of 1:100 dilution of NRS was followed by overnight incubation at 4 C. The amount of radioactivity was determined using a gamma counter after spinning for 30 minutes at 3000 RPM in a IEC DPR 6000 centrifuge (3000 x g) and removing the supernatant. Results are expressed as the

percentage (%) of binding (precipitatable counts when various amounts of cold hGH or sample dilutions are present in the reaction) versus total binding (total precipitatable counts when no cold hGH is added in the reaction) after subtracting the background values (Bo) from both binding (B) and total binding (T).

WESTERN BLOT ANALYSIS

Concentrated conditioned medium (600X, 35-80 μl) was boiled in sample buffer (10% glycerol, 2.3% sodium dodecyl sulfate (SDS), 0.625 M Tris-HCl pH 6.8, 0.175 M ß-mercaptoethanol) and resolved by SDS-PAGE (polyacrylamide gel electrophoresis) in a Bio-rad Protean 16 cm apparatus *(516)*. Transfer to nitrocellulose (0.20 μm, Schleicher & Schuell, Keene, NH) was performed at 250 mA for 90 minutes in a Bio-rad Transblot cell apparatus. For detection, BR-3-4 polyclonal antibody or NRS at a dilution of 1:250 were used as the primary antibodies and anti-rabbit IgG-Alkaline Phosphatase (AP) conjugate at a dilution of 1:3525 to 1:4400 (Promega Protoblot Immunoblotting System) was used as the secondary antibody. Non specific binding was blocked with 3% gelatin/TBST (10mM Tris-HCl pH 8.0, 150mM NaCl, 0.05% Tween-20) for 40 minutes and both primary and secondary antibodies were incubated for 40 minutes in 1% gelatin/TBST. Each antibody incubation was followed by 3 x 5-10 minute washes in TBST. Colour reaction was done according to the supplier (Promega, Madison, WI).

IMMUNOPRECIPITATION AND WESTERN BLOT ANALYSIS

Samples (600 μl) of 600 times concentrated conditioned or fresh media as well

as human transferrin (3 mg/ml) in immunoprecipitation buffer, were incubated with 5 μl BR-3-4 polyclonal anti-hGH serum or 5 μl NRS for 2 hours at 4^{0}C, followed by addition of 50 μl of a 30% volume/volume (v/v) solution of Protein A-Sepharose in immunoprecipitation buffer and incubation for 90 minutes at 4^{0}C with slow rotation on a multipurpose rotator (model 150 V, Scientific Industrie Inc., Spriengfield, MA, U.S.A.). After several washes with immunoprecipitation buffer and immunoprecipitation wash buffer [2.5 M potassium chloride (KCl), 0.05 M Tris-HCl, 0.15 M NaCl, 0.5% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM phenylmethylsulphonyl fluoride (PMSF), *(512,513)*] the pellets were boiled for 2-5 minutes in sample buffer (10 % w/v glycerol, 2.3 % w/v SDS, 0.625M Tris-HCl pH 6.8, 0.175M ß-mercaptoethanol) and after centrifugation the supernatants were resolved by 15% SDS-PAGE *(516)* in a Bio-rad Protean 16 cm apparatus. Transfer to nitrocellulose was performed as above. For detection, 3D5 monoclonal anti-hGH antibody at 1:2400 dilution was used as the primary antibody and anti-mouse IgG-AP conjugate at 1:7150 dilution (Promega Protoblot Immunoblotting system) as the secondary antibody. Blocking of non specific binding, primary and secondary antibody incubations, washes and colour reaction were performed as above.

TOTAL AND POLY A(+) RNA ISOLATION

Total RNA from sfRamos cells was prepared with the guanidine thiocyanate (GITC)-cesium chloride (CsCl) method *(517)*. Briefly, cell pellets were resuspended in 4M GITC and, after addition of the suspension to 2-6 ml of 6.6M CsCl, the resulting mixture was centrifuged in a Ti 60 or a SW50.1 rotor at 36000 RPM (130,000-200,000

x g) for 22 h at 21 C, in a L8-70M or a L5-65 Beckman ultracentrifuge. The resulting pellet was resuspended in water, ethanol precipitated and resuspended again in water. Poly A(+) RNA was prepared with the oligo(dT) cellulose affinity chromatography method *(518)*. Oligo(dT) cellulose (100-120 μl of a 60% v/v solution of oligo(dT) cellulose per 1 mg of total RNA) was added to 15 ml tubes containing total RNA in binding buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 1 mM EDTA pH 8.0, 0.1% SDS) and incubated for 40 minutes at room temperature with slow rotation. After centrifugation the supernatants were kept as the poly A(-) fraction. The pellets were washed with binding buffer and transferred to cellulose acetate spin columns (Spin-X, Costar, Cambridge, MA). Poly A(+) RNA was eluted after addition of 10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 0.05% SDS *(518)*, brief vortexing and incubation at 65 C for 1 minute. From 3.0 mg of total RNA, 79 μg were recovered as the poly A(+) fraction.

Total RNA from human placenta, hydatidiform moles and choriocarcinoma cell lines was prepared as previously described *(169)* by a modification of the method of Chomczynski and Sacchi *(519)* and also by the GITC-CsCl method *(517)*. Tissue samples were frozen and crushed before they were homogenized in glass homogenizers. For the former method, after the first propan-2-ol precipitation, the RNA pellet was resuspended in 0.4 ml of 10mM Tris-HCl pH 8.0/1 mM EDTA transferred to a microfuge tube and extracted with an equal volume of phenol saturated with sterile distilled H_2O, 0.1 volume of 2M sodium acetate pH 4.0 and 0.2 volumes of chloroform/3-methylobutan-1-ol mixture (24:1, v/v). The aqueous phase was reextracted with 3-methylobutan-1-ol and then precipitated with an equal volume of propan-2-ol. All RNA concentrations were determined by measuring absorption at a wavelength of 260 nM.

NORTHERN BLOT ANALYSIS

RNA samples were subjected to electrophoresis in 1.5% agarose-formaldehyde at 30 volts overnight (18-24 hours). After transfer to nitrocellulose membrane (Nitroplus 0.45 μm, MSI, Westbord, MA) for 18 hour with 20 x SSC (3 M NaCl, 0.3 M sodium citrate) the blot was hybridized with a random primed (Prime-a-Gene System, Promega) full length hGH-N cDNA probe, separated from free nucleotides after passing through a G-100 Sephadex column (specific activity 1.5 x 10^9 DPM/μg, 3 x 10^6 DPM/ml of hybridization solution). Prehybridization (42^{0}C for 3 hours) was performed in a solution of 50% v/v formamide, 5 x SSC, 5 x Denhardt's solution, 50 mM NaH_2PO_4 pH 6.5, 0.1% w/v SDS, 250 μg/ml sonicated salmon sperm DNA and 250 μg/ml yeast tRNA. Hybridization (42 ^{0}C, overnight) was performed in 50% formamide, 5 x SSC, 5 x Denhardt's solution, 10 mM NaH_2PO_4 pH 6.5 and 10% dextran sulphate *(520)*. Washes were 3 x 15 minutes at 65 ^{0}C with 0.1 x SSC/0.1% SDS. For autoradiography XAR film (Eastman, Kodak, Rochester, NY) was exposed for 1-4 days at -70^{0}C in a cassette with intensifying screens (DuPONT).

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR) AND PCR SEQUENCING

The RT-PCR *(521)* to detect the hGH family cDNAs was performed as follows: The reverse transcriptase (M-MLV Reverse Transcriptase, GIBCO-BRL) reaction was performed according to the supplier with certain modifications. Briefly, total or poly A(+) enriched RNA samples, 0.4-5 μg/ reaction, from sfRamos cells or normal human

pituitary and 0.4-3.9 μg/reaction, from human placenta, hydatidiform moles and choriocarcinoma cells were reverse transcribed in a final volume of 10 μl. Hexanucleotide primers (10 μM, University of Calgary, Regional DNA Synthesis Laboratory) were used instead of oligo$(dT)_{12-18}$ and incubation was at 37°C for 120-150 minutes. Two oligonucleotide primers [primer 1: 5'-CAG AAG TAT TCA TTC CTG CA-3' (sense), primer 2: 5'-TTT GGA TGC CTT CCT CTA G-3' (antisense)] corresponding to sequences in exon 3 and exon 4 of the hGH/CS genes *(Figure 2)* were used for amplification of the cDNAs generated by the reverse transcriptase reaction or of EcoRI digested HeLa genomic DNA. After an initial step at 94-95°C for 4-5 minutes the amplification (1 μl of the reverse transcriptase reaction or 1 μg of genomic DNA in 10 mM Tris-HCl pH 8.3, 1.5-2.5 mM magnesium chloride ($MgCl_2$), 50 mM KCl, 200 μg/ml gelatin, 0.2 mM each deoxynucleotriphosphates (dNTPs), 1 μM each primer and 2 U Taq polymerase (GIBCO-BRL) in a final volume of 50 μl was obtained with 36-40 cycles of denaturation at 94°C for 1 minute, annealing at 42°C for 40 seconds and extension at 72°C for 1 minute. In the final cycle the extension time was increased to 7 minutes. The products were resolved by 1-4 % agarose gel electrophoresis and visualised by ethidium bromide staining (4 μg/L of agarose gel and electrophoresis buffer).

For PCR sequencing, PCR generated fragments were excised from the agarose gel and were used as templates (fmol DNA Sequencing System, Promega). Briefly, the gel slices containing the PCR products were solubilized with QX1 solubilization solution, and 10 μl of a Qiaex silicagel particle solution (Qiaex gel extraction kit, Qiagen, Germany) were added. After a 10 minute incubation at 50°C during which the DNA was absorbed on the silicagel particles, the samples were centrifuged at top speed on a bench microcentrifuge for 30 seconds and the pellets were washed twice with QX2 high salt

buffer and twice with QX3 40% ethanol wash buffer. After the final wash and a 30 second microcentrifugation the DNA fragments were eluted from the pellets by addition

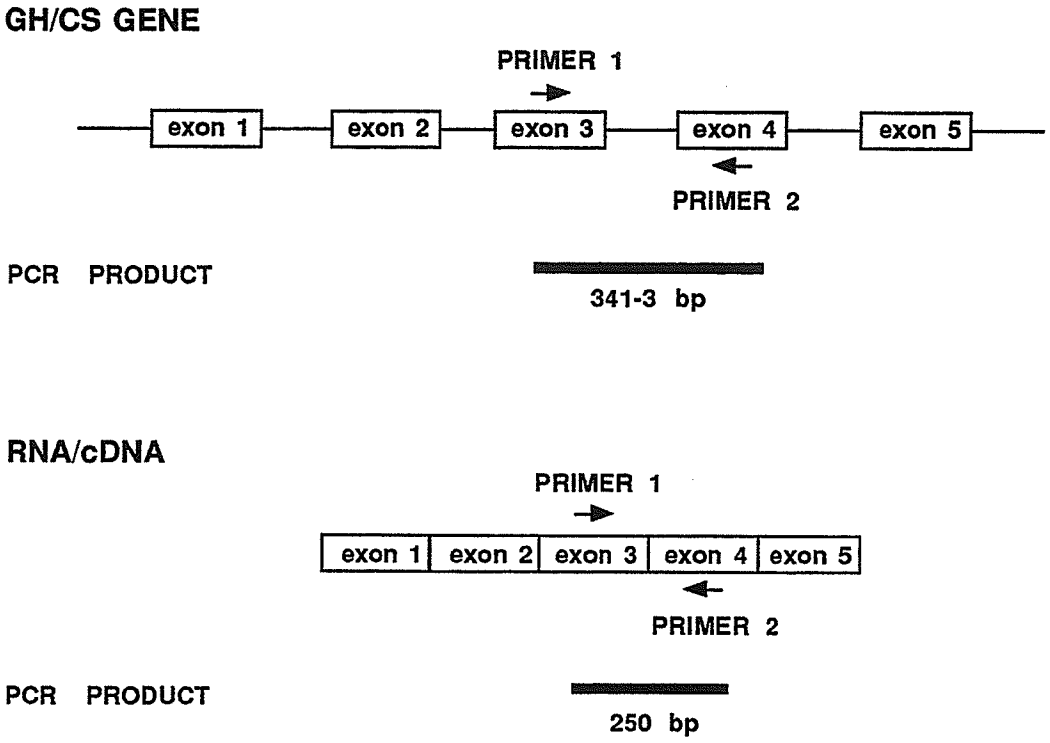


FIGURE 2: Basic structure of a hGH/CS gene and a hGH/CS mRNA; positions and direction of extension for the two oligonucleotide primers [primer 1: 5'-CAG AAG TAT TCA TTC CTG CA-3' (sense), primer 2: 5'-TTT GGA TGC CTT CCT CTA G-3' (antisense)] corresponding to sequences in exon 3 and exon 4 of the hGH/CS genes and predicted fragments deriving from genomic or mRNA/cDNA sequences are indicated *(see also Figure 17 p. 116).*

of 20-30 μl of TE, incubation at room temperature for 10 minutes and microcentrifugation for 30 seconds. The PCR-sequencing reaction was performed according to the supplier [500 ng of DNA per reaction, 25 ng of a hGH PCR primer, in 50 mM Tris-HCl pH 9.0 (at 25^{0}C), 2 mM $MgCl_2$, 10-200 μM of dNTPs/ddNTPs, 1.2 U sequencing grade *Taq* DNA Polymerase, 94^{0}C for 1 minute, 42^{0}C for 40 seconds, 70^{0}C for 40 seconds, for a total of 30 cycles]. The reaction was stopped with the addition of stop solution/loading buffer (10 mM NaOH, 95% v/v formamide, 0.05% bromophenol

blue, 0.05% xylene cyanole) and after heat denaturation at 80-95 ^{0}C for 5 minutes, the products were analyzed by 6.8-8.0 % polyacrylamide sequencing gel electrophoresis. The autoradiogram was developed after 3 hour exposure of XAR film at room temperature.

SOUTHERN BLOT ANALYSIS

Genomic DNA from HeLa, Ramos ATCC and sfRamos cells was isolated as previously described *(522)*. Briefly, cells were pelleted for 10 minutes at 2500 RPM on a bench top HN-S centrifuge (1000 x g). The supernatant was removed and 0.5 ml proteinase K solution (0.1M Tris-HCl pH 8.0, 0.1M NaCl, 5mM EDTA, 1% SDS, 100 μg/ml proteinase K) was added per 0.5 ml of packed cell volume. The proteinase K solution was added with a Gilson P1000 pipetman (Gilson Medical Electronics, France) bearing a tip with its end cut off. This allowed a larger tip diameter that facilitated the mixing of the very viscous samples once the proteinase K solution was added. Usually, 6-10 fast pipetting movements preceded the incubation of the samples at 37 ^{0}C for 18 hours. Twenty (20) μl of a 10mg/ml RNAase A solution were added subsequently and the samples were incubated at 65^{0}C for 15 minutes followed by phenol and phenol-chloroform extractions (two each, TE saturated phenol, pH 8.0) and one chloroform extraction. The samples were placed in 15 ml snap-cup opaque polypropylene tubes and 0.4 volumes of 5 M NaCl as well as 2.2 volumes of ice-cold 95% ethanol were added. A visible genomic DNA string, that was readily formed upon brief vortexing, was picked up directly using a Gilson P20 pipetman, placed in a microfuge tube, washed with ice-cold 70% ethanol, dried and 100-200 μl of TE pH 8.0 were added. The microfuge tubes were kept at 4 ^{0}C for DNA resuspension over a 72 hour period. The genomic DNA was

subsequently digested with *Eco*RI or *Bam*HI and subjected to electrophoresis in 1% agarose gel at 40 volts overnight (10 μg/lane). Denaturation of DNA in 1.5M NaCl/0.5M NaOH for 1 hour, neutralization twice in 3M NaCl/0.5M Tris-HCl pH 7.0 for 45 minutes, and transfer to nitrocellulose membrane with 20 x SCP (2M NaCl, 0.6M Na_2HPO4, 20mM Na_2EDTA pH 6.2) overnight, was followed by prehybridization (6.6 x SCP, 1% N-lauryl sarcosine, 0.1mg/ml sonicated, denatured salmon sperm DNA, 1 x Denhardt's) for 1-3 hours at 65 ^{0}C, and hybridization (prehybridization solution containing also 10% dextrane sulphate) overnight with a random primed (Prime-a-Gene, Promega) hGH-N cDNA probe (specific activity 1.9 x 10^9 DPM/μg, 2 x 10^6 DPM/ml of hybridization solution). For autoradiography XAR film was exposed for 72 hours at -70 ^{0}C in a cassette with intensifying screens (DuPONT).

RESTRICTION DIGESTION AND SOUTHERN BLOT ANALYSIS FOR THE IDENTIFICATION OF hCS, hCS-L, hGH-V AND hGH-N RNA

Fragments generated by RT-PCR were restriction digested with *Hph*I or *Rsa*I to yield a series of fragments which distinguish hCS (hCS-A and -B) from hGH-V, hCS-L and hGH-N gene products as well as processed RNA from DNA (*Table 7, p.109*). When necessary, the PCR generated fragments were first isolated by electroelution or excised from a gel slice, as described above (Qiaex gel extraction kit, Qiagen, Germany) before cutting. The digested material was analyzed by 4% agarose gel electrophoresis and ethidium bromide staining (4μg/L), photographed, denatured and neutralized on the gel [two 15 minute incubations in solution A (1.5 M NaCl, 0.5 M NaOH), followed by two 15 minute incubations in solution B (1 M NH_4acetate, 20 mM NaOH)] and transferred

to nitrocellulose membrane (Nitroplus 0.45 μm, MSI, Westbord, MA, USA) overnight with 20 x SSC. An oligonucleotide (5'-GGAATAGA[C/G]TCTGAGAA-3') corresponding to sequences in exon 3 of the hGH/hCS genes *(Figure 17, p. 116)* was used as a probe to distinguish hGH-V and hCS-L fragments generated by *Hph*I digestion of PCR products when necessary. The oligonucleotide was end-labeled with polynucleotide kinase to a specific activity of about 1 x 10^8 cpm/μg and used to probe blots at a concentration of 0.3-0.7 x 10^6 cpm/ml of hybridization solution. Prehybridization (46^{0}C for 3 hours) and hybridization (46^{0}C for 18 hours) were performed in a solution of 6x SSC, 5x Denhardt's solution, 50 mM NaH_2PO4 pH 6.5, 0.5% SDS and 100 μg/ml sonicated salmon sperm DNA. Washes were 15 minutes at 46^{0}C with 6x SSC and 15 minutes at 46^{0}C with 2 x SSC. For autoradiography, XAR films (Eastman, Kodak, Rochester, NY, USA) were exposed for 1-24 hours at -70^{0}C in cassettes with intensifying screens. Bands corresponding to predicted lengths (*Table 7, p.109*) were scanned to give a densitometric value. Relative hCS, hGH-V, hCS-L and hGH-N RNA levels in each of the trophoblast samples were expressed as a percentage of the total hGH/hCS RNA digested.

PCR SITE-DIRECTED MUTAGENESIS AND PLASMID CONSTRUCTION

A 1022 bp fragment of the hCS-B gene containing placental enhancer sequences as well as pSV1CAT1-1022 and pSV1CAT103-241 *(507)* were provided by Dr. G.F.Saunders (M.D. Anderson Cancer Center, Houston, TX). A 241 bp *Acc*I/*Pvu*II fragment containing the 22 bp TEF-1 enhancer element was isolated from the 1022 bp fragment and subcloned into PUC19. The PUC19 vector containing the 241 bp

AccI/PvuII fragment *(Figure 3A)* was used as template for the PCR site-directed mutagenesis *(523,524)*. Oligonucleotide primers (University of Manitoba, DNA Synthesis Laboratory) corresponding to sequences within the 241 bp fragment but bearing 5 bp mismatches were used for amplification of mutated (m) subfragments in separate reactions. These primers (RF-1m upstream primer: 5'-CTCATCAACTTACGTCGGACGGC-3'; RF-1m downstream primer: 5'-GCCGTCCGACGTAAGTTGATGAG-3'; DF-1m upstream primer: 5'-GAGATGCCTTTACGGTTTTCT-3'; DF-1m downstream primer: 5'-AGAAAACCGTAAAGGCATCTC-3'; mismatches are underlined) were used in combination with sequencing PUC19 primers corresponding to vector sequences flanking the polylinker *(Figure 3A)*.

After an initial step at 95 ^{0}C for 5 minutes the amplification (180 ng of plasmid template in 10 mM Tris-HCl pH 8.5, 2.0-2.5 mM $MgCl_2$, 50 mM KCl, 200 μg/ml gelatin, 200 μM each dNTP, 370 nM each primer and 2U Taq polymerase in a final volume of 0.1 ml) was obtained with 27-33 cycles of denaturation at 94^{0}C for 1 minute, annealing at 31-36^{0}C for 40 seconds and extension at 72^{0}C for 1 minute. In the final cycle the extension time was increased to 7 minutes.

The products were resolved by 1-4% agarose gel electrophoresis and visualised by ethidium bromide staining. When necessary, the PCR generated fragments were isolated by electroelution or excised from a gel slice (Qiaex gel extraction kit, Qiagen, Germany).

Mixing, heat denaturation, vortexing and annealing of the subfragments followed by amplification with the two external sequencing primers resulted in full length mutated products *(Figure 3B)*. These PCR products were restriction digested with *Eco*RI and

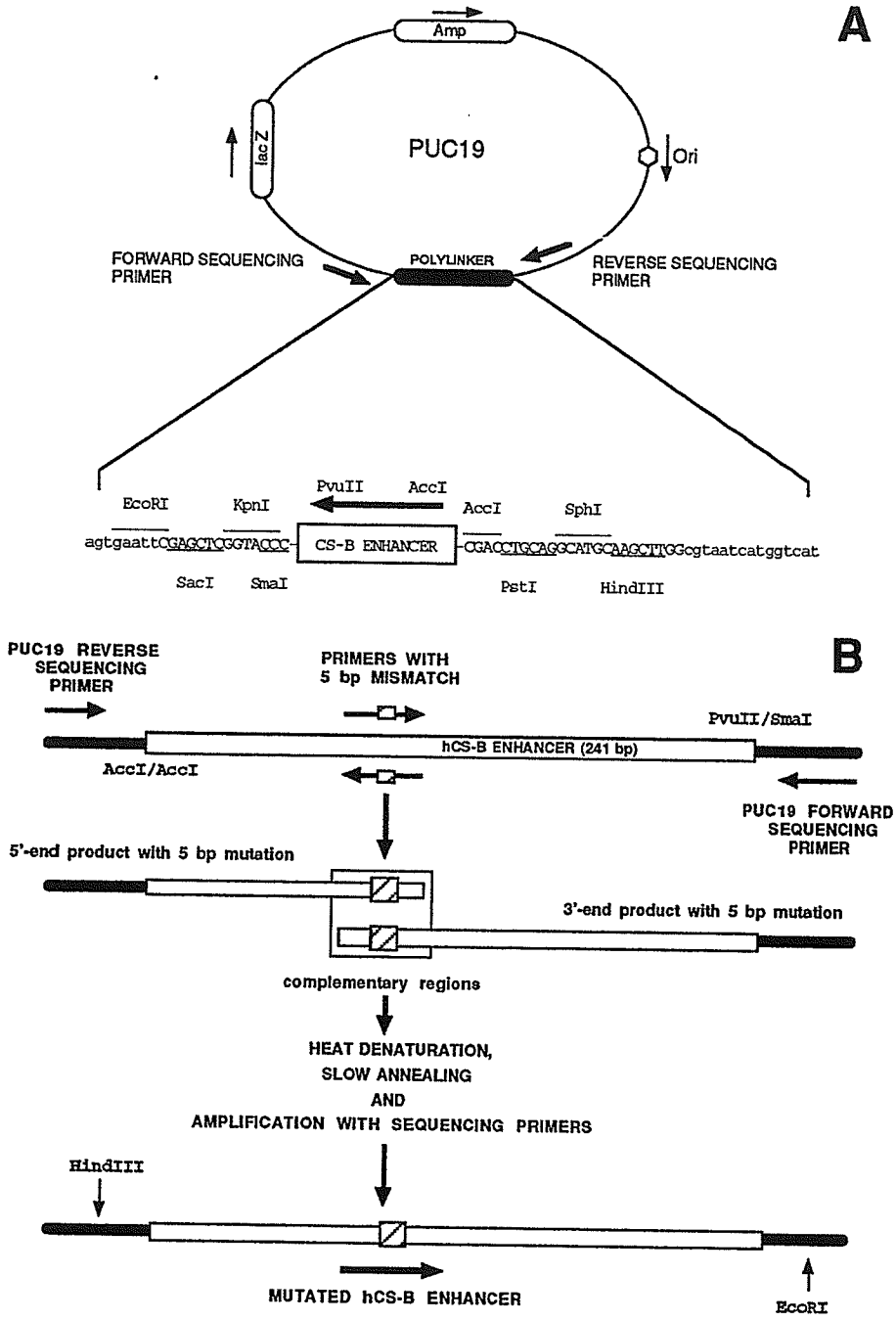


FIGURE 3: (A) Schematic representation of PUC19 vector containing a 241 bp *AccI/PvuII* hCS-B fragment that was used as template for the PCR site-directed mutagenesis. The 241 bp fragment, that contains the 22 bp TEF-1 enhancer element, was isolated from the 1022 bp hCS-B enhancer fragment and subcloned into the *AccI/SmaI* sites of PUC19. The direction of the 241 bp and the positions of sequences complementary to the PUC19 sequencing primers are indicated. (B) Oligonucleotide primers corresponding to sequences within the 241 bp fragment but bearing 5 bp mismatches were used in combination with the PUC19 sequencing primers corresponding to vector sequences flanking the polylinker *(Figure 2A)*, for amplification of mutated subfragments in separate reactions. Mixing, heat denaturation, vortexing and annealing of the subfragments followed by amplification with the two external PUC19 sequencing primers resulted in full length mutated products.

*Hind*III and subcloned into a PUC119 vector *(525)* for sequencing and confirmation of the mutation *(Figure 4)* as well as for generation of radiolabeled inserts to be used in gel shift assays. PUC19 or PUC119 vectors that contained the wild type or mutated 241 bp fragments were digested with *Hind*III and the resulting ends were blunted with Klenow (Pharmacia). The 241 bp fragments were released from their vectors after a second digestion with *Eco*RI that resulted in the generation of fragments bearing one blunted and one *Eco*RI end. Similarly, chloramphenicol acetyl transferase (CAT) constructs directed by the SV40 promoter (pCAT-Basic Vector; Promega, *Figure 5*) were digested at sequences downstream of the CAT gene and modified to provide vector fragments with one *Bam*HI (blunted) and one *Sal*I end. Ligation of the blunt ends of the vector and insert fragments was followed by repair of the *Sal*I and *Eco*RI ends and subsequent ligation and circularization to produce the insertion of the 241 bp fragments 3' of the CAT gene in the correct orientation *(Figure 5, see also Figure 24 p. 128)*. For hybrid CAT genes containing the RF-1 oligonucleotide, the 23 bp RF-1 oligonucleotide was inserted in SV40p35 *(Figure 24 p. 128)* in the forward orientation at the *BamH*I site 5' of the TEF-1 element maintaining the 5'-3' positioning of the two elements in the 241 bp enhancer fragment.

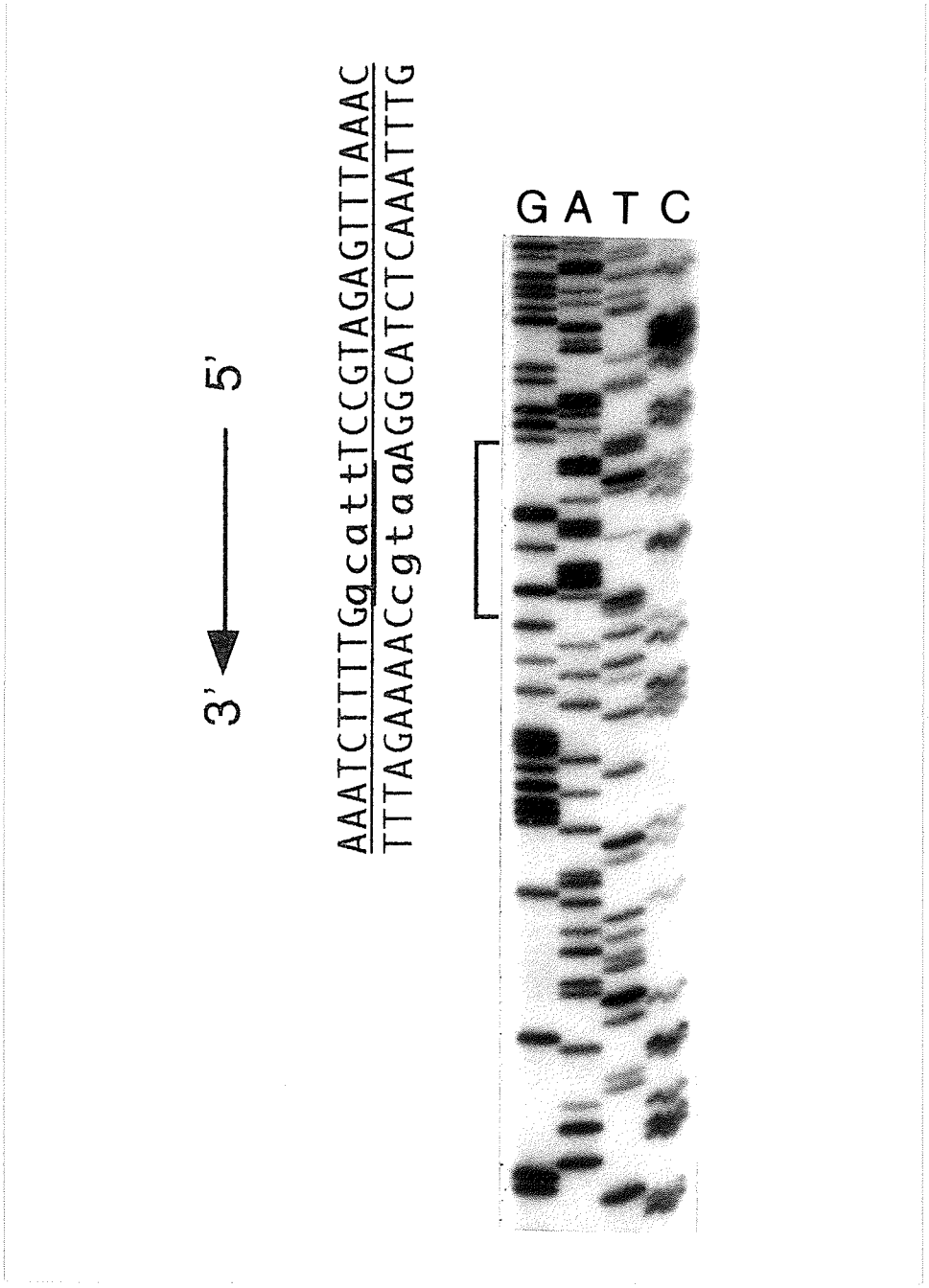


FIGURE 4: Sequencing and confirmation of a mutated DF-1 region within the 241 bp hCS-B enhancer. The presented sequence corresponds to the area indicated in the autoradiogram (half box). The direction of the sequence of the sense strand (shown on the left) is indicated. Substituted nucleotides are indicated by lower case letters.

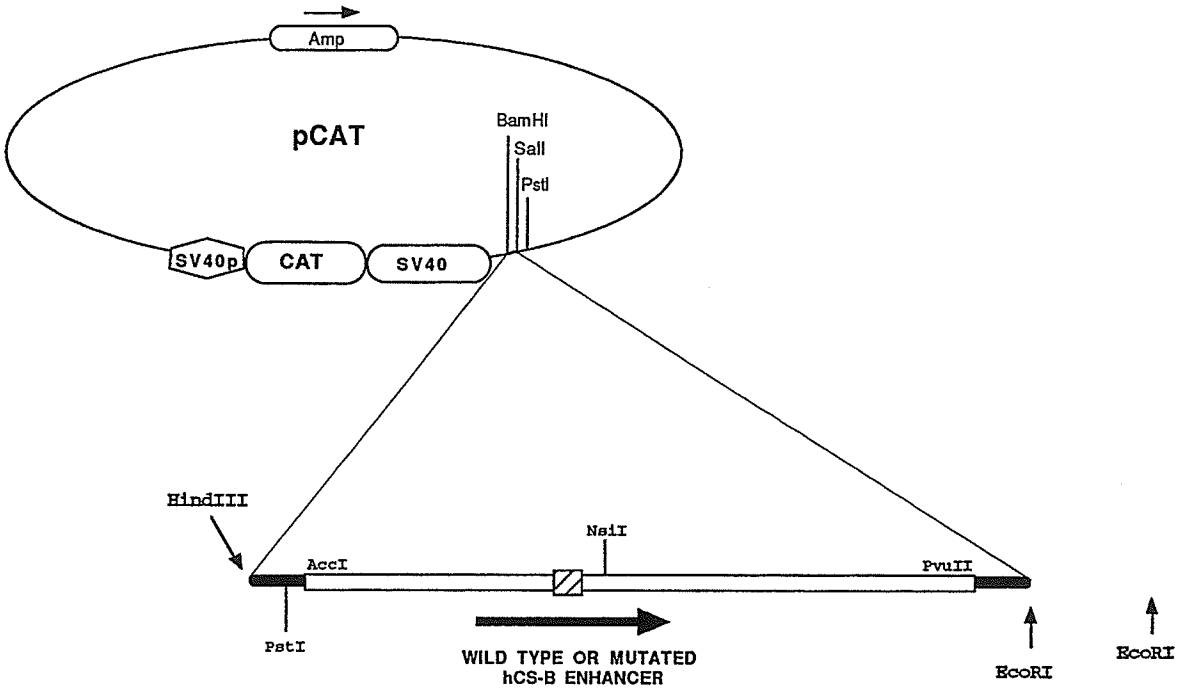


FIGURE 5: Construction of hybrid reporter CAT genes bearing hCS-B enhancer sequences. The 241 bp fragments were released from their vectors after a second digestion with *Eco*RI that resulted in the generation of fragments bearing one blunted and one *Eco*RI end. Similarly, chloramphenicol acetyl transferase (CAT) constructs directed by the SV40 promoter (pCAT-Basic Vector; Promega) were digested at sequences downstream of the CAT gene and modified to provide vector fragments with one *Bam*HI (blunted) and one *Sal*I end. Ligation of the blunt ends of the vector and insert fragments was followed by repair of the *Sal*I and *Eco*RI ends and subsequent ligation and circularization to produce the insertion of the 241 bp fragments 3' of the CAT gene in a 5' to 3' orientation.

The RF-1 oligonucleotide was also inserted in the forward orientation at the *Sal*I site 3' of SV40p/E *(Figure 24 p. 128)*. The 21 bp DF-1 oligonucleotide was introduced in the forward orientation at the *Sal*I site 3' of SV40p *(Figure 24 p. 128)*. An additional set of wild type (wt) primers (RF-1wt upstream primer: 5'-CTCATCAACTTGGTGTGGACGGC-3' and DF-1wt downstream primer: 5'-AGAAAACATCCTAGGCATCTC-3'; University of Calgary, Regional DNA Synthesis Laboratory) was used to amplify a 62 bp subfragment (nts 13-74) of the 241 bp fragment. PCR products intended for gel mobility shift competition assays were

isolated by electroelution and their concentration was determined by gel electrophoresis and comparison of the intensity of the ethidium bromide staining of the DNA bands with the intensity of known amounts of DNA markers.

PLASMID DNA TRANSFER

Human choriocarcinoma JEG-3 and BeWo cells, as well as cervical carcinoma HeLa cells were plated in 100 mm culture dishes at a density of 1-2 x 10^6, transiently transfected by calcium phosphate-DNA precipitation after 20-26 hours as described *(526)*. Briefly, DNA precipitation was obtained by adding 750 μl of a 250 mM $CaCl_2$ containing 15 μg of plasmid DNA and 0.75 to 5 μg of control CMV-luciferase plasmid *(527)*, used for normalization of the results for transfection efficiency) to 750 μl of 2xHEBS (2xHEBS: 280 mM NaCl, 50 mM Hepes-mw 238.3, 1.5 mM Na_2HPO_4) placed in a sterile 15 ml snap cap polystyrene tube. A 1 ml glass pipette connected to a filtered (0.2 μm) airline, was used to maintain a steady sterile air flow to the 2xHEBS solution during the addition of the 750 μl DNA/$CaCl_2$ solution that would be completed within 25-35 seconds, by directing small drops of the added solution to the 2xHEBS solution from a height of 0.5-1 cm over the bubbling surface. The addition of the DNA was followed by a 25-50 minute incubation at room temperature (20-25^{0}C). During this incubation period the culture media was renewed and cells were placed in DMEM/8% FBS. The DNA precipitate (480 μl per plate) was added uniformly. After 6-9 hours, the culture medium was aspirated, and 2 ml of 20% glycerol/DMEM were added to the plates at 10 seconds intervals (up to 15 plates per set). The medium was aspirated after 2.5 minutes at 10 second intervals, as well, and 5 ml PBS were added per plate, following the same time

frame. Before aspirating the PBS the plates were swinged briefly to facilitate glycerol removal. This last step was repeated once maintaining the same time frame. After aspirating the PBS, the original culture medium (growth medium) was added to the cells.

The cells were harvested 46-54 hours after the addition of the DNA, as follows: After aspirating the culture medium the cells were washed with 5 ml PBS-CMF (Ca^{++}-Mg^{++} free) and then incubated in 5 ml PBS-CMF/1 mM EDTA for 5-10 minutes at room temperature. Cells were lifted from the bottom of the plates using a Pasteur glass pipette and transferred to 15 ml polystyrene orange cup tubes (Corning) placed on ice. The cells were pelleted for 2 minutes at 3000 RPM in a HN-S bench top centrifuge (1500 x g). The supernatant was aspirated and the pellets were resuspended in 150 to 400 μl of 0.1% v/v Triton X-100/100 mM Tris-HCl pH 7.8, transferred to 1.5 ml microfuge tubes and incubated for at least 15 minutes on ice. Centrifugation for 15 minutes at 4^0C was followed by transfer of the supernatant (cell extracts) to a new microfuge tube.

DETERMINATION OF PROTEIN CONCENTRATIONS

Protein concentrations were determined by the Bradford assay *(528)* with BSA as a standard. For the Bradford assay, 5 μl of cell extracts that were diluted 1:5 were added to 795 μl of H_2O in 12 x 75 mm borosilate glass tubes. After vortexing, 200 μl of undiluted Protein-Assay Dye Reagent (Bio-rad) were added and the tubes were vortexed for 10-15 seconds and were kept at room temperature for 15-20 minutes. Protein concentrations were determined by absorption at a wave length of 595 nm (A_{595}) (Hitachi U-100 spectrophotometer). A Gilson P1000 pipette gun, was used to transfer the solution slowly to the cuvette, to avoid the formation of air-babbles that compromise the accuracy

of the optical density (OD) reading. Calculation of the protein concentration was based on comparison to BSA standards (2-10 μg, Sigma) and regression analysis.

CAT ASSAYS

CAT activity was measured using a modification of the two-phase fluor diffusion assay *(529)* as described previously *(169)*. Cell extracts (25-100 μg) were added to 0.1% v/v Triton X-100/100 mM Tris-HCl pH 7.8 (final volume 200 μl) in a 7 ml glass scintillation vial. The vials were placed at 70^{0}C for 10 minutes, allowed to cool to room temperature for 5-10 minutes, and then 75 μl of a ^{3}H acetyl CoA cocktail (3.3 mM chloramphenicol, 100 mM Tris-HCl pH 7.8, 0.5 μCi) were added. An organic phase cocktail (3 ml of Fisher-Scintelene) was added by directing the stream to the vial wall and allowed to slide down slowly. This step was done in the absence of strong light and the vials were subsequently covered with aluminum foil and incubated at 37^{0}C for 30 minutes before counting for 3-5 rounds, every 30-35 minutes. Quantitative values for CAT activity were determined by regression analysis to give cpm/mg of cell extract protein. CAT activity is expressed as fold increase over the basal SV40 promoter activity. Correction for transfection efficiency and DNA uptake was obtained by cotransfection with a CMV-1/luciferase gene hybrid construct and assessment of the luciferase activity of the cell extracts. For the luciferase assay, 20 μl of cell extracts, placed at the bottom of a 12 x 75 mm borosilate glass tubes, were analyzed in a photon counting luminometer (ILA911 Luminometer, Tropix Inc., Bedford, MA) with an automatic injector, supplying 100 μl of a substrate-buffer solution per sample (Promega Luciferase Assay System).

PREPARATION AND FRACTIONATION OF NUCLEAR EXTRACTS

Nuclear extracts were prepared from JEG-3 and HeLa cells according to published protocols *(530)* from 20-40 large (150 mm) plates. After washing the cells with PBS-CMF, 5 ml of PBS-CMF/1mM EDTA were added to each plate. The plates were kept for 5-10 minutes at room temperature and the cells were lifted from the bottom of the plates using a Pasteur glass pipette and transferred to 50 ml orange cup tubes (Corning) placed on ice. The cells were pelleted at 2500 RPM in a swing out rotor in a HN-S bench top centrifuge (1000 x g) for 5 minutes at room temperature or 4°C. The supernatant was aspirated and the pellets were resuspended in 40 ml of PBS. Five (5) μl of the cell suspension was added to 995 μl of a 5M urea/2M NaCl solution for determination of cell number by A_{260} measurement, before the cells were repelleted. The cells were resuspended in five (5) packed cell volumes of buffer A (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM PMSF) and were incubated on ice for 10 minutes. After centrifugation at 2500 rpm (1000 x g) for 10 minutes at 4°C, the supernatant was discarded and the pellets resuspended in two (2) packed cell volumes of buffer A and transferred to a 12 ml Dounce homogenizer. An average of 10-15 strokes with pestle A was sufficient to disrupt the cell membrane, as determined by microscopy, and was followed by centrifugation at 2500 RPM for 10 minutes at 4 °C in 15 ml oakridge centrifuge tubes. The supernatant was removed and the samples recentrifuged at 14500 RPM in a JS21 Beckman centrifuge in a JA21 rotor (24,000 x g) for 20 minutes at 4 °C. The pellets were resuspended in 2 ml of buffer C (20 mM Hepes pH 7.9, 25% v/v glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF) for each 0.1 A_{260} units determined as above, and homogenized further (15-20

strokes with pestle A). The samples were transferred again to the oakridge tubes, mixed on a Nutator for 30 minutes at 4 ^{0}C, and centrifuged at 14500 RPM in a JS21 Beckman centrifuge in a JA21 rotor (24,000 x g) for 30 minutes at 4^{0}C. The supernatant from this step was inserted in a 6-8000 MW cut-off dialysis tubing and dialysed against at least 50 volumes of buffer D (20 mM Hepes pH 7.9, 20% v/v glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF) for at least 5 hours at 4^{0}C. After removing the insoluble materials by centrifugation at 14500 rpm in a JS21 Beckman centrifuge in a JA21 rotor (24,000 x g) for 30 minutes at 4^{0}C the nuclear extracts were aliquoted (80 μl per tube) and maintained at -70 C. Some experiments used heparin agarose fractionated nuclear extracts prepared by Dr. Mark W. Nachtigal according to Lefevre *et al (204).* After isolation of nuclear proteins in buffer C, the lysate was dialyzed against TM buffer (50 mM Tris-HCl, 1 mM EDTA, 1mM DTT, 12.5 mM MgCl2, 20% glycerol, 0.1 mM PMSF, 100 mM KCl) *(509).* A heparin agarose (Sigma) column was made (1ml heparin agarose per 4 ml nuclear extract) and equilibrated with TM buffer. the dialysed material was added to the column and allowed to pass through by gravity flow. The column was washed once with one column volume of TM buffer, 3 column volumes of TM buffer with 200mM KCl and proteins were eluted with 3 column volumes of 400mM KCl TM buffer. The 400mM fractions were dialysed against buffer D using a 10 well Protodialyser (Enprotech). Fractions were quick frozen in a dry ice-ethanol bath and stored at -70 ^{0}C. Protein concentrations were determined by the Bradford assay *(528)* with bovine serum albumin as a standard.

GEL MOBILITY SHIFT ASSAY

A 100 bp *Hind*III-*Nsi*I fragment was excised from a pUC19 (wild type) or pUC119 (RF-1 or DF-1 site mutants) vectors containing the 241 bp in their polylinker sequences. The 100 bp fragment includes a 1-80 bp (*Acc*I-*Nsi*I) region that does not contain the 22 bp TEF-1 enhancer element. The gel mobility shift assay was performed essentially as described by Baldwin *(531)*. Crude or heparin-agarose purified JEG-3 nuclear extracts (2-22 μg), crude HeLa nuclear extracts (3-20 μg) and crude rat pituitary GC nuclear extracts (22 μg, provided by Dr. Mark W. Nachtigal) were incubated with ^{32}P-end-labelled DNA fragments or synthetic oligonucleotides (0.2-1.0 ng; 1-2 x 10^4 cpm) in the presence of 2 μg of poly dI-dC. Incubation of the reaction (in 10 mM Hepes-NaOH pH 7.9, 50 mM KCl, 0.5 mM EDTA, 10% glycerol, 1 mM DTT) on ice for 15 minutes and at room temperature for 20 minutes was followed by electrophoresis in non-denaturing 4% polyacrylamide gels with 1:60 bis to acrylamide crosslinking ratio. For competition, PCR generated fragments (10-60 ng), double stranded synthetic oligonucleotides (1.5 ng - 1 μg) or poly dI-dC (2-10 μg) were added to nuclear extracts on ice prior to the addition of the radiolabeled DNA.

DNAase I PROTECTION ANALYSIS

A *Hind*III-*Eco*RI fragment, that contained the full length wild type 241 bp enhancer, was radiolabeled at the *Hind*III site and used for DNAase I protection analysis. The *Hind*III-*Eco*RI fragment was excised from the pUC19 vector containing the wild type 241 bp enhancer in its polylinker sequences. A first restriction digestion with *Hind*III was

followed by Klenow treatment and a final digestion with *Eco*RI. Subsequently, the radiolabeled fragment was purified by electroelution, phenol-chloroform and chloroform extracted, ethanol precipitated and resuspended in H_20. The radiolabeled probe (1-3 ng; 1-2 x 10^4 cpm) was incubated with 0-59 μg of JEG-3, BeWo, HeLa or GC nuclear extracts, in a total volume of 20 μl buffer D, in 1.5 ml microfuge tubes placed on ice for 15-30 minutes. Subsequently, the microfuge tubes were transferred to a block heater set at 25^{0}C and, after 1 minute, 24 μl of a DNAase I solution (2.4 u/ml, 4.1 mM $MgCl_2$, 8.2 mM $CaCl_2$) were added to the reactions that were then incubated for 90 seconds at the same temperature. At the end of this incubation period 160 μl of a stop solution (125 mM Tris-HCl, 15.6 mM EDTA, 187.5 mM NaCl, 1.25% SDS (w/v), 12.5 μg/ml tRNA, 0.625 mg/ml proteinase K) were added and the reactions incubated at 37 ^{0}C for 25 minutes. Phenol-chloroform and chloroform extractions, ethanol precipitation and resuspension of the pellets in 5 μl of loading buffer [80% formamide (v/v), 1 mM EDTA, 0.1% xylene cyanole (w/v), 0.1% bromophenol blue (w/v)] was followed by 8% polyacrylamide sequencing gel electrophoresis at a constant power of 50 watts. For autoradiography XAR film was exposed for 12-72 hours at -70 ^{0}C in a cassette with intensifying screens (DuPONT).

STATISTICAL ANALYSIS

Student's t-tests were used for determination of statistical significance of the effects of antibodies in the Nb2 assay, as well as of the effects of enhancer sequences and their mutations on basal CAT activity.

To assess intra- and inter-assay variability of the assessment of relative levels of

individual members of the hGH family by RT-PCR and restriction digestion, 1 μg quantities of the same RNA sample (BeWo cells) were reverse transcribed and amplified in 3 separate assays ($n1=5$, $n2=5$ and $n3=10$ where n equals the number of assessments per assay; stock solutions were identical within an assay but prepared fresh for each assay) and the relative densitometric values of the hGH-V and hCS-A/B bands determined *(Figure 22 p. 124)*. The intra-assay coefficient of variation was 7.2% or better for hGH-V and 2.6% or better for hCS-A/-B. The inter-assay coefficient of variation was 9.3% for hGH-V and 3.2% for hCS-A/-B (Statview, Abacus Concepts, Inc., Berkeley, CA).

Both unpaired t-tests and analysis of variance (ANOVA) were used to assess the statistical significance of differences observed between normal and abnormal trophoblasts. When the results of the ANOVA indicated the presence of a significant difference, a post-hoc test was applied (Fisher's Protected Least Significant Difference; Statview, Abacus Concepts, Inc., Berkeley, CA). Significance was defined by $p<0.05$, however, all the differences observed in this study were significant at the $p<0.01$ level or better.

RESULTS

A Studies on the characterization of a lactogenic activity present in the conditioned media of sfRamos cells

The immune functions of GH and PRL have been a controversial subject for many years. Reports, on the effectiveness of immune functions in hypophysectomised animals, suggest the proliferative and/or differentiating effects of pituitary GH and PRL on cells of the immune system *(for reviews see references 187,191-193)*.

In contrast, it has been less clear whether several reported PRL-like activities *(22-29)* were indeed produced by (and act as autocrine factors for) lymphocytes. Both the actual synthesis as well as the identity of these PRL-like molecules has been questioned. A characteristic example was the case of the L2 murine T-lymphocyte line *(306)*. L2 cells, were able to sequester biologically active bovine PRL, deriving from the FBS in their culture media, however, they did not synthesize endogenous murine PRL *(306)*. In the first well documented case of hPRL synthesis and secretion by cells of immune origin, in the IM-9-P3 human lymphoma cell line, no autocrine effect in the growth of these cells was observed *(277)*. This finding has increased the interest in putative, yet non characterized PRL-like autocrine activities, distinct from PRL, that could serve as growth factors for immune cells.

A particularly interesting report on a hPRL-like autocrine activity present in lymphoma cells appeared at this time. A 29K hPRL-like peptide molecule was reported

to be secreted by a Burkitt lymphoma cell line (Ramos) under serum-free (sf) conditions *(502)*. Conditioned medium from these cells had a proliferative effect on the Nb2 rat lymphoma cells, known to respond with growth to lactogenic hormones (PRL, hGH and hCS) which interact with the PRL (lactogenic) receptor present on their surface *(152)*. Additionally, an autocrine effect on sfRamos cells was observed and a polyclonal antibody against hPRL inhibited the autonomous growth of these cells.

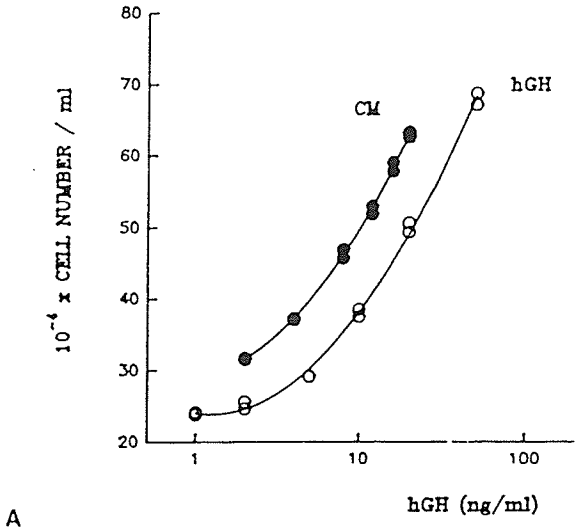
In order to reexamine the **hypothesis** that a PRL-like activity was produced by these cells, I initiated a series of experiments with the **objective** of confirming the presence of a lactogenic activity and proceeding with its characterization. The latter would include cloning the respective gene and developing the tools (specific DNA probes and antibodies) for studying the distribution, biological effects of its product(s) and the factors that determine its gene expression. The experimental design included an initial step aimed at confirming the presence of a biologically active lactogenic molecule in the conditioned media of sfRamos cells by the use of the Nb2 assay. Further, due to the use of only one polyclonal anti-hPRL serum during the studies that led to the identification of this lactogenic activity *(502)*, a more complete characterization was necessary. This would be obtained by the use of polyclonal as well as monoclonal anti-hPRL and/or anti-hGH antibodies, in an attempt to neutralize the lymphoproliferative effect of sfRamos conditioned medium in the Nb2 assay. The characterization of this lactogenic activity by the Nb2 bioassay would be followed by protein and RNA analyses.

I. A lactogenic activity was detected in concentrated conditioned media from sfRamos cells by the Nb2 assay

A lactogenic activity in concentrated conditioned media from sfRamos cells was indeed detected by the Nb2 assay. All preparations showed a mitogenic effect on Nb2 cells and the dilution curve of the concentrated conditioned media was parallel to hGH standard curve *(Figure 6A)*. Concentrated (300x) fresh media not exposed to cells or human transferrin (1.5-3.0 mg/ml) did not stimulate Nb2 cell proliferation *(Figure 6B)*.

II. The lactogenic activity detected in concentrated conditioned media from sfRamos cells by the Nb2 assay is GH- and not PRL-like

The lactogenic activity in concentrated conditioned media from sfRamos cells was determined by the Nb2 assay in the presence and absence of monoclonal antibodies against hGH (3D5) or hPRL (9C3). A monoclonal anti-PRL antibody (9C3) had no significant effect ($P > 0.05$) but a monoclonal anti-hGH antibody (3D5) completely blocked ($P < 0.005$) this activity *(Figure 6B)*. Two additional monoclonal (2A1 and 3B1) and two polyclonal (BR-3-4 and anti-hGH #1) antibodies against hGH also blocked the stimulatory effect of conditioned media in the Nb2 assay *(Results for anti-hGH #1 in Figure 6C)*. A polyclonal antibody (AR-38-7) against hPRL had no effect *(not shown)*. These results indicated the presence of bioactive hGH in the conditioned media of sfRamos cells.



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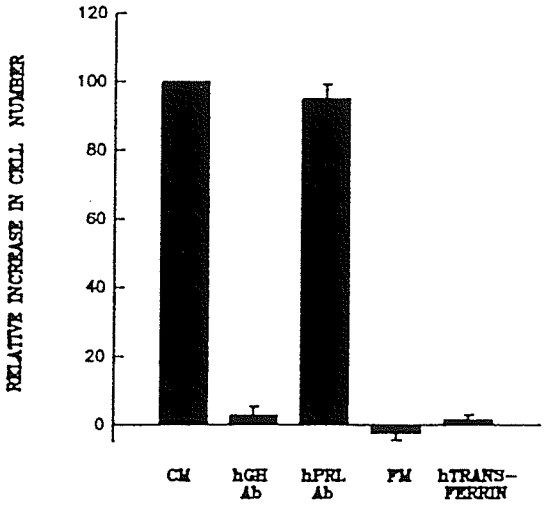

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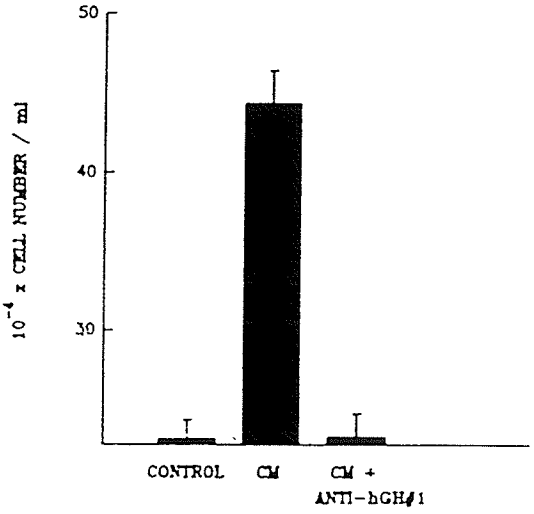


C

FIGURE 6: Effect of concentrated conditioned media (CM) from sfRamos cells on Nb2 cells in the presence and absence of Abs against hGH or hPRL. (A) CM from sfRamos cells was mitogenic when tested in the Nb2 assay. CM (1000x) was diluted to generate 800x, 600x, 400x 200x, 100x CM and tested in the Nb2 assay (100 μl samples). The dilution curve of the CM is parallel to the hGH standard curve (50 μl samples). ● CM dilution curve, o hGH standard curve. The mean value of the hGH bioactivity in concentrated conditioned media for this experiment is 22.8 ng/ml of 1000X CM. The corresponding value for hGH bioactivity in conditioned medium (not concentrated) is 22.8 pg/ml. (B) CM (150-500x) were examined for lactogenic activity in the Nb2 assay in the presence and absence of antibodies. The increase in cell number when CM only was added is arbitrarily set at 100% (n=10). All other differences in cell number between the control (no CM) plates and the sample plates (CM + antibodies, concentrated fresh media, human transferrin) are expressed as a percentage of this increase. The increase in cell number in response to CM was greatly inhibited ($P<0.005$) when monoclonal anti-hGH Ab (3D5, 1:4000 dilution) was added, (n=10) or remained unchanged ($P>0.05$) when monoclonal anti-hPRL Ab (9C3, 1:4000 dilution) was added (n=7). When concentrated fresh media (FM, n=4) or human transferrin (n=2) only was added, no increase in cell number was observed (C) The anti-hGH #1 antibody, previously shown to selectively inhibit the action of the hGH-N gene product and not that of the hCS or the hGH-V, in the Nb2 assay, at a dilution of 1:2000, completely blocked (n=2) the proliferative effect of 250-400X CM (50-100 μl samples) on Nb2 cells (n = number of determinations; each determination is the mean of a pair of duplicates). Positive and negative standard error bars are indicated.

III. Immunoreactive hGH is detected in concentrated conditioned media of sf Ramos cells by RIA

Concentrated conditioned medium was tested in an RIA for hGH using a polyclonal anti-hGH Ab (BH12). Human GH immunoreactivity was detected, and the dilution curve was parallel to the hGH standard curve *(Figure 7)*. No hPRL immunoreactivity was detected by a time-resolved fluoroimmunoassay *(Pharmacia, not shown)* in preparations of concentrated conditioned media that contained hGH immunoreactivity at levels similar to those shown in *Figure 7*.

Human GH immunoreactivity was not due to contamination of the culture media or the protein supplement (human transferrin) with hGH since no hGH immunoreactivity was detected in concentrated fresh media (400x) or in human transferrin 5 mg/ml by hGH 2-site immunoradiometric assays *(Pharmacia, not shown)*.

The hGH values in concentrated conditioned media determined by the Nb2 assay and the double antibody RIA are very similar. The ratio of the values determined by the Nb2 assay and RIA for the preparation shown in *Figure 7* is 0.98 and falls in the range reported for the Nb2/RIA ratio for hGH determinations *(152)*. The Nb2 value used to calculate the Nb2/RIA ratio was the mean of two determinations (in duplicate in two different assays) at the linear portion of the hGH standard curve and the RIA value the mean of the 1:1, 1:2, 1:4 dilution values.

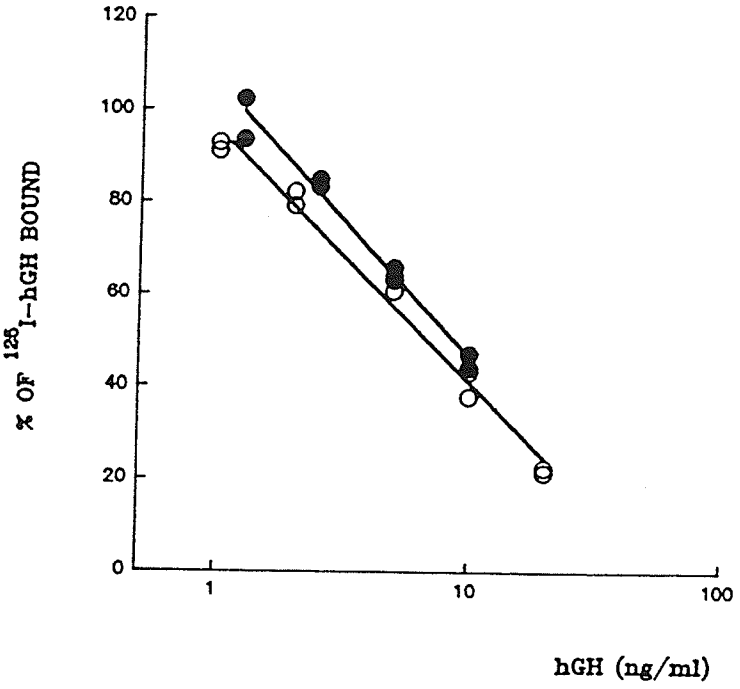


FIGURE 7: Human GH activity in concentrated conditioned media from sfRamos cells is detected by RIA. The dilution curve of 250 times concentrated conditioned medium is parallel to the hGH standards. Results are expressed as the % specific binding of ^{125}I-hGH. ○ hGH standard curve; ● concentrated conditioned media dilution curve. The values estimated through the hGH standard curve are 7.7, 3.8 and 1.8 ng/ml of 250 times concentrated medium for 1:1, 1:2 and 1:4 dilutions respectively. The mean estimate by RIA for the hGH concentration in the conditioned medium (non concentrated) is 30 pg/ml (data from a hGH RIA performed by Ni Quan).

IV. No specific hGH band was detected by Western blot analysis of concentrated sfRamos conditioned media

Concentrated conditioned medium was resolved by SDS-PAGE and after transfer to nitrocellulose BR-3-4 anti-hGH polyclonal antibody or normal rabbit serum were used for immunodetection *(Figure 8)*. A predominant band migrating below the 30K marker and a number of less intense bands were observed with anti-hGH antibody (lane 2) as well as with normal rabbit serum (NRS) (lane 3).

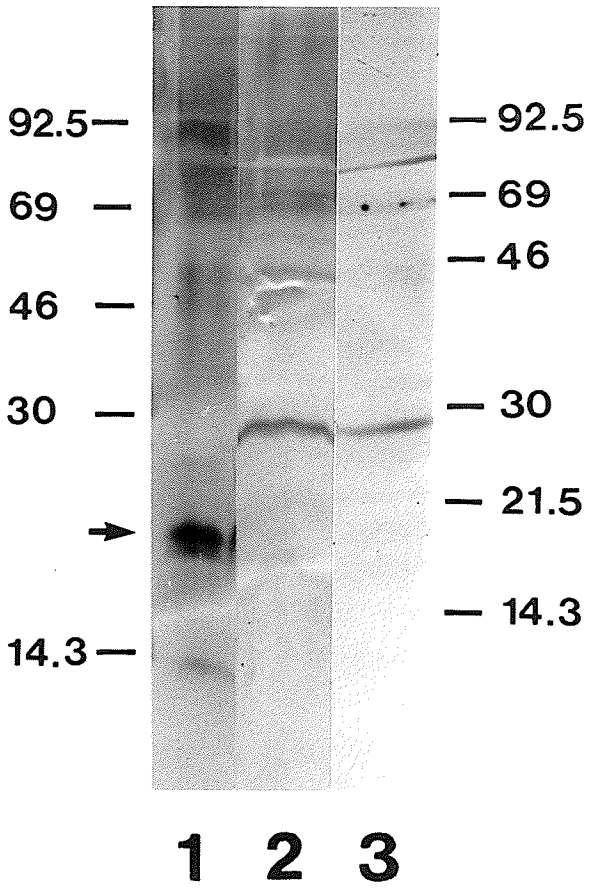


FIGURE 8: Detection of non specific bands in sfRamos concentrated conditioned medium (CM) by Western blot analysis. HGH control and concentrated conditioned medium (600X) were resolved by SDS-PAGE (lane 1 and 2: 15% SDS-PAGE, lane 1, 10 ng hGH, lane 2, 80 μl CM, lane 3: 12.5% SDS-PAGE, 35 μl CM) and after transfer to nitrocellulose, BR-3-4 anti-hGH polyclonal antibody (lane 1 and 2) or normal rabbit serum (lane 3), were used for immunodetection. A predominant band migrating below the 30K marker and a number of less intense bands were observed with anti-hGH antibody as well as with normal rabbit serum. The positions of the 22K hGH control and of the protein markers (rainbow mix, Amersham, Oakville, Ontario) are indicated. Markers for lane 1 and 2 are indicated at the left and for lane 3 at the right.

V. A specific 22K hGH band was detected by Western blot analysis after immunoprecipitation of concentrated sfRamos conditioned media

The direct application of concentrated conditioned media on SDS-PAGE *(Figure 8)* failed to detect a specific hGH band by Western blotting. Immunoprecipitation of 600 times concentrated conditioned media (CM) with BR-3-4 anti-hGH polyclonal antibody, followed by Western blot analysis with 3D5 anti-hGH monoclonal Ab as the primary antibody, was used as a more sensitive and specific method *(Figure 9)*.

The presence of a 22K band [lane 2, migrating at the same level with pituitary hGH (lane 1)] was revealed. The 22K band was absent when immunoprecipitation was performed with NRS (lane 3) or when 600 times concentrated fresh media or human transferrin (3 mg/ml) were immunoprecipitated with BR-3-4 anti-hGH antibody (not shown).

Additional bands (50, 42, 29 and 23K) are due to protein A, heavy and light chains or IgG fragments since they are also present when no CM but only anti-hGH polyclonal antibody and protein A are added to the immunoprecipitation buffer (not shown). The detection of these bands is likely due to the affinity of the anti-mouse IgG secondary antibody for Protein A and to its cross reactivity to the rabbit IgG. Immunoprecipitation with anti-hPRL polyclonal antibody (AR-38-7) followed by Western blot with anti-hPRL monoclonal antibody (9C3) did not detect the 22K or any other specific band (*Figure 10*).

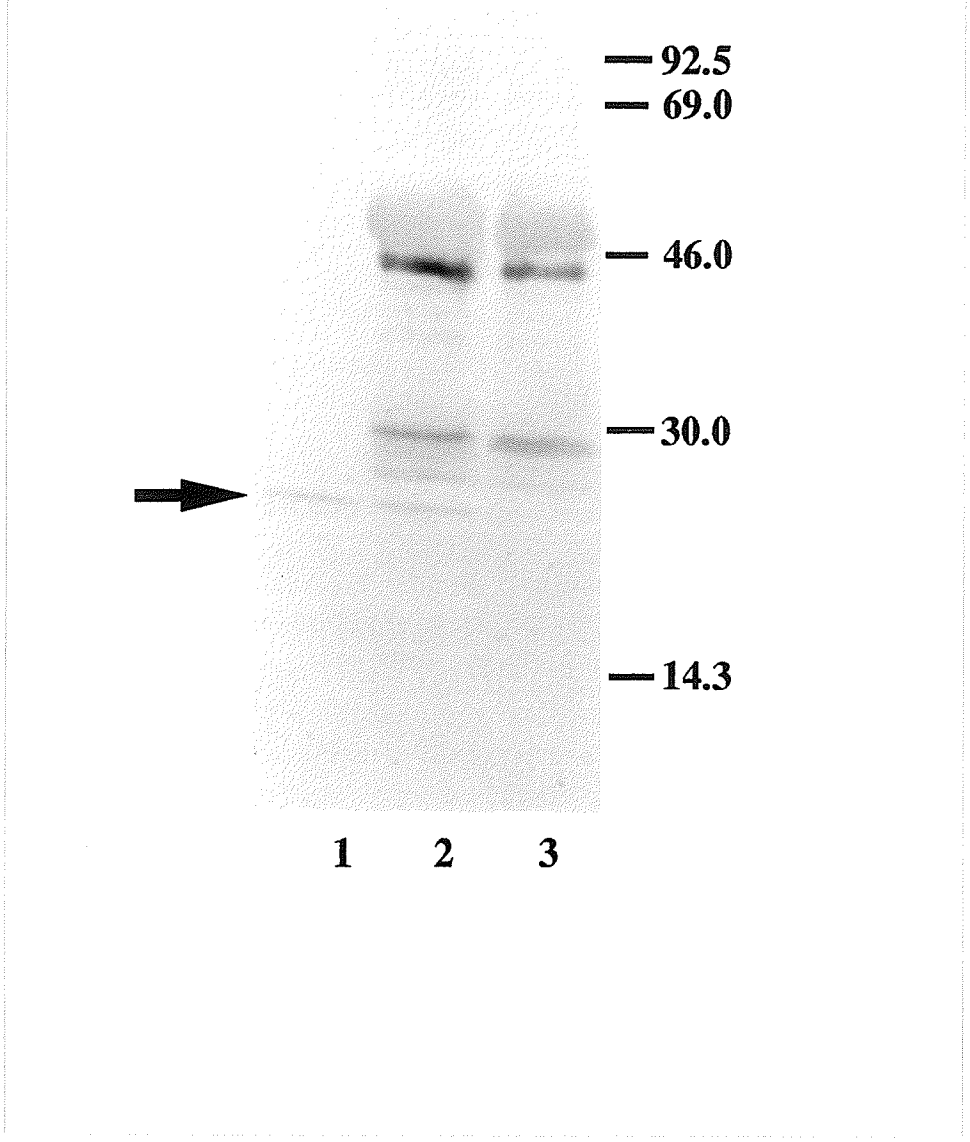


FIGURE 9: Detection of a 22K hGH band by Western blot analysis. Concentrated conditioned medium (600x) was immunoprecipitated with BR-3-4 polyclonal anti-hGH antibody (lane 2) or normal rabbit serum (lane 3) and subjected to SDS-PAGE. After transfer to nitrocellulose, 3D5 monoclonal antibody was used to detect hGH-immunoreactivity. A 22K band, migrating similarly to purified hGH (left side arrow, lane 1, 8 ng hGH), was observed when concentrated conditioned medium was immunoprecipitated with anti-hGH (lane 2) but not when immunoprecipitated with NRS (lane 3). An intense 42K band represents Protein A released from the sepharose beads while the bands at about 50, 29 and 23K and a number of fainter non specific bands (present in both lanes 2 and 3) likely represent heavy and light chains as well as IgG fragments of the antibodies used for immunoprecipitation. The positions of the molecular weight markers are indicated.

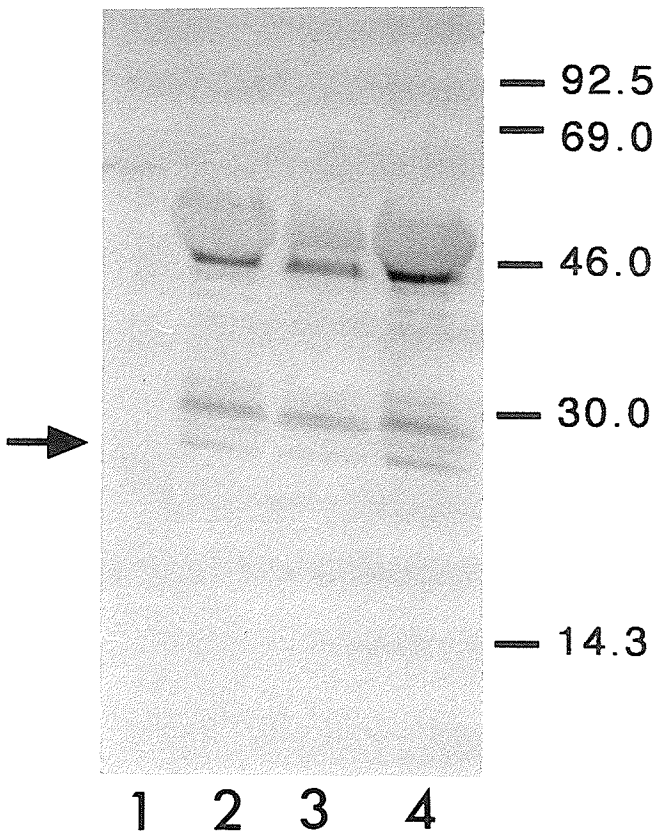


FIGURE 10: The 22K hGH band is not detected by western blot analysis using anti-hPRL monoclonal antibody after immunoprecipitation with anti-hPRL polyclonal serum. Concentrated CM (600X) was immunoprecipitated with AR-38-7 polyclonal anti-hPRL serum (lane 2) or normal rabbit serum (lane 3) and subjected to SDS-PAGE. After transfer to nitrocellulose, 9C3 monoclonal antibody was used to detect hPRL-immunoreactivity. A 23K (arrow) hPRL band was virtually undetectable when either purified human hPRL (lane 1, 10 ng hPRL) or immunoprecipitated hPRL (lane 4, AR-38-7 polyclonal anti-hPRL serum was used to precipitate 10 ng hPRL present in 600 μl of a 20 mg/ml BSA solution) were tested, suggesting that amounts less than 10 ng fall below the sensitivity of this assay. A 23K band was also not detected in the immunoprecipitated CM (lane 2). A 22K band was not observed suggesting that it was specific for both the anti-hGH polyclonal serum and monoclonal antibody. Further, no other specific band was detected by this Western blot analysis. An intense 42K band represents Protein A released from the sepharose beads while the bands at about 50, 29 and 23K and a number of fainter non specific bands (present in lanes 2-4) likely represent heavy and light chains as well as IgG fragments of the antibodies used for immunoprecipitation. The positions of the molecular weight markers are indicated.

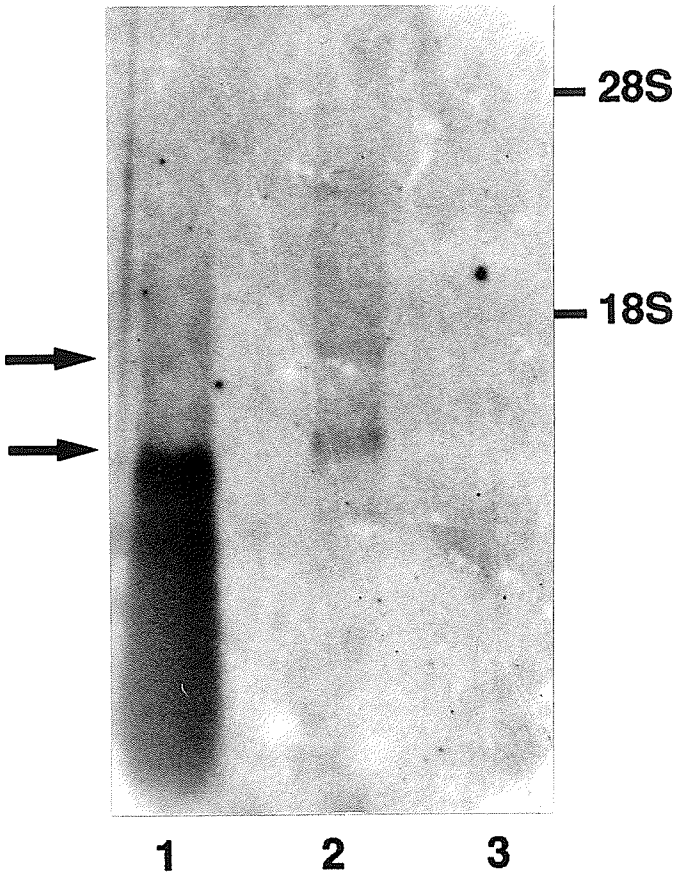
VI. HGH hybridization is detected by northern blot analysis of poly A (+) enriched sfRamos RNA

Poly A(+) enriched RNA (25 μg, lane 2) and Poly A(-) RNA (25 μg, lane 3) from sfRamos cells and human pituitary total RNA (2 μg, lane 1) were subjected to electrophoresis, transferred to nitrocellulose membrane and hybridized with a radiolabelled hGH-N cDNA probe. Two major transcripts were detected by autoradiography (*Figure 11*). A more abundant transcript migrates coincident with pituitary hGH mRNA (approximately 1.0 Kb) while a second transcript, also present in pituitary RNA blots, migrates at about the 1.6 Kb level.

VII. RT-PCR coupled with diagnostic restriction digestion and PCR-sequencing revealed identity of the sfRamos GH activity with hGH-N.

The predicted 250 bp fragment specific for the hGH-N cDNA was generated by the RT-PCR reaction when sfRamos total (lane 3) or poly A(+) enriched (lane 4) or human pituitary total (lane 2) RNA were used as templates (*Figure 12*). The fainter 341 bp bands in lanes 3 and 4 are the result of genomic DNA contamination of the RNA samples since they correspond to the predicted 341 bp band generated when HeLa genomic DNA is used as a template for the PCR reaction (lane 6). The 341 bp fragment spans intron C which explains the difference in size from the RT-PCR generated 250 bp fragment. An additional sfRamos poly (A)+ RNA preparation (*Figure 13*, lane 5) and total RNA from the parental Ramos ATCC cells cultured under serum free conditions for 72 hours (lane 3) also generated the 250 bp band.

FIGURE 11: Detection of hGH mRNAs in poly A(+) fraction of sfRamos RNA. Two RNA transcripts were identified (arrows, lane 2) when poly A(+) RNA (25 μg) was subjected to Northern blot analysis: a more abundant 1.0 Kb migrating at the same level with the pituitary hGH transcript (lane 1) and a less abundant 1.6 Kb transcript. The 1.6 Kb transcript can also be detected in human pituitary RNA as a minor species, with longer exposure. Poly A(-) RNA (25 μg, lane 3) did not show any hybridization. The positions of 28S and 18S are indicated.



*Rsa*I restriction digestion of RT-PCR generated products (*Figure 14*, lane 2, sfRamos total RNA, lane 4 and 6, two different preparations of sfRamos poly (A)+ RNA) revealed the presence of hGH as the predicted 190 bp and 60 bp hGH fragments (see *Table 7 p. 109*) were produced. *Hph*I digestion did not generate a pattern consistent with the expression of hCS or hGH-V (lane 3, sfRamos total RNA, lane 5 and 7, two different preparations of sfRamos poly (A)+ RNA). Finally, extraction of the 250 bp fragment, followed by PCR sequencing, revealed identity to the hGH-N cDNA. (*Figure 15, Table 8 p. 110.*)

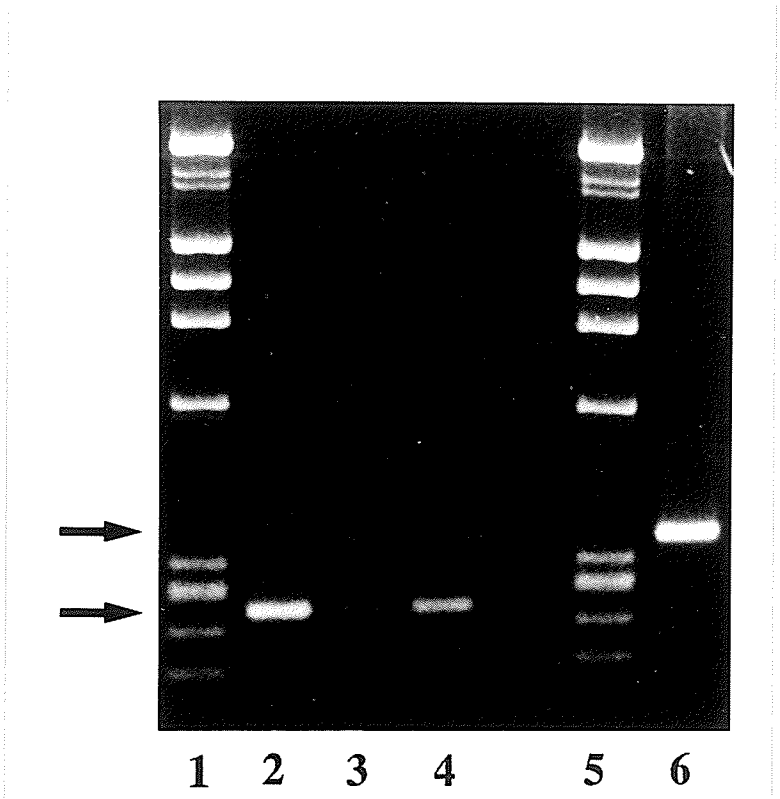
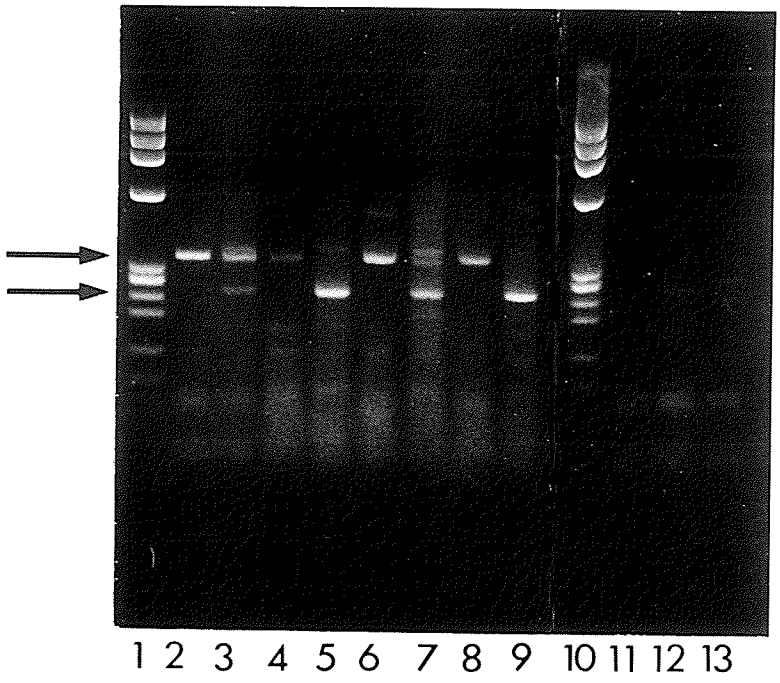


FIGURE 12: Detection of hGH cDNA fragments after application of RT-PCR on sfRamos and human pituitary RNA samples. Following the reverse transcriptase (RT) reaction two oligonucleotide primers corresponding to homolo-gous sequences in exons 3 and 4 of hGH gene family members were used to amplify the generated cDNAs. Both pituitary (lane 2, total RNA as template) and sfRamos (lane 3, total RNA as template, lane 4, poly A(+) enriched RNA as template) samples generated the predicted 250 bp band (arrow). The fainter 341-343 bp bands in lanes 3 and 4 are the result of genomic DNA contamina-tion of the RNA samples since they correspond to the predicted 341-343 bp fragments generated by amplification of *Eco*RI digested HeLa cell genomic DNA (lane 6, arrow). Molecular weight markers (Φx 174 *Hae*III and λ *Hind*III mixture, Pharmacia) are shown in lanes 1 and 5 and correspond to sizes of 23.1, 9.4, 6.6, 4.4 (not resolved, migrate as one band), 2.3, 2.0, 1.4, 1.1, 0.87, 0.60, 0.56, 0.31 0.28, 0.234 and 0.194 Kb.

FIGURE 13: RT-PCR reactions (lanes 3,5,7,9) and (-)RT-PCR controls (lanes 2,4,6,8) for sfRamos RNA samples presented in *Figure 12* (lanes 6-7, total RNA, lanes 8-9 poly (A)+ RNA) as well as from an additional sfRamos poly (A)+ RNA preparation (lanes 4-5) and total RNA from Ramos ATCC cells cultured under free conditions for 48 hours (lanes 2-3). Lanes 1 and 11, Φx 174 *Hae*III molecular weight markers; lanes 11, 12 and 13 represent (-)RT, RT and PCR buffer controls, respectively. The upper arrow corresponds to the amplification of the genomic 341 bp band and the lower of the 250 bp RT-PCR hGH cDNA product.



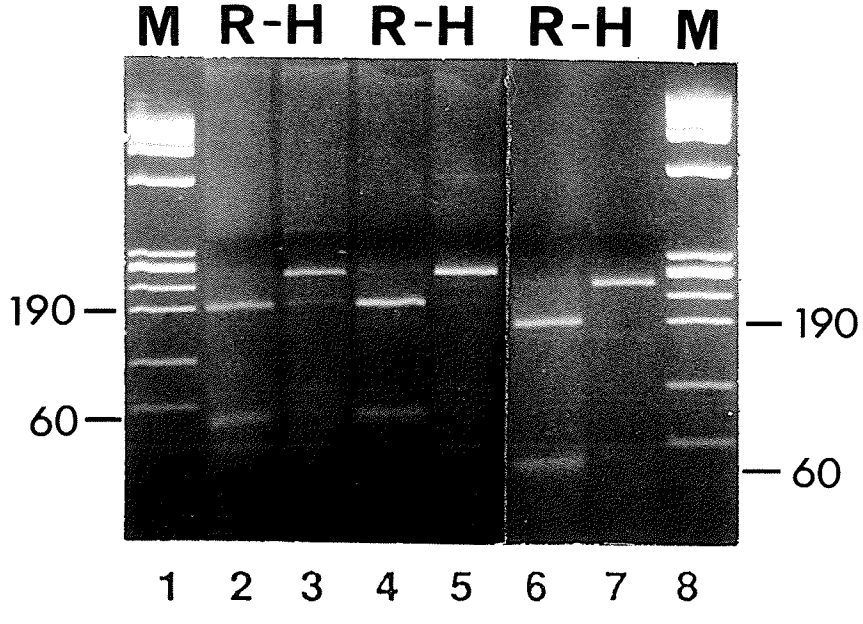


FIGURE 14: Restriction digestion of the RT-PCR generated products from the RNA samples presented in *Figure 13* confirmed the presence of hGH as the predicted 190 bp and 60 bp fragments (see *Table 7*) were produced by *Rsa*I digestion (lane 2, sfRamos total RNA, lane 4 and 6, two different preparations of sfRamos poly (A)+ RNA). *Hph*I digestion did not generate a pattern consistent with the expression of hCS or hGH-V (lane 3, sfRamos total RNA, lane 5 and 7, two different preparations of sfRamos poly (A)+ RNA). Lanes 1 and 8, Φx 174 *Hae*III molecular weight markers.

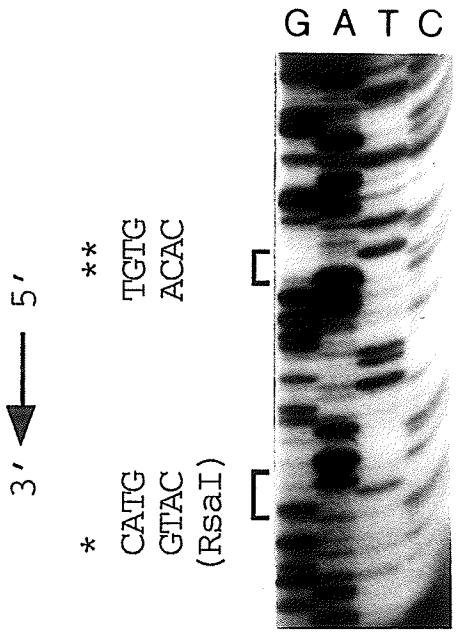


FIGURE 15: PCR sequencing reaction of sfRamos RT-PCR generated 250 bp fragments. The fragments were excised from the agarose gel and were used as templates for PCR-sequencing (fmol DNA Sequencing System, Promega). The sequencing products were analyzed by 6.8% polyacrylamide gel electrophoresis and the autoradiogram was developed after 3 hour exposure of XAR film at room temperature. A sequencing gel of the antisense strand is presented and the 5'-3' orientation that corresponds to the sense strand of the gene is indicated. Three short diagnostic sequences (diagnostic nucleotides are marked by an asterisk, see *Table 8*). The most 3' (sense strand) diagnostic sequence represents the *Rsa*I restriction site in the hGH cDNA.

Table 7. **Predicted hGH/hCS gene products of PCR and restriction endonuclease digestion**

hGH/hCS GENE	PCR PRODUCT	UNCUT (bp)	Hph I (bp)		Rsa I (bp)	
		EXON 3 PROBE +	EXON 3 PROBE +	-	EXON 3 PROBE +	-
hCS-A	RNA	250	207	43	250	
	GENOMIC DNA	342	299	43	342	
hCS-B	RNA	250	207	43	250	
	GENOMIC DNA	343	300	43	343	
hGH-V	RNA	250	87	163	250	
	GENOMIC DNA	341	87	254	341	
hCS-L	RNA	250	164	43	168	82
	GENOMIC DNA	341	255	43	259	82
hGH-N	RNA	250	250		190	60
	GENOMIC DNA	342	342		282	60

Fragments detected (+) or not detected (-) by the exon 3 probe are indicated

Table 8. Diagnostic sequence comparison of the RT-PCR generated cDNA from sfRamos RNA to the sequences of the hGH/CS cDNAs

Diagnostic sequence comparison of the RT-PCR generated cDNA from sfRamos RNA to the sequences of the hGH family cDNAs. The sequence corresponds to nucleotides 966 to 1010 in exon 4 of the hGH-N gene, relative to the transcription initation site as nucleotide 1.

TGC	AGT	TCC	TCA	GGA	GTG	TCT	TCG	CCA	ACA	GCC	TGG	TGT	ACG	sfRamos
---	---	---	---	---	---	---	---	---	---	---	---	---	---	hGH-N
---	--C	---	---	---	-C-	---	---	---	---	---	---	---	-T-	hGH-V
---	G--	---	---	---	--A	C--	--A	---	---	A--	---	---	-T-	hCS-L
---	G--	---	---	---	--A	-G-	---	---	---	A--	---	---	-T-	hCS-A
---	G--	---	---	---	--A	-G-	---	---	---	A--	---	---	-T-	hCS-B

VIII. The activation of the hGH-N in sfRamos cells is not the result of a gross alteration of the hGH gene family locus as shown by Southern analysis.

Genomic DNA from sfRamos and Ramos ATCC cells was analyzed by restriction digestion (*Eco*RI or *Bam*HI), blotting and hybridization with a radiolabelled hGH-N cDNA probe in order to investigate the possibility of a gross deletion, insertion or rearrangement in any of the genes of the hGH family locus. SfRamos and Ramos ATCC DNA showed identical patterns (*Figure 16*) to HeLa (human cervical carcinoma) DNA, described previously to contain an intact hGH gene family locus (*505.*).

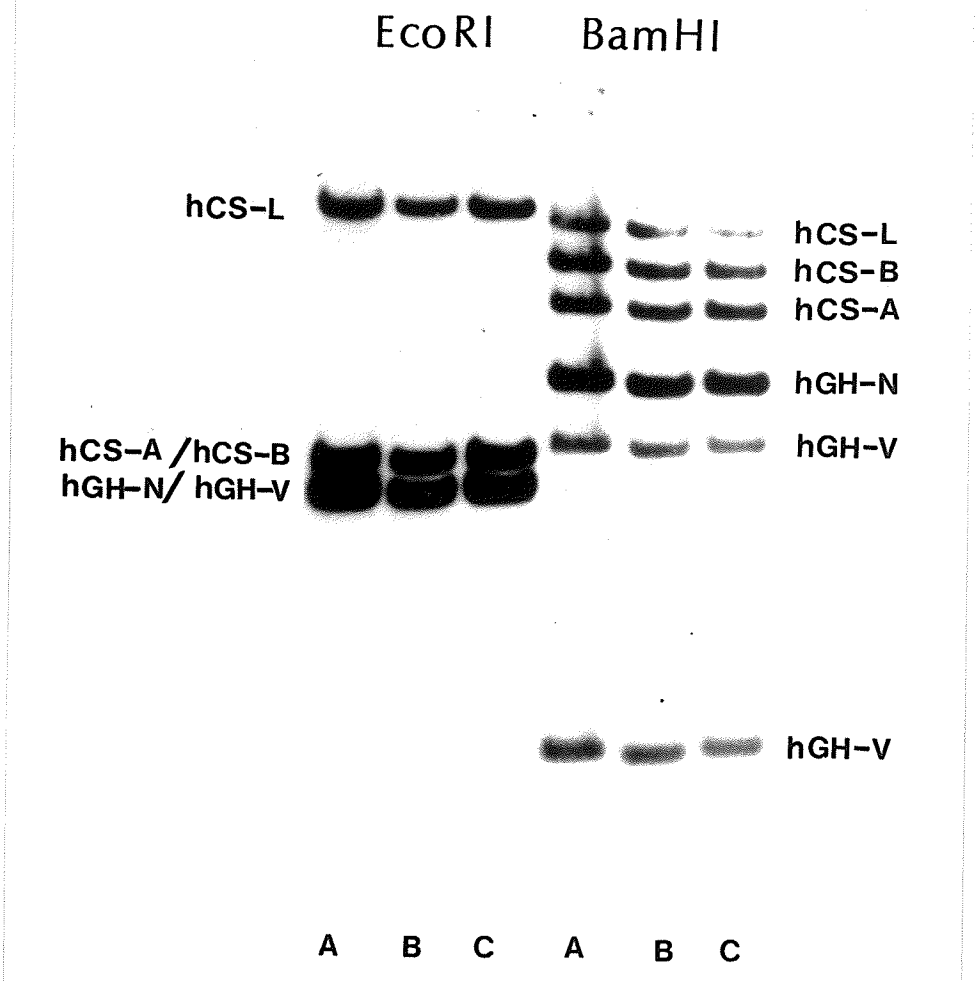


FIGURE 16: Genomic DNA analysis of the hGH family locus DNA from HeLa (A), sfRamos (B) and Ramos ATCC (C) cells was digested with *Eco*RI or *Bam*HI and resolved by 1% agarose gel electrophoresis. After transfer to nitrocellulose, hybridization with a radiolabelled hGH-N cDNA probe was performed. The specific gene fragments are indicated. No gross rearrangement is observed in any of the genes of the hGH family locus in sfRamos cells. HeLa cells have been previously shown to have an intact hGH gene family locus and, as such, were used as a normal control.

B. Studies on the expression of the members of the hGH/CS family in normal and abnormal trophoblasts.

This result and the complete nature of the approach used to demonstrate the expression of hGH in these lymphoma cells contributed significantly to our current knowledge since for quite a long time the expression of GH in lymphocytes was a controversial subject. The result was also provocative as it suggested the importance of hGH for the survival of these tumor cells. Furthermore, it emphasized the complexity and diversity of the mechanisms that regulate the expression of the hGH gene family. At the time, it was broadly accepted that the members of the hGH family are regulated in a very tight tissue-specific manner that allows their expression exclusively in pituitary (hGH-N) or in placenta (hGH-V and hCS genes). Pituitary-specific expression is determined by the transcription factor GHF-1/Pit-1 that interacts with elements in the 5' flanking region of the hGH-N gene. Placenta-specific expression is believed to be regulated by sequences in the 3' flanking region of the hCS-B gene where a single DNA element representing a binding site for a transcriptional enhancer factor (TEF-1) was identified. The expression of hGH in a lymphoid cell line, provides evidence for the existence of diverse mechanisms that control the expression of the hGH family in extrapituitary and extraplacental tissues. It also provides an interesting model for the study of human GH family tissue-specific gene expression as it involves mechanisms for both transcriptional activation (hGH-N) and transcriptional blockade (hGH-V and hCS genes). This pattern of hGH expression in lymphocytes is similar to that seen in the pituitary. Synthesis in the pituitary is dependent on the presence of the tissue-specific factor Pit-1/GHF-1. The similarity between GH expression in lymphocytes and the

pituitary is therefore interesting since Pit-1/GHF-1 appears absent or at very low levels in lymphoid cells *(206,532-534)*.

These findings corroborated the need for reevaluation of certain beliefs based on the earlier scientific experience in molecular biology. The cloning of the first tissue specific transcription factors (such as Pit-1/GHF-1) seemed to provide relatively simple models for the tissue specific expression of certain genes. Currently, however, it is recognized that more complex mechanisms are necessary for exclusive and efficient gene expression in a particular cell type. Additional factors are necessary for regulation of temporary or cell specific gene expression and in many cases combined action of transcription factors with partial tissue specificity confers very tight tissue-specific expression.

The hGH/hCS gene family is a perfect example of this complexity. Apart from sharing >90% nucleotide sequence homology in their coding and flanking regions *(76)*, all five genes contain Pit-1 elements in their promoter regions *(76)* but only hGH-N is expressed in pituitary. Further, the hCS-A and hCS-B genes are expressed at much higher levels than the hGH-V and hCS-L genes in human placenta *(76,78)*.

Several questions are raised evaluating these expression patterns: (a) What prevents the promoters of the placental members of this family from being functional in pituitary since they contain the binding sites for the pituitary specific transcription factor? (b) What are the DNA elements and transcription factors that through their interactions regulate tissue specific and temporal gene activation of the placental members of the hGH/CS family and determine the levels of expression of each of these genes? (c) Do the same factors determine placental or even embryonic development?

To investigate these questions we would need to use systems that would allow the

dissection of the different components of these transcriptional mechanisms and assess the functional importance of putative regulatory DNA sequences and transcription factors. Aberrations of normal regulatory pathways may lead to the disruption of normal developmental processes and/or the formation of tumors. Very frequently, these events are associated with a profound modification of the biochemical phenotype and the loss of the differentiation characteristics of particular cell populations.

Abnormalities of the placental development are, indeed, accompanied by the disruption of normal gene expression in this tissue. Human GH/CS expression in hydatidiform moles and choriocarcinomas is greatly reduced compared to the normal tissue *(371)*. It has not been clarified yet whether the placental hGH/CS genes follow parallel expression profiles in normal placenta or whether there are individual gene-expression patterns that may underline distinct regulatory mechanisms. Hydatidiform moles and choriocarcinomas presumably represent a broad spectrum of cell populations at various stages of differentiation. If distinct mechanisms exist for the expression of individual placental members of the hGH/CS family this should be reflected in different trophoblast tumor populations.

To investigate this hypothesis, **I examined the expression of individual members of the hGH/hCS gene family in normal placenta, hydatidiform moles and choriocarcinoma cell lines.** This might also allow the identification of hGH/CS expression profiles as potential markers for placental abnormalities and distinct cell subpopulations as components of systems appropriate to study the cell specific expression of hGH/CS genes and the transcription factors that may regulate placental development.

Previous attempts to assess human placental growth hormone variant (hGH-V) and chorionic somatomammotropin (hCS) RNA in choriocarcinoma cell lines had been

hampered by low levels of expression and limited sensitivity of RNA blotting analysis. Studies in Cattini's laboratory had reported the expression of hGH-V and hCS but not hGH-N in BeWo choriocarcinoma cells by the use of differential hybridization of specific oligonucleotides for individual hGH/hCS transcripts *(505)*. However, no demonstration of hGH-V versus hCS expression in JAR and JEG-3 cells was obtained due to the low levels of messenger RNA *(505,535)*.

In the present studies I applied the more sensitive method of RT-PCR and simplified the procedure to a single set of primers in conjunction with restriction digests to distinguish hCS, hGH-V, hCS-L and hGH-N RNA from choriocarcinoma BeWo, JAR and JEG-3 cell lines and samples of three hydatidiform moles (HM-1, -2 and -3). The primers were also designed to distinguish processed RNA from any contaminating DNA *(Figure 17, Table 7)*.

I. Human CS, hCS-L and hGH-V RNA are detected in term placenta by RT-PCR.

The predicted 250 bp fragment specific for the hCS/hGH cDNAs (see *Table 7*) was generated by the RT-PCR reaction when total RNA from human placenta was used as the template (*Figure 18*, lane 1). Restriction digestion of the 250 bp fragment with *HphI* (*Figure 18A*, lanes 2 and 3) generated a pattern consistent with the expression of hCS (hCS-A and -B) genes in human placenta. Two bands, 207 and 43 bp corresponding to the digested hCS 250 bp fragments are clearly visible. Weaker 164/163 and 87 bp bands were also detected by ethidium bromide staining (small arrows). The 163 bp band,

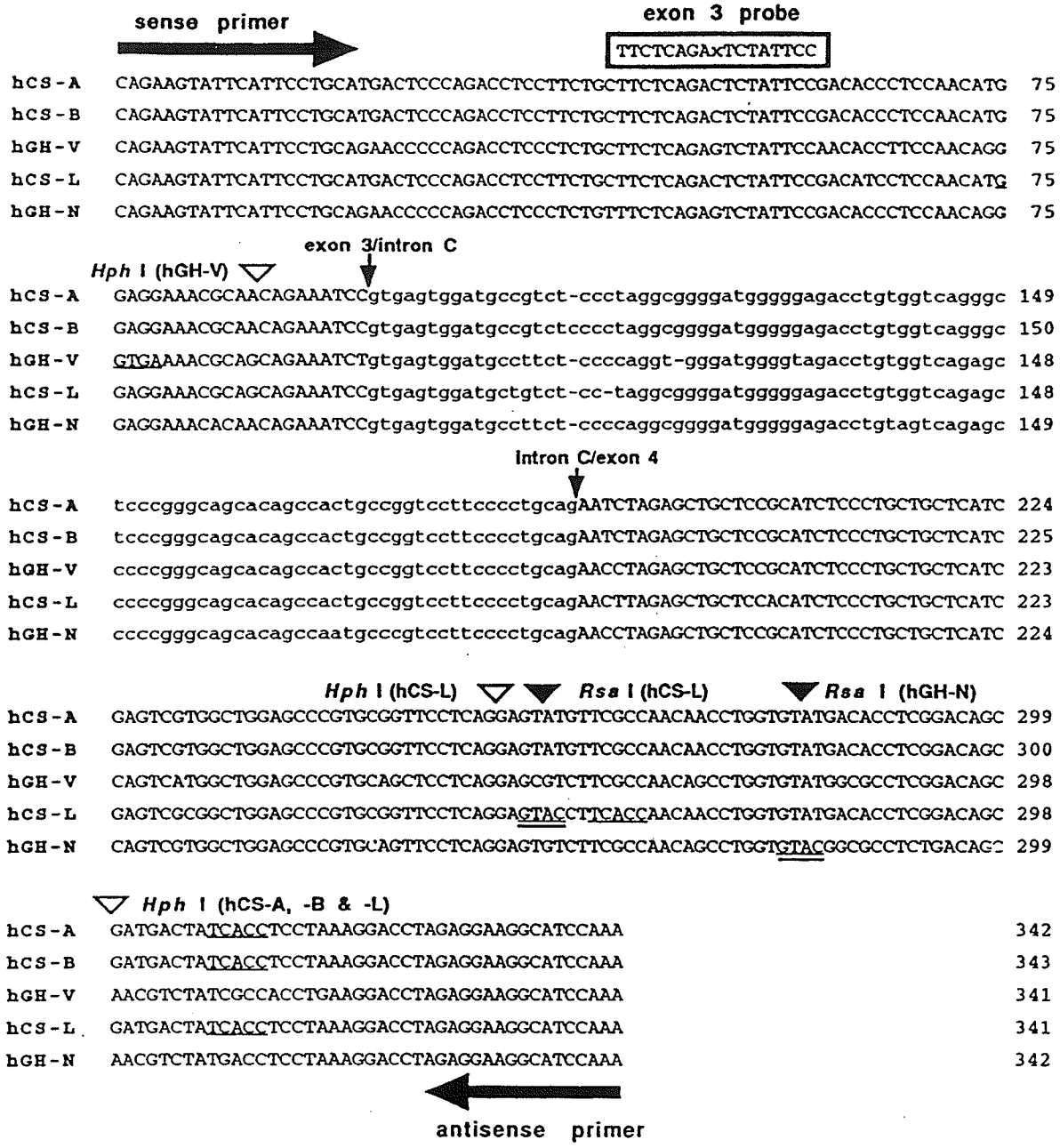


FIGURE 17: Comparative representation of the hGH/hCS regions amplified by RT-PCR. The positions of the sense and antisense primers as well as the location of an oligonucleotide probe (boxed) used for specific hybridization with the RT-PCR products are indicated. The restriction enzymes used for diagnostic cuts and their respective target genes (in parentheses) are indicated. The enzyme recognition sequences are underlined for *Hph*I or double underlined for *Rsa*I, and the nucleotide position of a restriction cut is indicated by an open (*Hph*I) or a filled (*Rsa*I) triangle. Exon/intron boundaries are indicated by arrows and exon and intron sequences are represented by upper and lower case letters, respectively.

corresponding to the generated 3' fragment of the hGH-V cDNA and the 164 bp band corresponding to the restriction digestion generated 5' hCS-L fragment, comigrate and cannot be distinguished by ethidium bromide staining. Fragments were transferred to nitrocellulose and probed with an oligonucleotide complementary to sequences within the 207 bp hCS-A and -B, the 87 bp hGH-V and the 164 bp hCS-L fragments (*Figure 17 and Table 7*). The 163 bp hGH-V and the 43 bp hCS-A, -B and -L fragments are not detectable by this oligonucleotide. The revealed 87 bp hGH-V and 164 bp hCS-L fragments indicate very low hGH-V and even lower hCS-L mRNA levels in this tissue (*Figure 18B*). The 207 bp band diagnostic for the presence of hCS-A and -B RNA shows, as expected, very strong hybridization. A degree of biological variation was observed in normal term placenta samples with hCS-A/-B representing 94.5-95.8% and hGH-V representing 3.2-4.7% of the total hGH/hCS RNA.

II. HGH/CS RNA can be detected by RT-PCR in samples of hydatidiform mole and choriocarcinoma cell lines.

The predicted 250 bp fragment was generated by the RT-PCR reaction when total RNAs from three hydatidiform mole samples (HM-1, -2 and -3) and three choriocarcinoma cell lines (BeWo, JAR and JEG-3) were examined for the presence of hCS/hGH-V transcripts (*Figure 19A*). A 341-343 bp band present in PCR reactions in the absence of reverse transcription (*Figure 19B*, lanes 2-10) is the result of genomic DNA contamination of the RNA samples since it corresponds to the predicted 341-343 bp band generated when human genomic DNA is used as a template for the PCR reaction

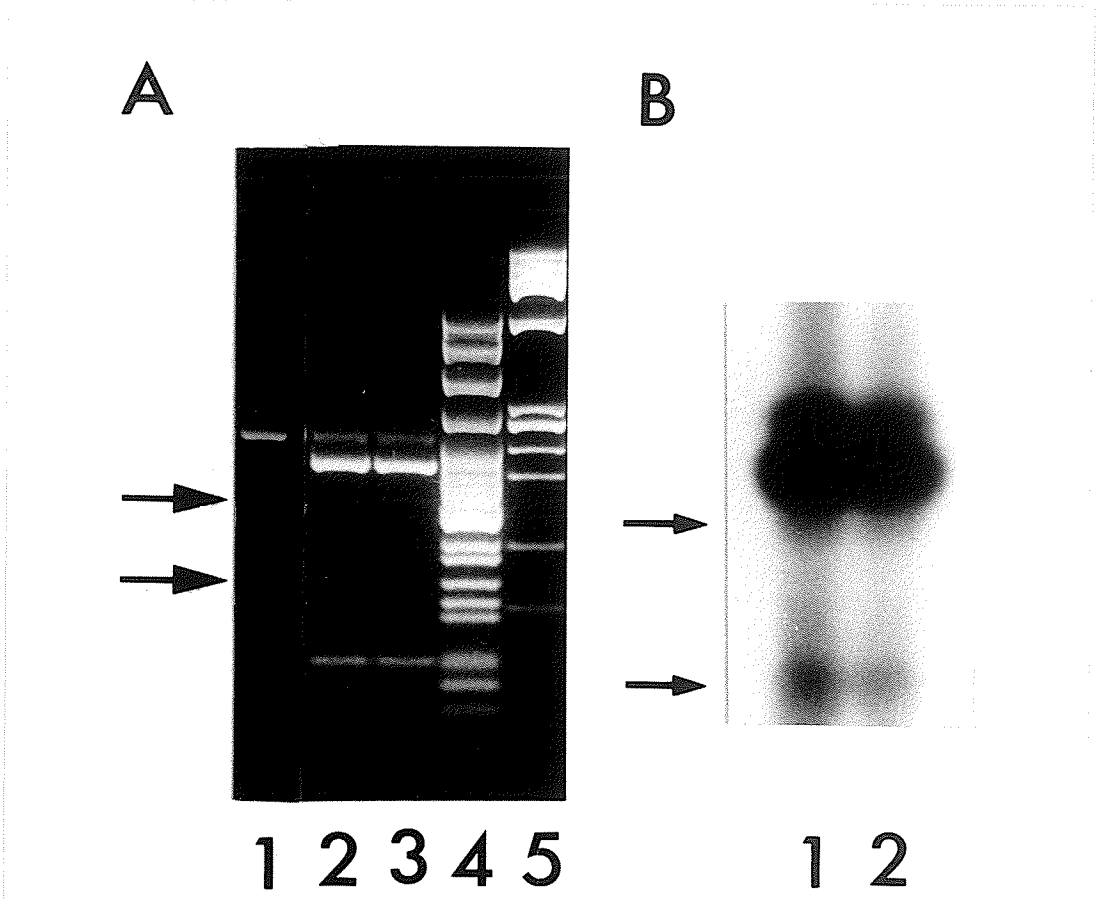


FIGURE 18: Detection of hCS, hCS-L and hGH-V RNA in human term placenta. (A) The predicted 250 fragment specific for the hCS/hGH cDNAs (see *Table 7*) was generated by the RT-PCR reaction when total RNA from human placenta was used as the template (lane 1). Restriction digestion of the 250 bp fragment with *Hph*I generated a pattern consistent with the expression of hCS (hCS-A and -B) genes in human placenta (lanes 2 and 3). Two bands, 207 and 43 bp corresponding to the digested hCS 250 bp fragments are clearly visible. Weaker 164/163 and 87 bp bands are also visible (small arrows). The 163 bp band corresponding to the generated 3' fragment of the hGH-V cDNA and the 163 bp band corresponding to the restriction digestion generated 5' hCS-L fragment co-migrate and cannot be distinguished by ethidium bromide staining. Lane 4, pBR322/*Hpa*I molecular weight markers; lane 5, Φx174/*Hae*III molecular weight markers. (B) Fragments were transferred to nitrocellulose and probed with an oligonucleotide complementary to sequences within the 207 bp hCS-A and -B, the 87 bp hGH-V and the 163 bp hCS-L fragments (see *Figure 17 and Table 7*). The 163 bp hGH-V and the 43 bp hCS-A, -B and -L fragments are not detectable by this oligonucleotide. The revealed 87 bp hGH-V and 163 bp hCS-L fragments (lanes 1 and 2) indicate very low hGH-V and even lower hCS-L mRNA levels in this tissue. The 207 bp band diagnostic for the presence of hCS-A and -B RNA shows, as expected, very strong hybridization. Incomplete digestion of the 250 bp band is observed.

(see *Figure 20A*, lane 2). The 341-343 bp fragment spans intron C which explains the difference in size from the RT-PCR generated 250 bp fragment *(Figure 17)*. The 341-343bp fragment is absent from the RT-PCR reactions likely due to competition by the more abundant 250 bp fragment generated by reverse transcription.

III. HCS, hCS-L and hGH-V but not hGH RNA are detected in samples of hydatidiform mole and choriocarcinoma cell lines.

To avoid contributions of contaminating DNA or unprocessed RNA, the 250 bp fragment was excised and digested with *Hph*I to produce different patterns of relative expression for the hGH-V versus the hCS and hCS-L cDNAs (*Figures 20 and 21*). The major products seen from hydatidiform mole samples (HM-1, HM-2 and HM-3; *Figure 21A*, lanes 4, 10 and 12 respectively) as well as choriocarcinoma BeWo (*Figure 20A*, lane 5; *Figure 21A*, lane 6) and JEG-3 (*Figure 21A*, lane 14) cell lines were the 207 and 43 bp bands corresponding to hCS RNA (*Table 7*). By contrast, the major bands detected in JAR cells were 163 and 87 bp (5' restriction digestion generated fragment; *Table 7*) suggesting higher levels of hGH-V than hCS RNA (*Figure 20A, lane 4; 21A, lane 8*).

For greater sensitivity in detecting hGH-V RNA and to determine whether hCS-L RNA was also present in the samples of hydatidiform mole and choriocarcinoma cells, *Hph*I digested RT-PCR product was blotted and probed with an oligonucleotide complementary to exon 3 sequences. This probe hybridizes to the 164 bp hCS-L fragment as well as the 87 bp but not 163 bp hGH-V fragments (*Figure 17 and Table 7*). HGH-V RNA was detected in all hydatidiform mole (*Figure 21B*, lanes 8, 10 and 12)

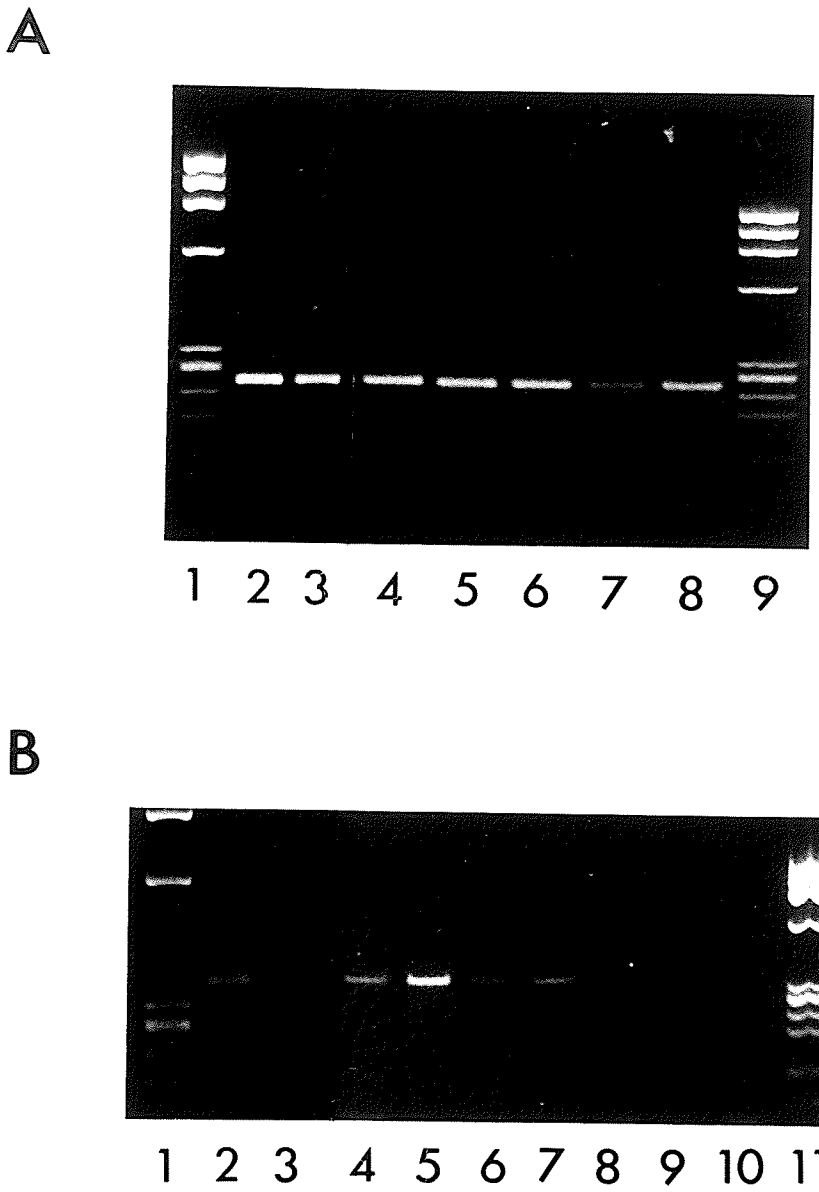


FIGURE 19: Detection of hGH/CS RNA in samples of hydatidiform mole and choriocarcinoma cell lines.

(A) The predicted 250 bp fragment was generated by RT-PCR when total RNAs from hydatidiform mole samples (HM-1, lane 8; HM-2, lanes 2 and 4; HM-3, lane 5) and choriocarcinoma cell lines (BeWo, lane 3; JAR, lane 7; JEG-3, lane 6) were examined for the presence of hCS/GH-V transcripts. Lanes 1 and 9 contain Φx174/*Hae*III molecular weight markers.

(B) A 341-343 bp band present in PCR reactions in the absence of reverse transcription (HM-1, lane 7; HM-2, lane 4; HM-3, lane 2; BeWo, lane 5; JAR, lane 6; JEG-3, lane 3) is the result of genomic DNA contamination of the RNA samples since it corresponds to the predicted 341-343 bp band generated when human genomic DNA is used as a template for the PCR reaction (see *Figure 20A*, lane 2). The 341-343 bp fragment is absent from the RT-PCR reactions likely due to competition by the more abundant 250 bp fragment generated by reverse transcription. Lanes 8-10 represent RT and PCR reaction buffer controls. Lanes 1 and 11 contain Φx174/*Hae*III molecular weight markers. DNA contamination of the RNA samples since it corresponds to the predicted 341-343 bp band generated when HeLa genomic DNA is used as a template for the PCR reaction (*Figure 20A*, lane 2). The 341-343 bp fragment spans intron C which explains the difference in size from the RT-PCR generated 250 bp fragment (*Figure 17*). The 341-343 bp fragment is absent from the RT-PCR reactions likely due to competition by the more abundant 250 bp fragment generated by reverse transcription.

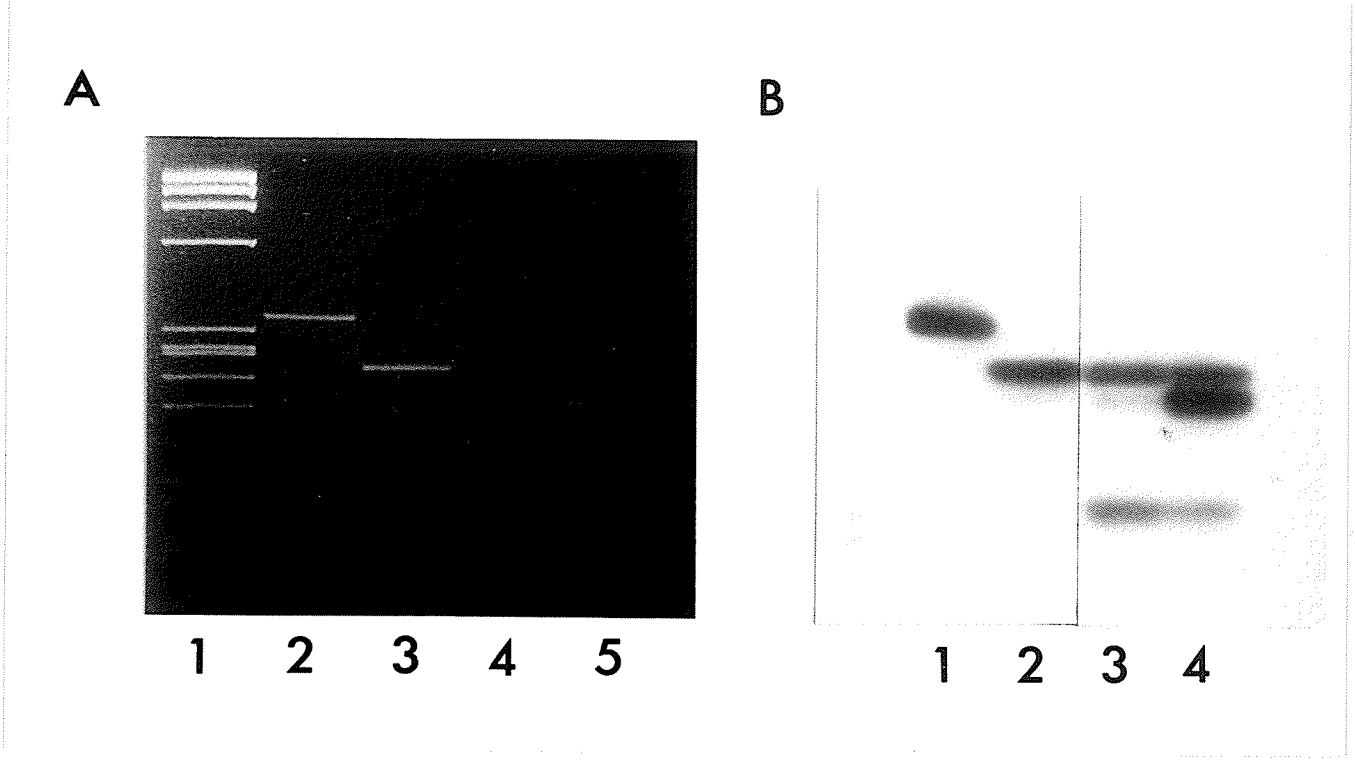


FIGURE 20: (A) PCR reactions using the hGH/hCS specific primers with human (HeLa cervical carcinoma) DNA as the template generated a 341-343 bp fragment (lane 2). The 341-343 bp fragment spans intron C (see *Figure 17*) which explains the difference in size from a BeWo derived RT-PCR generated 250 bp fragment (lane 3). After *Hph*I digestion the major products seen from BeWo choriocarcinoma cell lines (lane 5) was the 207 band corresponding to hCS RNA. The major bands detected in JAR cells were 163 and 87 bp suggesting higher levels of hGH-V than hCS RNA (lane 4). Lane 1 contains Φx174/*Hae*III molecular weight markers (B) The *Hph*I digested RT-PCR products were blotted and probed with an oligonucleotide complementary to exon 3 sequences. This probe hybridizes to the 207 bp hCS-A/hCS-B fragment and the 164 bp hCS-L fragment as well as the 87 bp but not 163 bp hGH-V fragments and also (see *Figure 17 and Table 7*). HCS-A/CS-B and hGH-V hybridization is observed in both samples (JAR, lane 3; BeWo, lane 4) with higher relative levels of hCS present in BeWo cells but higher hGH-V than hCS levels in JAR cells. A hybridization pattern (164 bp band) consistent with the presence of hCS-L in either sample was not observed even after prolonged exposures.

and choriocarcinoma cell samples (*Figure 20B*, lanes 3 and 4; *Figure 21B*, lanes 4, 6 and 14) and showed variability in relative levels (in hydatidiform moles hGH-V represented <2%, in BeWo cells 14.8-25.8%, in JAR cells 72.5-83.3% and in JEG-3 cells 20.7-36.8% of the total hGH/CS RNA). In contrast, hCS-L was detected in all three hydatidiform mole samples but not in any of the choriocarcinomas even after prolonged autoradiographic exposures. Also, with the exception of hydatidiform mole sample HM-2, hGH-V RNA levels were greater or equal to those of hCS-L.

IV. HGH RNA is not detected in hydatidiform moles and choriocarcinoma cell lines.

Direct assessment of hGH RNA expression is allowed by *Rsa*I digestion of the 250 bp RT-PCR generated fragment. Digestion of the 250 bp hGH fragment with *Rsa*I would generate two fragments of 189 and 59 bp while cutting the 250 bp hCS-L fragment would generate 167 and 81 bp fragments (*Table 7*). No *Rsa*I sites are present in the 250 bp fragments generated by the hCS-A, hCS-B and hGH-V transcripts (*Table 7*). HGH RNA was not detected with the hGH/CS exon 3 probe in samples from either hydatidiform mole or choriocarcinoma cell lines (*Figure 21B*).

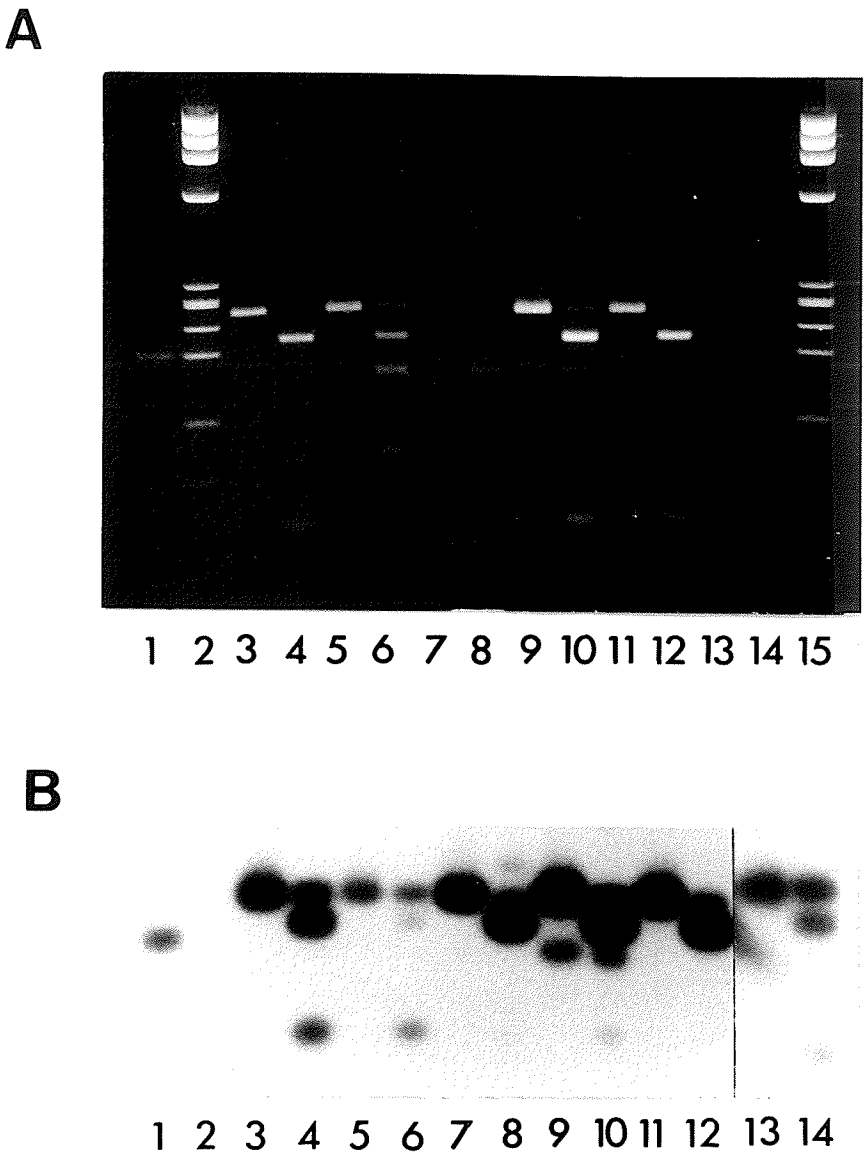


FIGURE 21. The PCR-generated 250 bp fragments were digested with *Hph*I and *Rsa*I to produce different patterns (see *Table 7*) for each of the hGH/hCS cDNAs. (A) After *Hph*I digestion the major products seen from hydatidiform mole samples (HM-1, HM-2 and HM-3, lanes 4, 10 and 12, respectively) as well as choriocarcinoma BeWo and JEG-3 cell lines (lanes 6 and 14, respectively) were the 207 and 43 bp bands corresponding to hCS RNA. The major bands detected in JAR cells were 163 and 87 bp suggesting higher levels of hGH-V than hCS RNA (lane 8). *Rsa*I digestion of the 250 bp RT-PCR derived product from HM-2 (lane 9) generated the 168 and 82 bp fragments predicted for hCS-L. The 190 and 60 bp bands predicted for the hGH RT-PCR product were not detectable by ethidium bromide staining in any of the hydatidiform mole or choriocarcinoma cells samples. No *Rsa*I sites are present in the 250 bp fragments generated by the hCS and hGH-V transcripts. Lanes 2 and 15; Φx174/*Hae*III molecular weight markers. (B) The *Hph*I and *Rsa*I digested RT-PCR products were blotted and probed with an oligonucleotide complementary to exon 3 sequences. This probe hybridizes to the 164 bp hCS-L fragment as well as the 87 bp but not 163 bp hGH-V fragments and also to the 207 bp but not the 43 bp hCS fragments.(see *Figure 17 and Table 7*). HCS hybridization is observed in all samples. HGH-V hybridization is detected in the choriocarcinoma cell samples (BeWo, lane 4; JAR, lane 6 and JEG-3, lane 14) and in hydatidiform mole samples HM-2 (lane 10) and HM-1 (lane 8). HGH-V hybridization can also be detected in HM-3 (lane 12) after longer exposure (not shown). HCS-L hybridization is detected in hydatidiform mole sample HM-2 (lane 10) and in HM-3 and HM-1 after prolonged autoradiographic exposure (not shown). The hybridization pattern (190 bp band) that is generated by *Rsa*I digestion of the 250 bp fragment produced by amplification of an sfRamos generated hGH cDNA (lane 1) was not detected with the hGH/CS exon 3 probe in samples from either hydatidiform mole or choriocarcinoma cell lines (lanes 3,5,7,9,11,13). Thus a certain degree of incomplete digestion that is observed after *Hph*I digestion in all samples tested should not be attributed to the presence of hGH RT-PCR products. The hybridization pattern (168 bp band) detected in HM-2 sample (lane 9) is consistent with the detection of hCS-L.

V. Intra- and inter-assay variability of the RT-PCR and restriction digestion analysis for the measurement of the relative levels of individual members of the hGH family

To assess intra- and inter-assay variability of the determination of relative hGH/CS levels by RT-PCR and restriction digestion, 1 μg quantities of the same BeWo RNA sample were reverse transcribed and amplified in 3 separate assays and the relative densitometric values of the hGH-V and hCS-A/B bands determined (*Figure 22*).

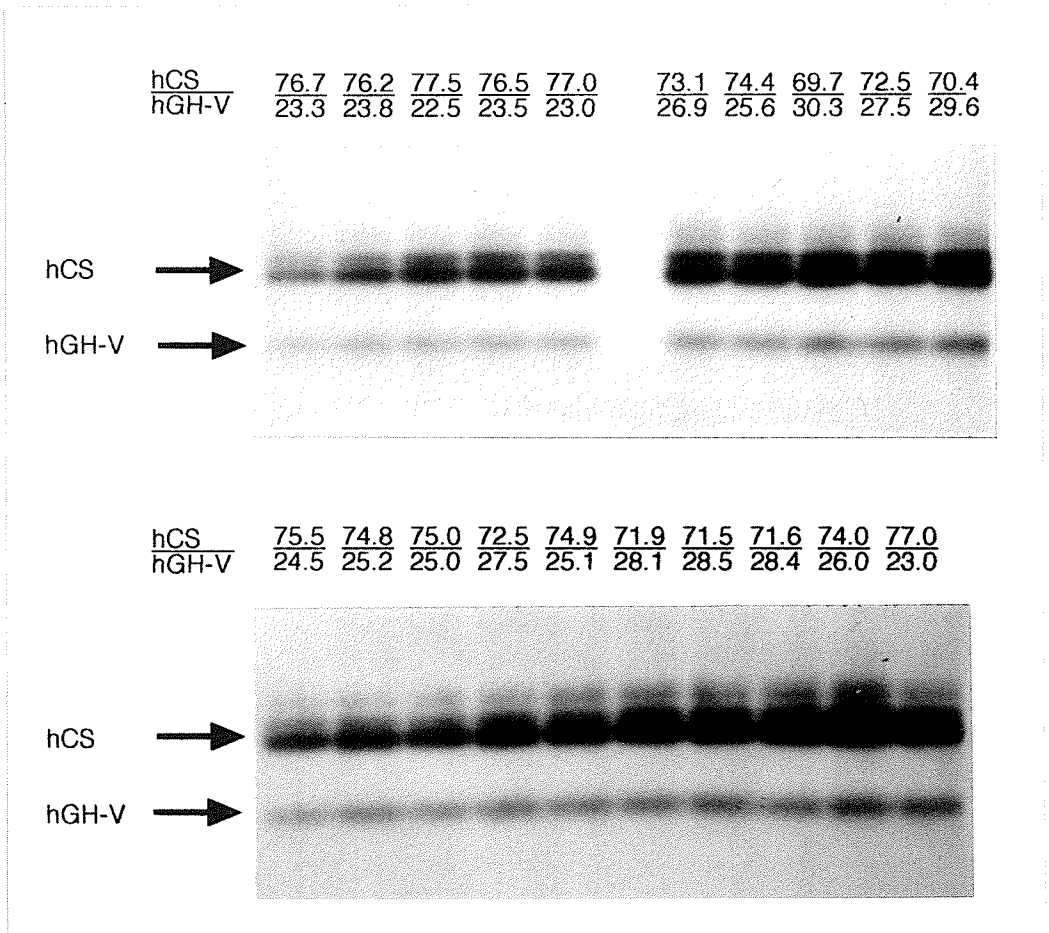


FIGURE 22: Assessment of the intra- and inter-assay variability of the RT-PCR and restriction digestion analysis. BeWo mRNA (1 μg quantities of the same sample) were reverse transcribed and amplified in 3 separate assays. Relative densitometric values of the hGH-V and hCS bands are indicated at the top of each lane. The intra-assay coefficient of variation was 7.2% or better for hGH-V and 2.6% or better for hCS-A/-B. The inter-assay coefficient of variation was 9.3% for hGH-V and 3.2% for hCS.

C. Characterization of regulatory elements in the hCS-B enhancer region

A conclusion derived from the RT-PCR analysis in cells of placental origin is that the expression of hCS (hCS-A/B) is impaired to a greater extent than the expression of hGH-V or hCS-L in placental abnormalities particularly in choriocarcinomas. Since hCS represents the primary placenta product it may be hypothesized that factors absent or defective in placental abnormalities control the gene expression of the hCS-A and hCS-B genes. A variety of factors could account for defective gene expression including transcription factors as well as factors involved in RNA processing and mRNA stability. However, extreme differences between the expression of a gene in two different cell subpopulations (such as the difference between the hCS expression in normal and abnormal trophoblasts) likely reflect involvement of tissue-specific restrictive or activating transcriptional mechanisms. Since tissue specific gene expression determines differentiation and reflects the functional competence of a tissue cell population, impairment of placenta-specific gene expression may also reflect the impairment of pathways and related transcription factors involved in the development and differentiation of the placental tissue.

To investigate the presence and explore the mechanisms of such placenta-specific transcriptional pathways, I pursued studies on the placenta-specific expression of the hCS-B gene and specifically on the identification and characterization of 3' flanking DNA elements of this gene. A 1022 base pair (bp) region of 3' flanking DNA located 2.2 kilobases (kb) downstream of the hCS-B gene, was shown to direct placenta-specific expression *(506,497)*. Fragments of this 1022 bp region, designated nucleotides (nts) 1-1022, were tested for enhancer activation of a heterologous simian

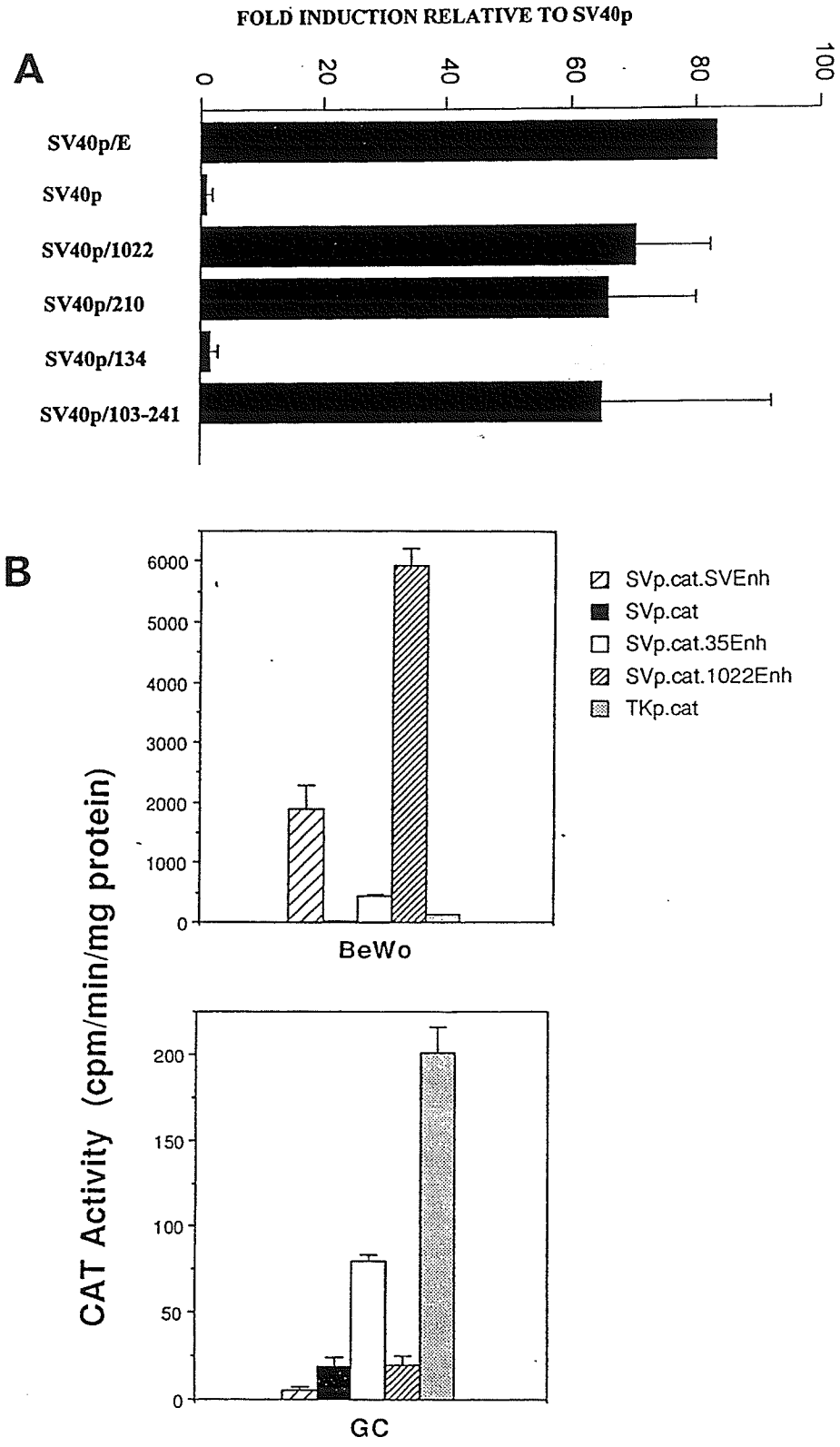


FIGURE 23: Fragments of a 1022 bp 3' region of the hCS-B gene, designated nucleotides (nts) 1-1022, were tested for enhancer activation of a heterologous simian virus 40 (SV40) promoter in placental JEG-3 and pituitary GC cells. **(A)** Fragments spanning nts 1-210 and 103-241 retained 94% and 93% of the enhancing activity of the full length 1022 bp sequence, respectively. A fragment containing nts 1-134 retained only 2.4% of the enhancing activity of the 1022 bp sequence.*[Results from Walker et al (507)]* **(B)** Comparison of the activity of a 35 bp TEF-1 element with the activity of the full length 1022 bp enhancer in BeWo and GC cells *(Results from Nickel and Cattini, unpublished).*

virus 40 (SV40) promoter in human choriocarcinoma JEG-3 cells *(507, Figure 23A)*. Fragments spanning nts 1-210 and 103-241 retained 94% and 93% of the enhancing activity of the full length 1022 bp sequence, respectively *(507)*. In addition, a fragment containing nts 1-134 retained only 2.4% of the activity of the 1022 bp sequence.

Based on these results (*Figure 23A*) the enhancing activity was localized within a 138 bp fragment corresponding to nts 103-241 of the 1022 bp sequence. A single DNA-protein interaction spanning 22 bp (5'-TAATTAGACTGGAATGTTGTCCA-3'), and attributed to a factor termed TEF-1 *(536,537)*, was demonstrated in the region 1-210 at nts 117-139 by DNAase I protection experiments *(507)*.

Introduction of the 22 bp TEF-1 element 3' of hybrid chloramphenicol acetyl transferase (CAT) genes followed by transfection of placental and non placental cell lines, resulted in a "loose" tissue-specificity and reduced enhancer activity compared to the 1022 bp fragment *(507,497, Nickel and Cattini unpublished results, Figure 23B)*. Although it enhanced hCS promoter activity preferentially in placental cells, the 22 bp element also stimulated promoter activity in non placental cells *(497)*. Further the 22 bp element showed substantially lower enhancing activity compared to the 1022 bp fragment in placental cells *(507,497, Nickel and Cattini unpublished results, Figure 23B)*.

These data led to the **hypothesis** that additional sequences within the 1022 bp fragment are responsible for the tight control of the placental-specific expression, permitting and augmenting the enhancing activity of the 22 bp TEF-1 enhancer element exclusively in placental cells. The **objective** of the following studies was a detailed characterization of the 3' enhancer region and the identification of DNA elements and transcription factors that interact with these DNA elements and contribute to

placenta-specific expression.

The region 1-134 was shown by Walker *et al (507)* to retain only 2.4 % of the enhancing activity of the 1022 bp fragment, but was generated by digestion within the DNAase I protected region. Since this, presumably, disrupted the TEF-1 site (nts 117-139), I questioned whether sequences within this fragment might have some functional importance which would be dependent on an intact TEF-1 element. If this were the case, disruption of TEF-1 resulting from the digestion used to generate the 1-134 fragment would "mask" any possible effects of sequences within this fragment on transcriptional activation.

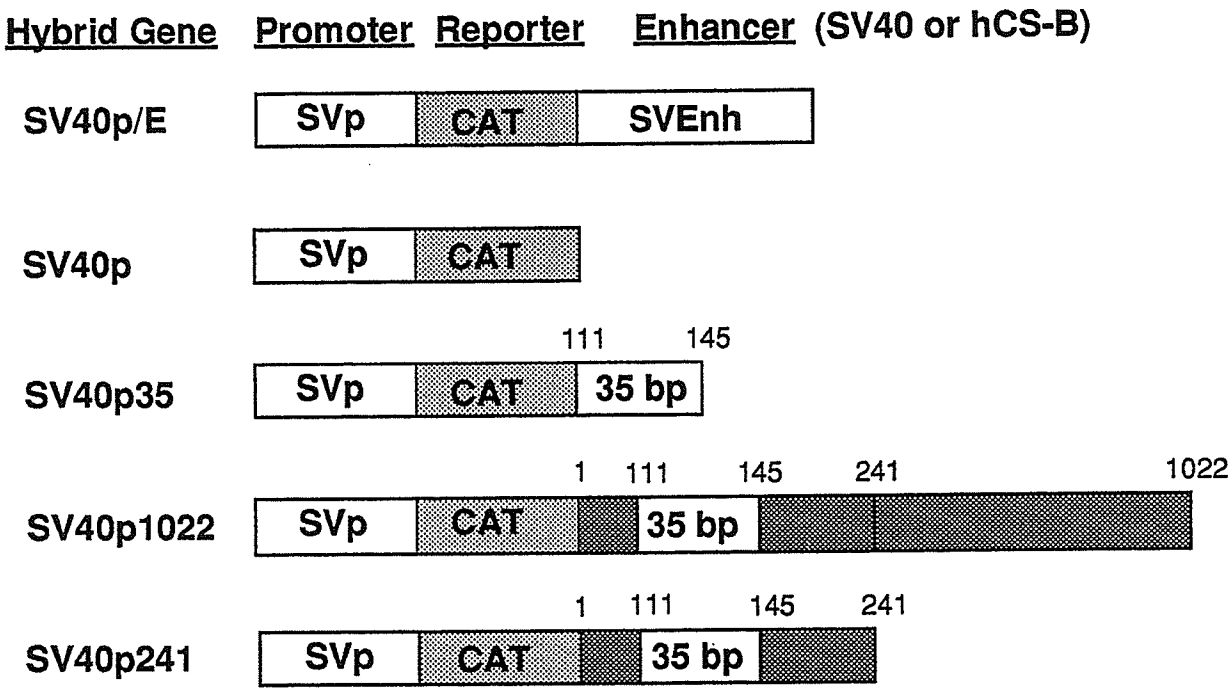


FIGURE 24: Hybrid CAT genes directed by the SV40 promoter used to assess the function of hCS-B gene 3' flanking sequences in placental and non-placental cells. The 3' flanking sequences of the hCS-B gene tested include: (i) a 35 bp fragment containing the 22 bp TEF-1 enhancer element described by Walker *et al*; (ii) a 1022 bp fragment which was shown to exhibit placenta-specific enhancing activity; and (iii) a 241 bp region of the 1022 bp fragment that includes the 22 bp TEF-1 element. An additional construct, SV40p/E, directed by SV40 promoter (p) and containing the SV40 enhancer (E) was used as a positive control.

I. A 241 bp fragment (nts 1-241) that includes the TEF-1 element has the same placenta enhancing activity as the 1022 bp region (nts 1-1022).

Hybrid CAT reporter genes directed by the SV40 promoter were used to assess the function of hCS-B gene 3' flanking sequences in human placental JEG-3 cells. The 3' flanking sequences of the hCS-B gene tested include: (a) a 35 bp fragment including nts 111-145 and containing the 22 bp TEF-1 enhancer element; (b) the complete 1022 bp fragment and (c) the 241 bp fragment spanning nts 1-241 of the 1022 bp fragment, which includes the 22 bp TEF-1 element (*Figure 24*). An additional construct containing the SV40 promoter and enhancer but no hCS-B sequences was used as a positive control. Hybrid CAT gene activity was assessed after transfection of JEG-3 cells (*Figure 25*). The enhancing activity of 241 bp fragment that contains the previously described 22 bp TEF-1 enhancer element was 7.4-fold higher (325- versus 44-fold induction; $P<0.0001$, $n=18$ and $n=9$ respectively) than that seen with the synthetic 35 bp oligonucleotide that contains TEF-1 alone. This increased stimulation with the 241 bp versus the 35 bp fragment was also seen when the hCS-A promoter (-496/+1) was used (*Nickel and Cattini, unpublished observations*). In contrast, there was no difference between the activity of the 241 bp fragment and the larger 1022 bp fragment of 3' hCS-B sequences (325- versus 332-fold induction of the basal SV40 promoter activity; $P>0.05$, $n=18$ and $n=9$ respectively). The same pattern was obtained after transfection of human placental choriocarcinoma BeWo cells (*not shown*).

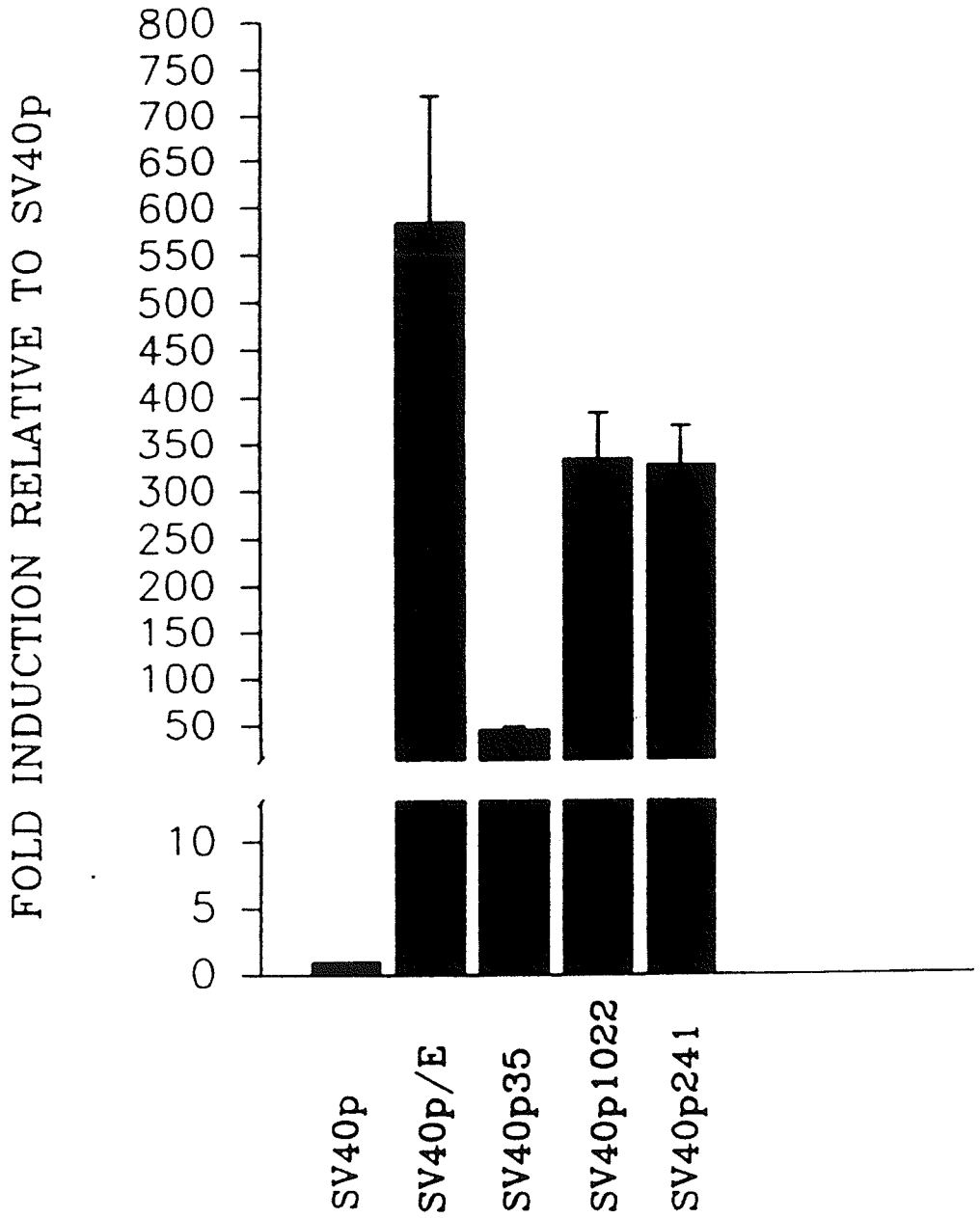


FIGURE 25: The transcriptional activities of hybrid reporter (CAT) genes directed by the SV40 promoter (*see Figure 24*) were assessed after transfection of placental JEG-3 cells. CAT activity from at least 9 determinations is expressed as fold stimulation over basal SV40 promoter activity. The enhancing activity of the 241 bp fragment that contains the previously described 22 bp TEF-1 enhancer element is 7.4-fold higher than the activity of a synthetic 35 bp oligonucleotide that contains the 22 bp element alone. There was no difference between the effect of the 241 and 1022 bp 3' hCS-B sequences on SV40 promoter activity.

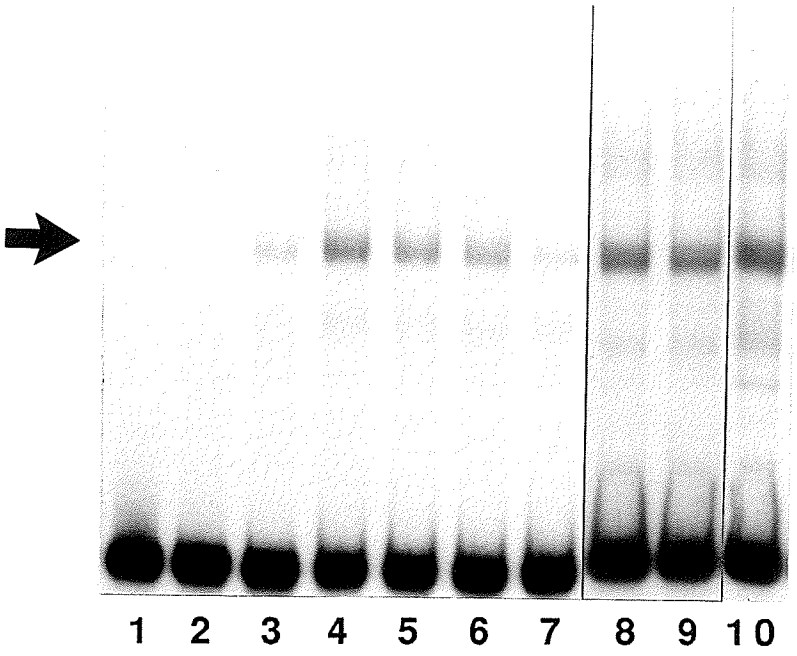
II. Placental protein-DNA interactions occur within sequences of the wild type 241 bp fragment distinct from the 22 bp enhancer motif.

Since DNAase I protection experiments had failed to demonstrate DNA-protein interactions other than TEF-1 in the 1-210 region *(507)*, I pursued the identification of new interactions by the use of gel mobility shift assays. For our analysis we used a subfragment of the 1-210 region that does not contain the TEF-1 element (nts 117-139) and, thus, would allow the identification of protein-binding DNA sequences distinct from the TEF-1 enhancer. Gel mobility shift assays were performed with a fragment that includes only the 1-80 (*Acc*I-*Nsi*I) region. Increasing amounts (0-8.3 μg) of JEG-3 heparin-agarose fractionated nuclear extracts were incubated with the 1-80 region in the presence of 2 μg poly dI-dC (*Figure 26*). A major complex was evident with higher levels of protein. The formation of this complex is competed by increasing amounts (10-60 ng) of an unlabelled 62 bp fragment (nts 13-74) of the 80 bp region (lanes 5-7) but not by 1 μg of double stranded synthetic oligonucleotides corresponding to either the D8 or the Pit-1/GHF-1 binding sites in the 5'-flanking of the human prolactin *(538)* or growth hormone gene *(509)*, respectively.

III. The 22 bp TEF-1 element interacts with factors that participate in complexes formed on DNA elements within the 1-80 bp region.

To test for a possible interaction between the 1-80 region and TEF-1, the principal enhancer element within the 241 bp fragment, a *Hind*III-*Nsi*I fragment

FIGURE 26: Gel mobility shift assays performed with a radiolabelled 100 bp *Hind*III-*Nsi*I fragment that includes only the 1-80 bp (*Acc*I-*Nsi*I) region (part of the 241 bp fragment) that does not contain the 22 bp TEF-1 enhancer element. Increasing amounts of JEG-3 placenta choriocarcinoma heparin-agarose fractionated nuclear extracts (lane 1, free probe; 2, 2.3 μg; 3, 4.2 μg; 4, 8.3 μg protein) were incubated with the 100 bp fragment in the presence of 2 μg poly dI-dC. A major complex is evident with larger amounts of protein (arrow). The formation of this complex is competed by a 62 bp region corresponding to nts 13-74 of the 1-80 fragment (lanes 5-7, 8.3 μg protein, 2 μg poly dI-dC; lane 5, 10 ng; 6, 20 ng; 7, 60 ng specific competitor) but not by 1 μg of non related double stranded synthetic oligonucleotides corresponding to either the D8 or the Pit-1/GHF-1 binding sites in the 5'-flanking of the human prolactin or growth hormone gene, respectively (lane 8, 8.3 μg of protein, 2 μg poly dI-dC, 1 μg D8 oligonucleotide competitor; lane 9, 8.3 μg of protein, 2 μg poly dI-dC, 1 μg Pit-1/GHF-1 oligonucleotide competitor). Lane 10, control lane for lanes 8 and 9 (8.3 μg of protein, 2 μg poly dI-dC).



containing only the 1-80 bp region was radiolabelled and incubated with JEG-3 (*Figure 27*, lanes 1-2) and HeLa (lanes 3-4) nuclear extracts in the absence (lanes 1, 3) or presence (lanes 2, 4) of 1 μg of a 35 bp synthetic double stranded oligonucleotide. This oligonucleotide contains the 22 bp TEF-1 enhancer element found in the hCS-A and -B 3'-flanking sequences *(507,497)*. The presence of the 35 bp TEF-1 oligonucleotide strongly inhibited the formation of the major complex in the presence of JEG-3 nuclear

extracts. Lower complexes were relatively unaffected but an additional faint higher complex was also inhibited. Although relatively high amounts of competitor were used the effect was specific since oligonucleotides corresponding to either the D8 or the Pit-1/GHF-1 binding sites *(538,509)* failed to inhibit the formation of the major complex (see *Figure 26*, lanes 8-10). Due to the presence of TEF-1 binding activity in HeLa cells *(507)* we performed the same competition experiment in the presence of HeLa nuclear extracts. A different binding pattern was obtained with HeLa extracts and inhibition of the formation of a slowly migrating complex by the 35 bp oligonucleotide was observed (*Figure 27*). Other HeLa complexes were either moderately modified or unaffected by the addition of the 35 bp TEF-1 oligonucleotide.

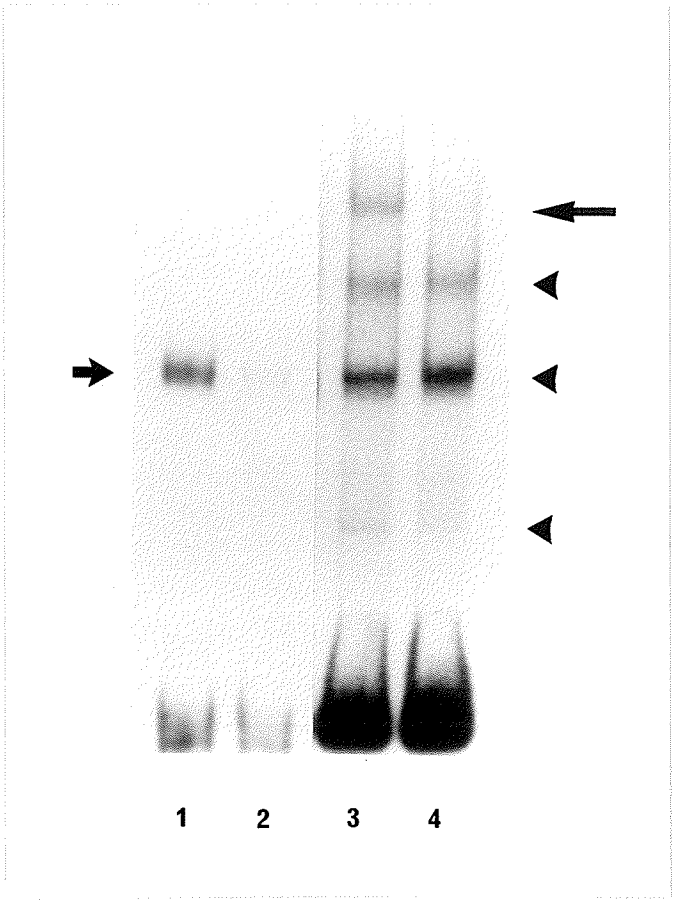


FIGURE 27: The 22 bp TEF-1 element competes with elements within the 1-80 bp *Acc*I-*Nsi*I fragment for certain DNA-protein interactions. radiolabelled 100 bp *Hind*III-*Nsi*I fragments containing the 1-80 bp region were incubated with JEG-3 (lane 1-2) and HeLa (lanes 3-4) nuclear extracts in the absence (lanes 1, 3) or presence (lanes 2, 4) of a 35 bp synthetic double stranded oligonucleotide that contains the 22 bp TEF-1 enhancer element. The major complex formed in the presence of JEG-3 nuclear extracts was inhibited by 1 μg of TEF-1 oligonucleotide competitor (lane 2, left arrow) by not by 1 μg of double stranded synthetic oligonucleotides corresponding to either the D8 or the Pit-1/GHF-1 binding sites in the 5'-flanking of the human prolactin or growth hormone gene, respectively (see *Figure 26*, lanes 8-10). Inhibition of a slowly migrating complex formation by the 35 bp oligonucleotide was observed in the presence of HeLa nuclear extracts (lane 4, right arrow). Other HeLa complexes were either moderately reduced or unaffected by the addition of the 35 bp oligonucleotide (arrowheads).

IV. Modification of nts 63-67 (DF-1 site) eliminates the enhancer function in placental cells, while disruption of nts 24-28 (RF-1 site) stimulates promoter activity in placental and non placental cells.

The interaction between the 1-80 bp and TEF-1 regions (*Figure 27*) suggested that factors associated with the 1-80 bp region play an important role in placental enhancer function. Sequence analysis of the 1-80 bp region revealed similarities with the p sequences *(76,509)* that are present 2 kb upstream of the promoters of all four placental members of the growth hormone gene family, but not upstream of the pituitary growth hormone gene. We showed that regions of these sequences (PSF-A and PSF-B) repress hCS-A promoter function in pituitary cells providing a mechanism for a pituitary-specific repression of the otherwise active hCS-A promoter *(509)*. Two sequences, within the 1-80 bp region, that we term as the RF-1 and DF-1 regions, represent a PSF-A like sequence and a sequence found immediately downstream to the PSF-B site, respectively. Both sequences are present in the reverse orientation within the 1-80 bp region *(Figure 28A)*. In order to examine the functional significance of these sequences for enhancer activity, 5 bp substitutions were made in the 241 bp enhancer fragment in the DF-1 (nts 63-67) and RF-1 (nts 24-28) regions by polymerase chain reaction site-directed mutagenesis *(Figure 28B)*. The DF-1 site mutation eliminated the enhancer activity (97.5% or 39-fold reduction, $P<0.0001$, $n=18$) of the wild type 241 bp fragment in JEG-3 cells *(Figure 29A)*. The remaining activity was also 5.3-fold lower ($P<0.0001$, $n=18$ and $n=9$, respectively) than the enhancing activity of the 35 bp that includes TEF-1 alone. In contrast, modification of the RF-1 site showed a statistically significant

potentiating effect on the enhancer activity of the 240 bp fragment (615- versus 325-fold increase of the basal SV40 promoter activity, $P<0.0001$, $n=15$ and $n=18$ respectively) in JEG-3 cells (*Figure 29A*). The same pattern was obtained after transfection of BeWo placental cells although the differences between the RF-1 site mutated and the wild type 241 bp fragments (316- versus 218-fold, $P>0.05$, $n=15$ and $n=14$, respectively) as well

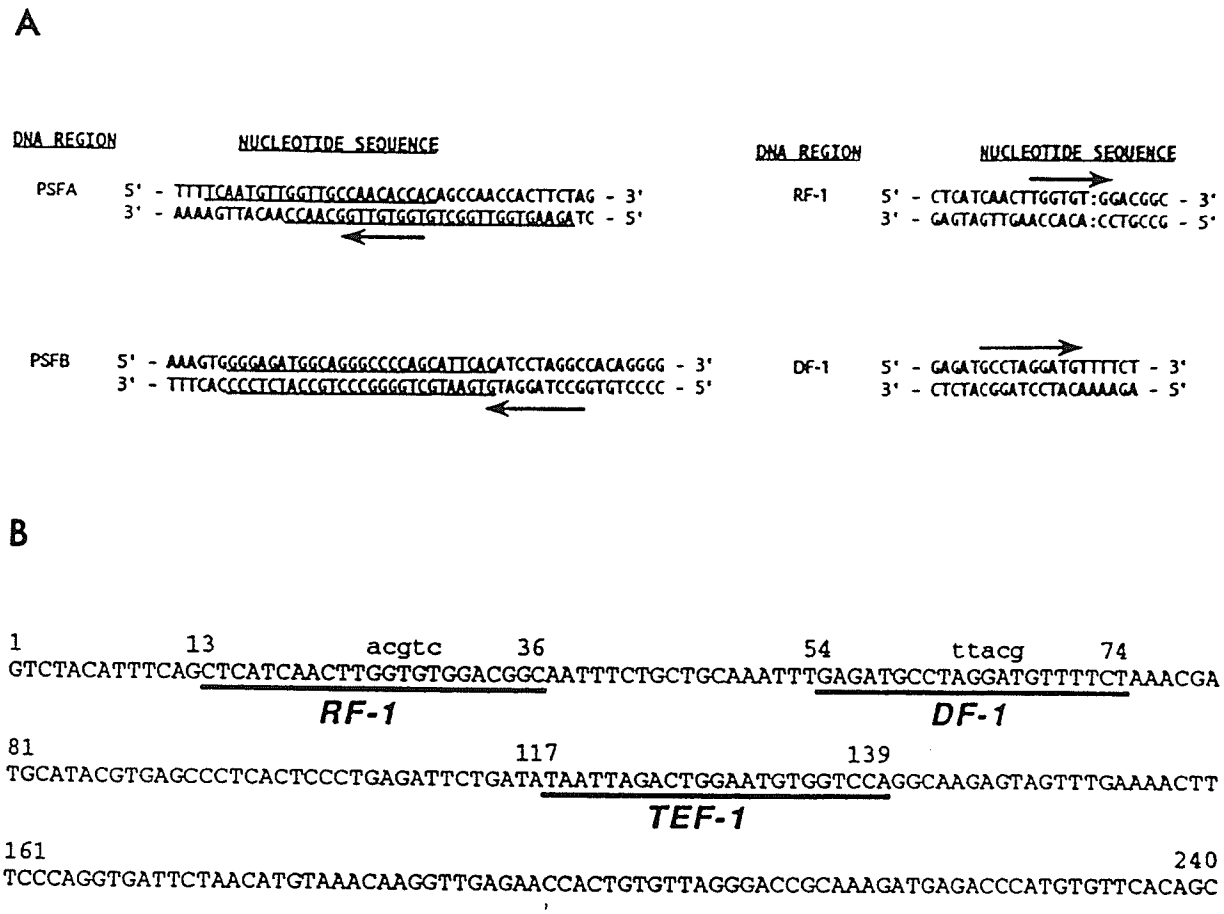
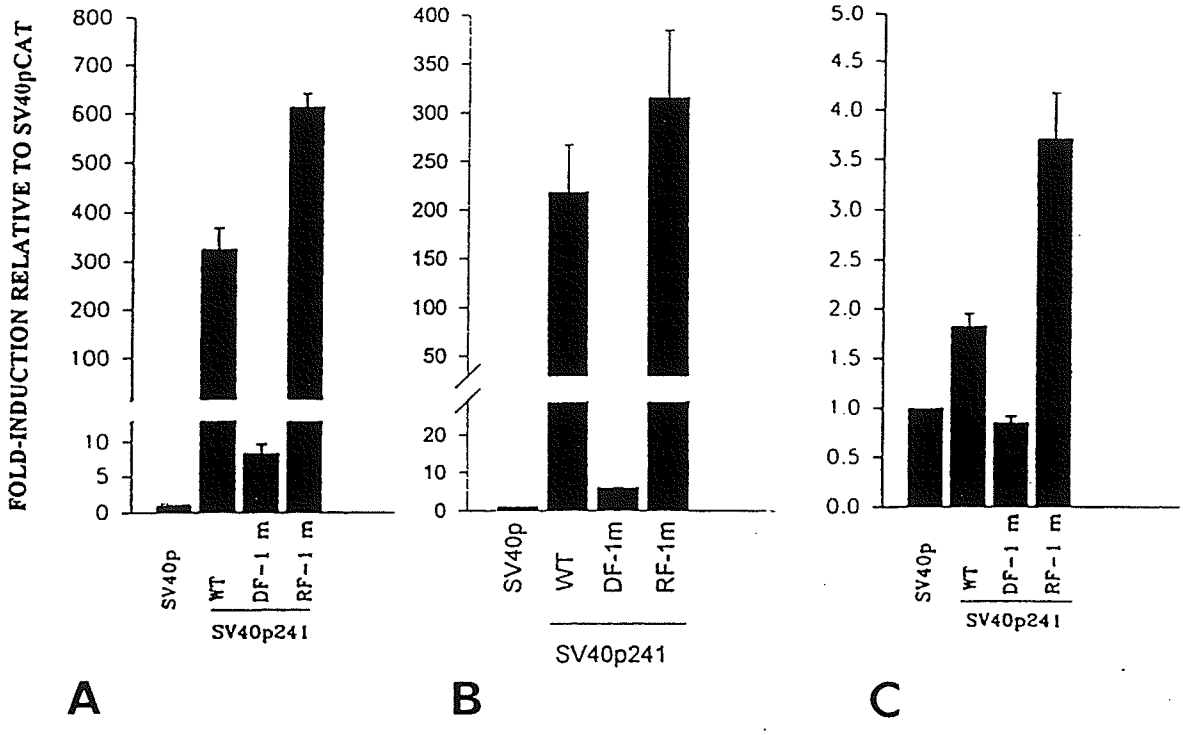


FIGURE 28: (A) Comparison of the p sequences with sequences (RF-1 and DF-1 sites) in the 1-80 bp fragment. Underlined are the regions (PSF-A and PSF-B) identified by DNAase I protection assays. All sequences are shown in their natural orientation in the hGH gene family locus. The 5' to 3' direction of the upper strands match the 5' to 3' direction of the genes. Arrows indicate the orientation of and span the homologous regions within the p sequences and the 1-80 bp region. The RF-1 and DF-1 sites are placed in the reverse orientation relative to the PSF-A and PSF-B sites. Positions and orientations of PSF-A and -B within the hGH gene locus are described in *Figure 40*. (B) Sequence map of the 241 bp enhancer region and introduction of mutations to the RF-1 and DF-1 regions. The TEF-1, DF-1 and RF-1 sequences are underlined. Mutations were introduced by PCR site-directed mutagenesis (*Materials and Methods*). Nucleotide substitutions introduced in the DF-1 and RF-1 regions are indicated with small letters. Position and orientation of the 241 bp hCS-B enhancer region within the hGH gene locus are shown in *Figure 40*.

FIGURE 29: Effect of mutations on the enhancer activity of the 241 bp fragment in placental JEG-3 (A) and BeWo (B), as well as, cervical tumor HeLa cells (C). The DF-1 and RF-1 sites in the 1-241bp fragment were modified with 5 bp substitutions by PCR site-directed mutagenesis *(Figure 3)*. Wild type and mutated 241 bp fragments were tested downstream of a hybrid CAT gene directed by the SV40 promoter. CAT activity is expressed as fold stimulation over basal SV40 promoter activity. (A),(B) Disruption of the DF-1 site dramatically reduced the enhancer activity of the wild type 241 bp fragment in placental cells. (C) The wild type 241 bp fragment induced a 82% increase of the basal SV40 promoter activity but RF-1 site mutated 241 bp fragment increased the basal SV40 promoter activity after transfection of HeLa cells by 3.7 fold.



as between the DF-1 site mutated 241 bp fragment and the 35 bp TEF-1 element alone (6- versus 16-fold, $P<0.05$, $n=12$ and $n=8$ respectively) were not statistically significant at the 0.01 level (not shown).

In non placental HeLa cells (*Figure 29B*), the wild type 241 bp fragment caused a small (82%, $P<0.0001$, $n=12$) increase and the DF-1 site mutated 241 bp fragment a slight, but not statistically significant at the 0.01 level, decrease (15.5%, $P<0.05$, $n=12$) of the basal SV40 promoter activity. By contrast, the RF-1 site mutated 241 bp fragment induced a 3.7-fold increase of the basal SV40 promoter activity ($P<0.0001$, $n=12$).

V. Disruption of DF-1 site within the 1-80 bp fragment modifies placental protein-DNA interactions.

To investigate the mechanism by which the DF-1 site mutation affected enhancer activity and whether this mutation disrupts binding events occurring on the larger 1-80 bp fragment, gel mobility shift assays were performed with radiolabelled fragments containing only the 1-80 bp region (*Figure 30*). The wild type (lanes 1-4) and DF-1 site mutated (lanes 5-8) probes were used (*Figure 30*). Increasing amounts of JEG-3 crude nuclear extracts (0-16 μg protein) were incubated with the 1-80 bp region in the presence

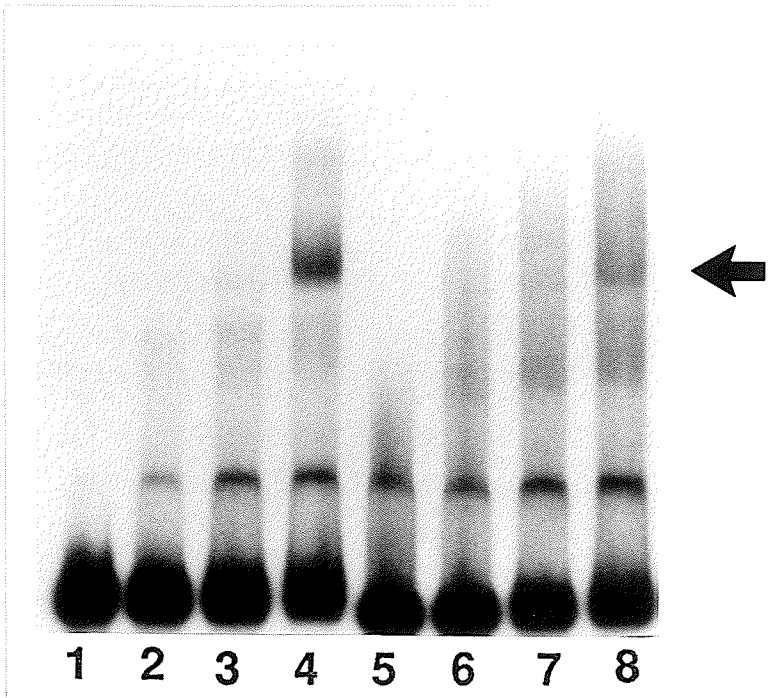


FIGURE 30: Gel mobility shift assay of radiolabelled wild type or mutated 1-80 bp fragments with placental JEG-3 nuclear extracts. A 100 bp fragment containing the wild type (lanes 1-4) or DF-1 site mutated (lanes 5-8) 1-80 bp region was incubated with increasing amounts of crude JEG-3 nuclear extracts (lanes 1 and 5, free probe; lanes 2 and 6, 4 μg; lanes 3 and 7, 10 μg; lanes 4 and 8, 16 μg protein) in the presence of 2 μg poly dI-dC. The major complex that is evident with 16 μg of protein after incubation with the wild type probe (lane 4; arrow) is reduced when the same amount of protein was incubated with the mutated probe (lane 8).

of 2 μg poly dI-dC. The major complex formed after incubation of the wild type probe with 16 μg of protein (lane 4) is reduced when the mutated probe was incubated with the same amount of protein (lane 8). A relative increase in the formation of the lower complexes was also observed (*Figure 30*).

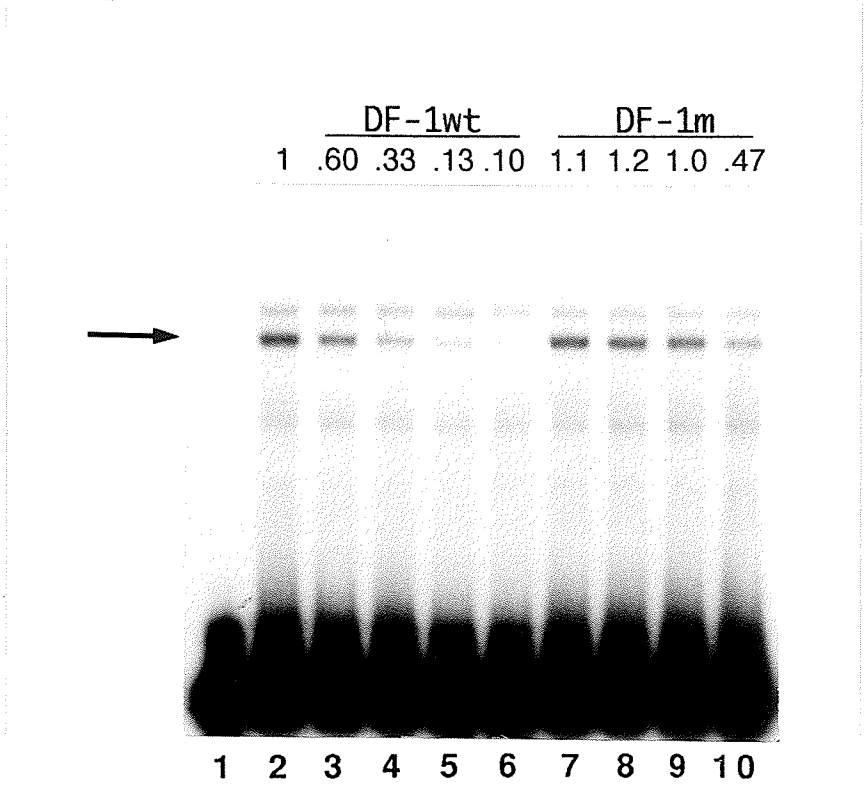
VI. Oligonucleotides that span the DF-1 and RF-1 regions interact with placental or non placental nuclear proteins.

A synthetic oligonucleotide (nts 54-74) spanning the DF-1 region of the 1-80 bp fragment was labelled and incubated with JEG-3 nuclear extracts in the presence of 2 μg of poly dI-dC (*Figure 31 and 32A*). The major complex could be effectively competed by 1.5-250 ng of unlabelled DF-1 oligonucleotides (*Figure 31 and 32A*).

A mutated DF-1 oligonucleotide bearing the same substitutions that were introduced in the 241 bp was also used for competition experiments. The mutated DF-1 oligonucleotide was a much weaker competitor than the wild type oligonucleotide for complexes generated with the DF-1 wild type labelled oligonucleotide (*Figure 31 and 32B*). Densitometric analysis of gel mobility shift experiments in the presence of 1.5-50 ng of unlabelled competitors showed that the DF-1 mutated oligonucleotide was a 20-fold weaker competitor relative to the wild type oligonucleotide (*Figure 31*).

Interestingly, effective competition (*Figure 32C*) was also obtained by 25 ng and background levels by 250 ng of an oligonucleotide spanning the RF-1 region (nts 13-35). In contrast, only weak competition for a fainter lower mobility complex was observed with the 35 bp TEF-1 oligonucleotide or with greater than 6 μg of poly dI-dC.

FIGURE 31: A synthetic oligonucleotide (nts 54-74) spanning the DF-1 region of the 1-80 bp fragment was labelled and incubated with JEG-3 nuclear extracts in the presence of 2 μg of poly dI-dC (*Figure 31 and 32A*). Lane 1, no extract. The major complex (arrow, lane 2) could be effectively competed by 1.5-50 ng of unlabelled DF-1 oligonucleotides (lane 3, 1.5 ng; lane 4, 5 ng; lane 5, 15 ng; lane 6, 50 ng). A mutated DF-1 oligonucleotide bearing the same substitutions that were introduced in the 241 bp was also used for competition (lane 7, 1.5 ng; lane 8, 5 ng; lane 9, 15 ng; lane 10, 50 ng). The mutated DF-1 oligonucleotide was a much weaker competitor than the wild type oligonucleotide for complexes generated with the DF-1 wild type labelled oligonucleotide. Densitometric analysis (relative densitometric values of the major complexes are indicated at the top of the gel) revealed that the DF-1 mutated oligonucleotide was a 20-fold weaker competitor relative to the wild type oligonucleotide.



A radiolabelled DF-1 oligonucleotide was also incubated with HeLa nuclear extracts in the presence of 2 μg of poly dI-dC and increasing amounts of wild type or mutated DF-1 oligonucleotide competitors. The major complex seen with JEG-3 nuclear extracts (see arrow *Figure 32C*) was not present with HeLa nuclear extracts and, although two faint complexes appeared to be competed by the wild type but not by the mutant competitor, the DF-1 interactions with HeLa nuclear proteins seemed weak and rather not specific (*Figure 32D*).

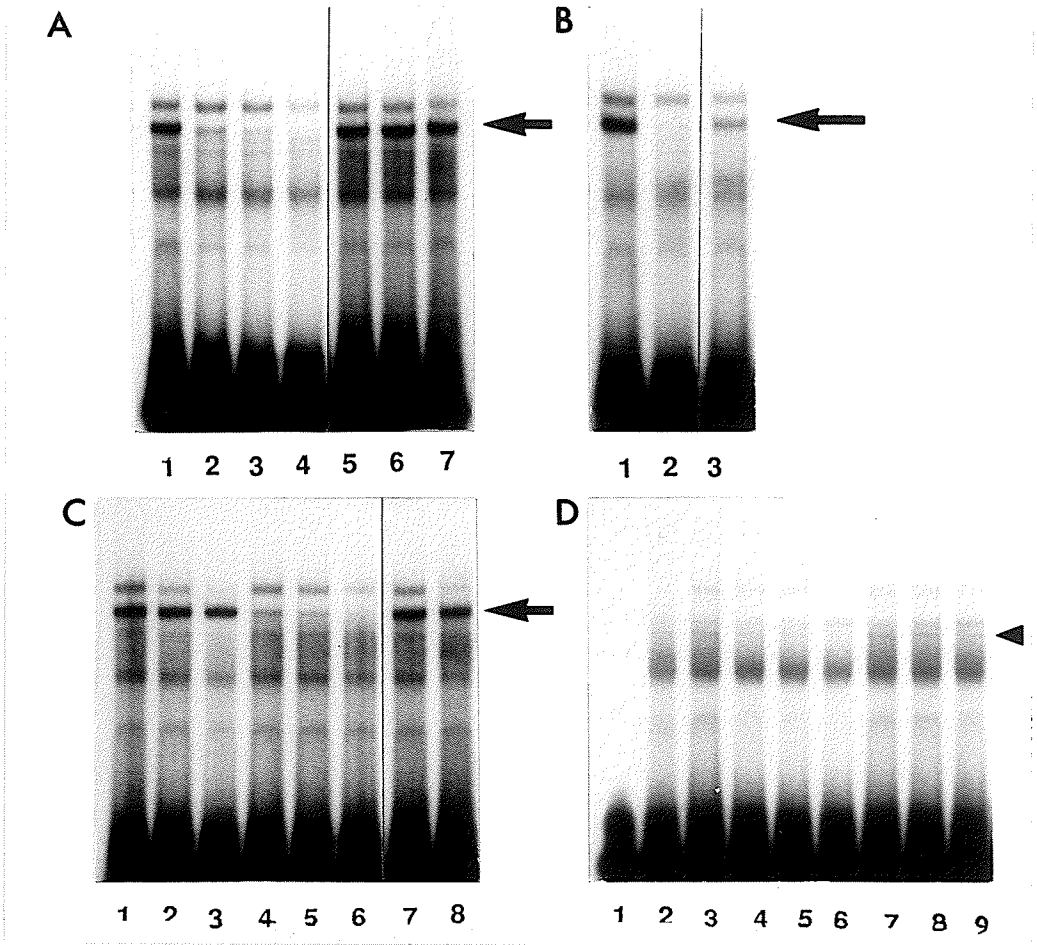


FIGURE 32: Characterization of DF-1 binding activities in placental and non placental nuclear extracts. (A) A 21 bp double stranded ^{32}P-end-labelled oligonucleotide (nts 54-75 of the 1-80 bp region) spanning the DF-1 region was incubated with 20 μg JEG-3 nuclear extracts in the presence of 2 μg of poly dI-dC. No specific competitor (lane 1), increasing amounts of DF-1 unlabelled oligonucleotide competitor (lane 2, 25 ng; lane 3, 50 ng; lane 4, 250 ng) or increasing amounts of TEF-1 unlabelled oligonucleotide competitor (lane 5, 25 ng; lane 6, 50 ng; lane 7, 250 ng) were used. A specific complex (arrow) is competed to background levels by the DF-1 oligonucleotide competitor. The upper complex is also competed but not as effectively by the DF-1 oligonucleotide competitor. (B) The ^{32}P-end-labelled DF-1 oligonucleotide was incubated with 20 μg of JEG-3 nuclear extracts in the presence of 2 μg of poly dI-dC. Wild type and mutated DF-1 oligonucleotides (100 ng) were used in competition assays. The DF-1 mutant oligonucleotide (lane 3) was a much weaker competitor for the prominent complex (arrow) than the DF-1 wild type oligonucleotide (lane 2). Densitometric analysis of competition assays using increasing (1.5-50 ng) amounts of wild type or mutant competitors (not shown) revealed a 20-fold difference in the efficiency of the two oligonucleotides to compete for the prominent complex. (C) The ^{32}P-end-labelled DF-1 oligonucleotide was incubated with 20 μg of JEG-3 nuclear extracts in the presence of increasing amounts of poly dI-dC (lane 1, 2 μg; lane 2, 6 μg; lane 3, 10 μg), RF-1 oligonucleotide competitor (lane 4, 50 ng; lane 5, 100 ng; lane 6, 250 ng) or TEF-1 oligonucleotide competitor (lane 7, 100 ng; lane 8, 1 μg). The prominent complex (arrow) is unaffected by 10 μg of poly dI-dC or by 1 μg of TEF-1 oligonucleotide but is effectively competed by 50 ng of RF-1 oligonucleotide. Competition for the upper complex is observed with 250 ng of RF-1 or 1 μg of TEF-1 oligonucleotides and, to a lesser extent, with 6 μg of poly dI-dC. (D) The ^{32}P-labelled DF-1 oligonucleotide was incubated with 10 μg (lane 2) and 20 μg (lanes 3-9) of

When a RF-1 oligonucleotide was radiolabelled and incubated with JEG-3 nuclear extracts a major complex migrating similarly to the one generated by the DF-1 oligonucleotide was observed (*Figure 33A*). The formation of this complex was competed by as low as 5 ng and reduced to background levels by 50 ng of unlabelled RF-1 oligonucleotide (*Figure 33A*).

Interestingly, this complex was also competed by 50 ng and reduced to background levels by 250 ng of unlabelled DF-1 oligonucleotide but not by 1 μg of a TEF-1 oligonucleotide (*Figure 33B*). A DF-1 mutated oligonucleotide was a 5-fold weaker competitor than the wild type oligonucleotide (*Figure 33B*).

Incubation of radiolabelled RF-1 oligonucleotide with HeLa nuclear extracts revealed a pattern of interactions different from the pattern generated with JEG-3 nuclear extracts (*Figure 33C*). Competition experiments were performed using the wild type or a mutated (bearing the same modifications as in the RF-1 site mutated 241 bp fragment) RF-1 unlabelled oligonucleotide in the presence of HeLa nuclear extracts. A DNA-protein interaction was competed (*Figure 33C*) by 5-100 ng of the wild type (lanes 3-6) but not by 5-100 ng of the mutated RF-1 oligonucleotide (lanes 7-10).

No competition for RF-1 complexes with HeLa nuclear proteins was observed with increasing amounts of the wild type DF-1 (50-250 ng), the mutated DF-1 (50-250 ng) or the TEF-1 (100-500 ng) unlabelled oligonucleotides (*Figure 33D*).

FIGURE 32 (Cont.)
HeLa nuclear extracts in the presence of 2 μg of poly dI-dC. Increasing amounts of wild type or mutated DF-1 oligonucleotide competitor (lane 4, 15 ng; lane 5, 50 ng; lane 6, 250 ng of wild type DF-1 oligonucleotide competitor and lane 7, 15 ng; lane 8, 50 ng; lane 9, 250 ng of mutant oligonucleotide competitor). The major complex seen with JEG-3 nuclear extracts (see arrow *Figure 32C*) was not present with HeLa nuclear extracts and although a faint complex (arrowhead) as well as the upper complex appear to be moderately competed by the wild type but not the mutant competitor, the DF-1 interactions with HeLa nuclear proteins seem weak and rather not specific.

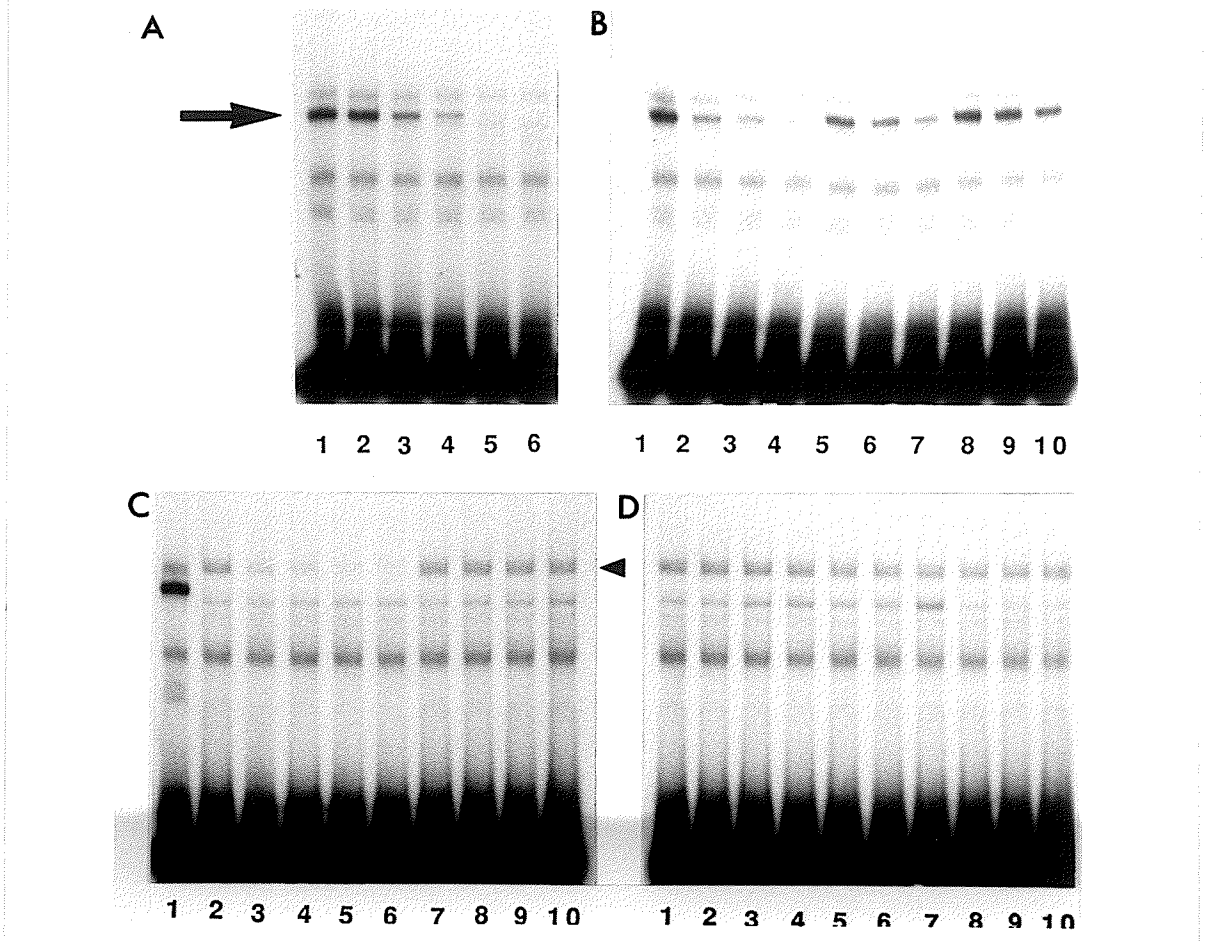


FIGURE 33: Characterization of RF-1 binding activities in placental and non placental nuclear extracts. **(A)** JEG-3 (lane 1, 10 μg, lane 2-6, 20 μg) nuclear extracts were incubated with a 23 bp double stranded ^{32}P-end labelled oligonucleotide (nts 13-35) that spans the RF-1 region. The prominent complex (arrow) is effectively competed by increasing amounts of RF-1 unlabelled oligonucleotide competitor (lane 3, 5 ng; lane 4, 15 ng; lane 5, 50 ng; lane 6, 100 ng). The upper complex is competed but much less effectively by the RF-1 oligonucleotide competitor. As determined by densitometric analysis of experiments using 5-100 ng of competitors, a mutated RF-1 oligonucleotide could also compete for the prominent complex but 2-fold less effectively than the wild type competitor. **(B)** JEG-3 (lane 1--10, 20 μg) nuclear extracts were incubated with the ^{32}P-end labelled RF-1 oligonucleotide. The prominent JEG-3/RF-1 complex could be effectively competed with increasing amounts of DF-1 unlabelled oligonucleotide (lane 2, 50 ng; lane 3, 100 ng; lane 4, 250 ng). A mutated DF-1 oligonucleotide (lane 5, 50 ng; lane 6, 100 ng; lane 7, 250 ng) was able to compete for this complex but at least 5-fold less effectively than the wild type oligonucleotide as determined by densitometric analysis. A TEF-1 oligonucleotide (lane 8, 100 ng; lane 9, 250 ng; lane 10, 1 μg) moderately reduced the formation of the prominent complex at the highest amount used. **(C)** JEG-3 (lane 1, 20 μg) and HeLa (lanes 2-10, 20 μg) nuclear extracts were incubated with the ^{32}P-end labelled RF-1 oligonucleotide. The prominent complex (left arrow) that is formed in the presence of JEG-3 nuclear extracts appears specific for this tissue since it is absent in the presence of HeLa nuclear extracts. Competition of HeLa complexes with increasing amounts of wild type (lane 3, 5 ng; lane 4, 15 ng; lane 5, 50 ng; lane 6, 100 ng) and mutated RF-1 oligonucleotides (lane 7, 5 ng; lane 8, 15 ng; lane 9, 50 ng; lane 10, 100 ng) revealed that mutated competitors were unable to compete for a complex (right arrow) that was reduced to background levels by wild type

VII. A DF-1 oligonucleotide has no direct effect on SV40 promoter activity.

15

The 21 bp DF-1 oligonucleotide was introduced in the forward orientation 3' of a hybrid CAT gene directed by the SV40 promoter. A slight and not statistically significant increase (22.6%, $P > 0.05$, $n=18$) of the basal SV40 promoter activity by the DF-1 oligonucleotide was observed after transfection of JEG-3 cells (*Figure 34*).

VIII. Introduction of the RF-1 oligonucleotide 3' of hybrid CAT genes has a direct negative effect on TEF-1 and SV40 enhancer activities.

The 23 bp RF-1 oligonucleotide was inserted 3' of a hybrid CAT gene directed by the SV40 promoter and containing the synthetic 22 bp TEF-1 enhancer element inserted also at the 3' end of the CAT gene (see *Figure 24 p. 129*). The RF-1 oligonucleotide was placed in the forward orientation 5' of the TEF-1 element maintaining the 5'-3' positioning of the two elements in the 241 bp enhancer fragment. The RF-1 oligonucleotide was also inserted in the forward orientation 3' of a control CAT hybrid gene containing the SV40 promoter and SV40 enhancer regions (see *Figure 24 p. 129*). The RF-1 oligonucleotide repressed both TEF-1 and SV40 enhancer activities in placental JEG-3 cells (*Figure 35*). The TEF-1 enhancer element increased basal SV40 promoter activity by 41-fold. In the presence of an RF-1 site, the TEF-1 enhancing effect

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FIGURE 33 (Cont.) oligonucleotide.
(D) HeLa nuclear extracts (lanes 1-10, 20 μg) were incubated with the ^{32}P-end labeled RF-1 oligonucleotide. No competition was observed with increasing amounts of a wild type DF-1 unlabelled oligonucleotide (lane 2, 50 ng; lane 3, 100 ng; lane 4, 250 ng), a mutated DF-1 oligonucleotide (lane 5, 50 ng; lane 6, 100 ng; lane 7, 250 ng) or a TEF-1 oligonucleotide (lane 8, 100 ng; lane 9, 250 ng; lane 10, 500 ng).

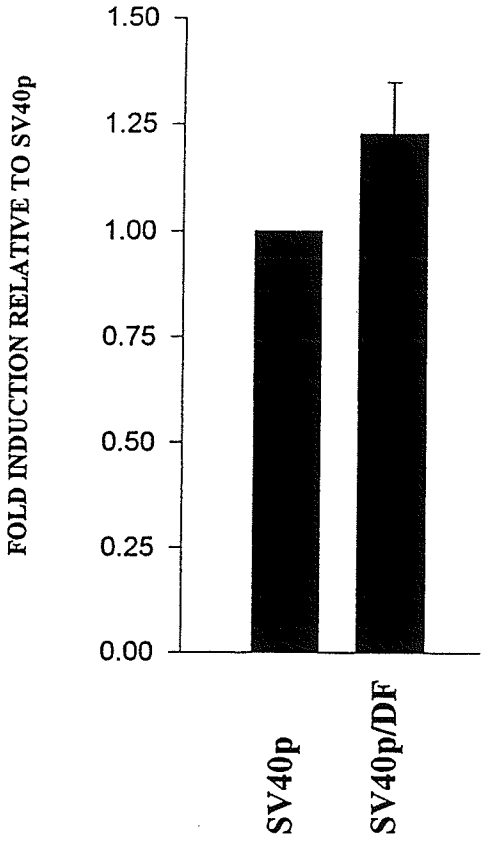

FIGURE 34: The 21 bp DF-1 oligonucleotide was introduced in the forward orientation 3' of a hybrid CAT gene directed by the SV40 promoter. A slight and not statistically significant increase (22.6%, P> 0.05, n=18) of the basal SV40 promoter activity by the DF-1 oligonucleotide was observed after transfection of JEG-3 cells.

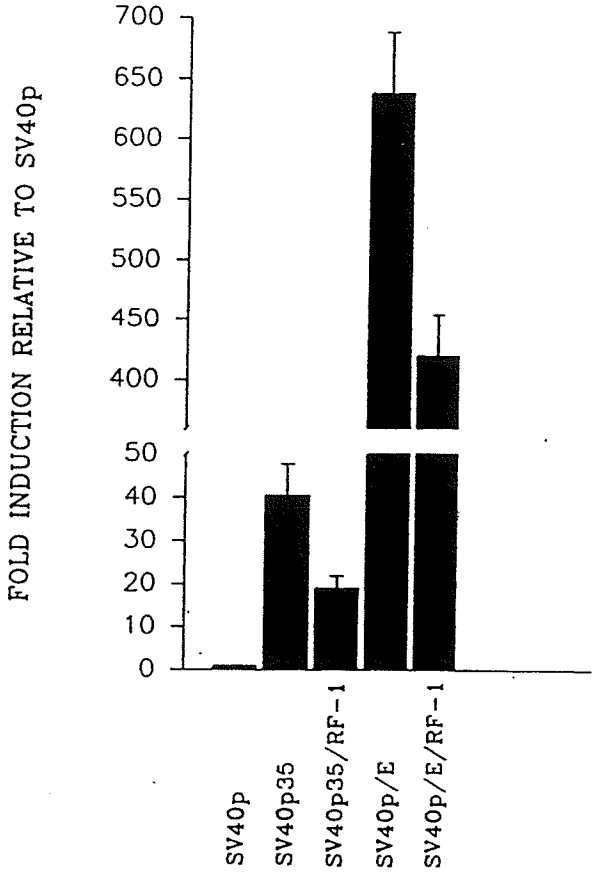

FIGURE 35: Effect of RF-1 on TEF-1 and SV40 enhancer activity. A 23 bp RF-1 oligonucleotide was inserted 3' of a hybrid CAT gene directed by the SV40 promoter and containing the synthetic 22 bp TEF-1 enhancer element inserted also at the 3' end of the CAT gene (see Fig. 1). The RF-1 oligonucleotide was placed in the forward orientation 5' of the TEF-1 element maintaining the 5'-3' positioning of the two elements in the 240 bp enhancer fragment. The RF-1 oligonucleotide was also inserted in the forward orientation 3' of a control CAT hybrid gene containing the SV40 promoter and SV40 enhancer regions (see Figure 24). CAT activity is expressed as fold stimulation over basal SV40 promoter activity. The RF-1 oligonucleotide showed a repressing effect on the function of both the TEF-1 and SV40 enhancers. The TEF-1 enhancer element increased basal SV40 promoter activity by 41-fold. In the presence of the RF-1 oligonucleotide, the TEF-1 enhancing effect on basal SV40 promoter activity was reduced to 19-fold (P< 0.001, n=18). Similarly the SV40 enhancer activity was significantly reduced in the presence of the RF-1 oligonucleotide (638-fold versus 420-fold, P< 0.0001, n=18).

on basal SV40 promoter activity was reduced to 19-fold ($P < 0.001$, $n=18$). Similarly the SV40 enhancer activity was significantly reduced in the presence of the RF-1 oligonucleotide (638- versus 420-fold, $P < 0.0001$, $n=18$).

IX. GC pituitary protein - RF-1 complexes can be competed by unlabelled RF-1, DF-1, PSF-A and PSF-B oligonucleotide competitors.

The identification of the RF-1 oligonucleotide as a repressor and the sequence similarity with PSF-A suggested that pituitary nuclear proteins may also interact with the RF-1 oligonucleotide and that RF-1 binding proteins be also interacting with PSF-A and or PSF-B. Mobility gel shifts of RF-1 radiolabelled oligonucleotides with rat pituitary GC nuclear extracts revealed the presence of a shifted band that could be effectively competed by 50 ng of RF-1 unlabelled oligonucleotide competitor (*Figure 36*). DF-1 could compete for this complex although somewhat less effectively. This was also the case for PSF-A and -B oligonucleotide competitors that effectively competed, at higher amounts (250 ng) for the same RF-1 complex (*Figure 36*).

X. Studies on hCS-B enhancer activity by two independent laboratories confirm the functional significance of DF-1

Simultaneously with a report on the results on hCS-B enhancer activity presented in this thesis [Lytras and Cattini *(539)*], two additional reports on the same subject, appeared in the literature *(540,541)*. Although different promoter systems (homologous hCS promoter, and a variety of heterologous promoters (TK promoter, SV40 promoter)

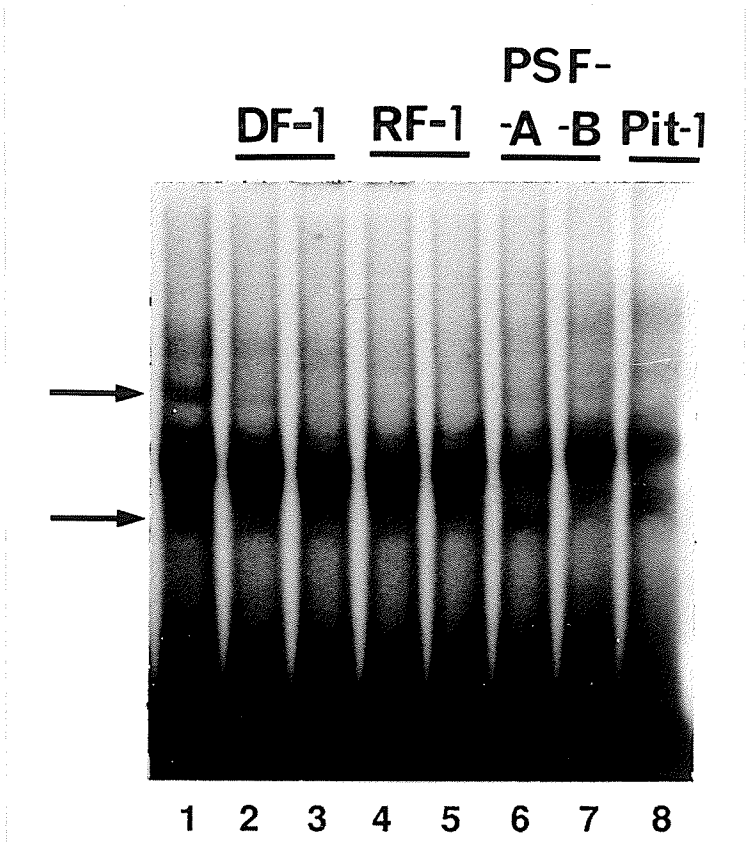

FIGURE 36: RF-1 complexes with GC pituitary nuclear proteins and possible interactions with PSF-A and or PSF-B. GC nuclear extracts (lane 1-8, 22 μg) were incubated with a 23 bp double stranded ^{32}P-end labelled oligonucleotide (nts 13-35) that spans the RF-1 region. A lower mobility complex (arrow) is effectively competed by increasing amounts of RF-1 unlabelled oligonucleotide competitor (lane 4, 50 ng; lane 5, 250 ng) The same complex is competed but less effectively by the DF-1 oligonucleotide competitor (lane 2, 50 ng; lane 3, 250 ng). PSF-A, PSF-B and pit-1 oligonucleotides (lane 6, 7 and 8 respectively, 250 ng) inhibited also the formation of this complex.

were used, almost identical results for the functional importance DF-1, were shared between the three studies. Jacquemin *et al (540)*, however, using a thymidine kinase (TK) promoter system, could also show strong enhancer function (78% of full enhancer activity) for direct repeats of a region (nucleotides 22-104) that included the DF-1 region, but not the TEF-1 region. A single copy of the 22-104 region showed reduced enhancer activity (3-fold, 17% of full enhancer activity) relative to the direct repeats.

Further, Jiang and Eberhardt *(541)* performed a detailed mutational analysis of

the protected regions in the context of the 241 bp CS-B enhancer using the homologous hCS promoter. A summary of the results of this mutational analysis as well as a comparison with our mutational analysis is presented in *Table 9.* Extensive deletional analysis was performed by both groups, that resulted in provocative results (*Table 10*). As the comparisons of these analyses suggest, an enhancing activity, equivalent to the intrinsic enhancing activity of the TEF-1 element when used alone, is present within nucleotides 22-102. This is not inconsistent with the results in this thesis that revealed minimal DF-1 effects on the SV40 promoter when a DF-1 oligonucleotide was used alone

Table 9. Comparison of mutational analyses of the hCS-B enhancer.

PROMOTER	CS (BeWo)		SV40 (JEG-3)	
MUTATED REGION OF 1-241 bp ENHANCER	MUTATED NUCLEOTIDES	% OF FULL ENHANCER ACTIVITY	MUTATED NUCLEOTIDES	% OF FULL ENHANCER ACTIVITY
FP-1: 4-27	5-12 (GT-IIC$^{7/8}$)	56%		
	24-31 (GT-I$^{8/9}$)	NO EFFECT	24-28 (RF-1)	189% (JEG-3) 203% (HeLa) 145% (BeWo/NS)
FP-2: 68-80	70-75 (OCT$^{8/8}$, IR)	0-13%	63-67 (DF-1)	2.5%
	80-85 (OCT$^{5/7}$)	30-35%		
FP-3: 115-140	126-133 (GT-IIC$^{8/9}$, TEF-1)	0%		
FP-4: 145-165	153-158 (Sph-I$^{7/8}$, IR)	51%		
FP-5: 199-239	195-205 (DR)	74% (sense) 11% (as)		
	226-236 (DR)	36%		

Table 10. Summary of deletional analyses of the hCS-B enhancer.*

PROMOTER	CS (BeWo)	TK (JEG-3)		SV40 (JEG-3)	
ENHANCER REGION	% OF FULL ACTIVITY (+1/+242= 12-fold)	ENHANCER REGION	% FULL ACTIVITY (-156/+147 =21-fold)	ENHANCER REGION	% FULL ACTIVITY (1/+241= 325-fold)
103-151 (TEF-1)	0-1%	-156-(+147) (df-1-4)	100%	1/1022	100%
57-242	**22.5%**	-88-(+147) (df-2-4)	78%	1/210 1/241	93.6% 97.9%
1-180	**0-5%**	**22-147** (df-3-4)	**81%**	**1/134**	**2.4%**
57-180	0-1%	-156-(-100) (df-1, x1,x2)	0%	**103/241**	**92.2%**
Δ70-157	0%	-88-(+58) (df-2, x1,x3)	0%	158/1022	0.8%
		22-102 (df-3 /AF-1+DF-1)	**17%**	**54/74** (DF-1)	**0%**
		22-102 (x2)	74%		
		97-147 (df-4 /TEF-1)	**16.7%**	**111/145** (TEF-1)	**13.5%**
		97-147 (x3)	89%		

* A minus numbering for the sequences used in the analysis of Jacquemin *et al (540)* is used relative to the first nucleotide of the 1-241 bp enhancer region.

(*Figure 34*), but raise the possibility that DF-1 may interact with other elements in the 22-104 region to confer enhancer activity that may act synergistically with TEF-1. This is supported by the fact that although, in the analysis of Jacquemin *et al (540)*, TEF-1 (nucleotides 97-147) had an enhancer activity equivalent to 16.7% of the full enhancer activity, when used alone [comparable with the enhancer activity of the 35 bp TEF-1 oligonucleotide (nucleotides 11-145) on the SV40 promoter as determined in this thesis

(13.5% of the full 241 bp enhancer activity)], by similarity to the 22-104 region, 3 copies of the TEF-1 region demonstrated 89% of the full enhancer activity. This suggests that homologous or heterologous enhancer complexes are necessary for full enhancer activity.

The 22-104 enhancer activity must be, presumably, inhibited by sequences overlapping or within nucleotides 1-22 and/or 102-134 since the 1-134 fragment demonstrates 7-fold lower enhancing activity [2.4% of the enhancing activity of the full length 241 bp enhancer *(507)*] than the 22-104 fragment [17% of the enhancing activity of the 241 bp enhancer *(540)*]. Repressor activity may be also present between nucleotides 57-103 as suggested by the comparison of the relative enhancer activity of the 57-242 fragment [22.5% of the enhancing activity of the full length 241 bp enhancer (541), to the relative enhancer activity of the 103-241 fragment (93-95%) *(507 and here in, not shown)*], providing that the difference in enhancing activity between the two fragments is not due to enhancer-promoter specific interactions.

XI. DNAase I protection mapping suggests that the DF-1 region may consist of three distinct and partially overlapping DNA elements

Two related, but distinct, DNAse I protection patterns were revealed by the studies of Jacquemin *et al (540)* and Jiang and Eberhardt *(541)* (*Figure 37*). By coincidence Jacquemin *et al (540)* used the same term (df, standing for ***Distal Footprint***) for the footprints that they identified in the enhancer region. However, our DF-1 region corresponds to the footprint df3 as reported by Jacquemin *et al (540)*, while in that report

FIGURE 37: Summary of DNAse I protection analyses of the hCS-B enhancer. Jacquemin *et al (540)*: df-1, df-2, df-3, df-4. Protected regions identified by Jiang and Eberhardt *(541)*: FP1, FP2, FP-3, FP4, FP5. The positions of RF-1, DF-1 and TEF-1 are indicated.

df1 and df2 correspond to protected regions upstream of the 241 bp enhancer region and df4 corresponds to the TEF-1 region. A shorter, extending from nts 67-83, and a longer, extending from nts 49-87, footprints were identified over the DF-1 region by Jiang and Eberhardt *(541)* and Jacquemin *et al (540)*, respectively. The extended footprint region identified in the latter study *(540)*, that overlaps and flanks the footprint identified by Jiang and Eberhardt *(541)*, implies that the larger protected region of Jacquemin *et al* corresponds to a different complex possibly containing additional, or even different, protein factors. Alternatively, different experimental conditions may correspond to distinct DNA-protein contact profiles; a stronger DNA-protein contact may result in a more extensive protected region.

In contrast, Jiang and Eberhardt that detected a protected region over the RF-1 region, Jacquemin *et al* did not detect such a region. Both groups, however, could detect a DNAse I protected region over the TEF-1 region in agreement with the results of Saunders's group *[Walker et al (507)]*.

By similarity to the results of Walker *et al (507)*, in our initial analysis with JEG-3 nuclear extracts we did not detect any DNAase I protected regions other than TEF-1. This led us, at that time, to use the mobility gel shift assay as a screening method for putative new DNA elements. Following the results of Jacquemin *et al (540)* and Jiang and Eberhardt *(541)*, however, we reexamined the 241 bp enhancer region for DNAase I protected regions. New batches of JEG-3 and BeWo nuclear extracts were tested and DNAase I protection patterns with both similarities and differences with the patterns generated by Jacquemin *et al (540)* and Jiang and Eberhardt *(541)* were observed.

Protection over the RF-1 region was seen only occasionally. Our DF-1 DNAase I

protection patterns were similar to those produced by both Jacquemin *et al (540)* and Jiang and Eberhardt *(541)*. A larger DF-1 protected region (similar to the region identified by Jacquemin *et al*) appeared to be disrupted by bands of relatively increased intensity, observed around the most 5' limit of the FP-2 footprint of Jiang and Eberhardt *(541)*. A clear demonstration of this phenomenon was obtained by footprinting analysis using GC nuclear extracts (*Figure 38*). Multiple protected regions were identified, one extending over nucleotides 17-42 that includes a TEF-$2^{7/8}$ site present within the RF-1 region *(see section E_{VI} in **DISCUSSION** p. 194)*, and partially overlaps with the footprint

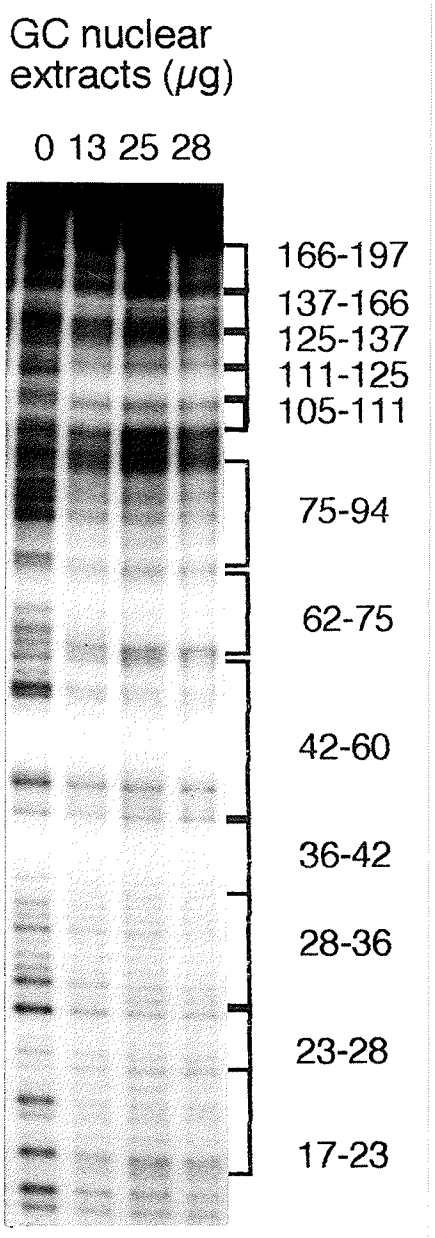

FIGURE 38: DNAase I protection analysis of the 241 bp hCS-B enhancer region. A 3'-end (antisense strand) radiolabelled 241 bp enhancer fragment was incubated on ice with increasing amounts of rat pituitary GC nuclear extracts and subsequently digested partially with DNAase I for 90 seconds at 25°C. Non protected nucleotides are indicated to mark the putative limits of DNAase I protected regions. Two protected regions (nucleotides 42-60 and 62-75) extending over DF-1, border at the upstream end (nucleotide 62) of the $ets^{7/8}$ site (nucleotides 63-70), suggesting that at least two distinct elements may exist within the DF-1 region. Footprints are also observed over RF-1 (nucleotides 17-42) and TEF-1 (nucleotides 105-137) as well as over two downstream regions (nucleotides 137-166 and 166-197). (Figure provided by J. Feng Zhang)

FP-1 (nucleotides 4-27) of Jiang and Eberhardt *(541)*. This protected region appeared to include a number of bands of increased intensity (nucleotides 23,28 and 36) that may represent the limits of distinct protected regions or hypersensitivity due to changes in the conformation of the DNA (such as DNA bending) because of the binding of a single protein.

Two protected regions (nucleotides 42-60 and 62-75) extending over DF-1, border at the upstream end (nucleotide 62) of an $ets^{7/8}$ site (nucleotides 63-70, *see section E_{VI} in DISCUSSION p. 194)*. This is of significance as nucleotides 55-62 represent a sequence similar (7/8 identical nucleotides) to a sequence (termed PSF-B1) present within the DNAase I protected region of PSF-B. In contrast, the 11 bp sequence which is present in the PSF-B region and corresponds to nucleotides 60-70 of DF-1 is unprotected in the context of the P sequences, however, it lays inverted at the downstream border of the protected region, 15 nucleotides from the end of PSF-B1 *(Figure 28A)*. These facts suggest that the DF-1 region (in this instance defined as the region that corresponds to the 21 bp DF-1 oligonucleotide) may contain at least two distinct elements, a $PSF\text{-}B1^{7/8}$ and an $ets^{7/8}$. However the mutational analysis of Jiang and Eberhardt *(541), (Table 9)* suggests that part of a third element (mutated at nucleotides 70-75) that corresponds to both an inverted $Oct^{8/8}$ (nucleotides 74-65) and one of two inverted repeats (nucleotides 70-79 and 159-151) present in the 241 bp enhancer, may exist within this DF-1 region. Interestingly our protected region over the $ets^{7/8}$ site (nucleotides 62-75, 62 and 75 being non protected nucleotides) extends to nucleotide 75 which is coincident with both the 5' end of the inverted $Oct^{8/8}$ and the 3' end of the DF-1 oligonucleotide.

Two additional protected regions (nucleotides 105-137 and 137-166) appear almost

coincident with DF4 (nucleotides 104-139, extends over TEF-1) of Jacquemin *et al (540)* and FP-3 (nucleotides 143-166) of Jiang and Eberhardt *(541)*. Our TEF-1 protected region appears disrupted by bands of increased intensity that may define the limits of distinct smaller protected regions (nucleotides 105-111, 111-125 and 125-137). The most central region (nucleotides 125-137) contains the GTIIC[8/9] TEF-1 element.

DISCUSSION

A. HUMAN GROWTH HORMONE FAMILY PROTEINS AND BASIC SYSTEMS FOR THE STUDY OF THEIR FUNCTIONS

The importance of growth hormone proteins for certain developmental, maturation and differentiation processes has been a controversial issue due to the fact that consistent and reproducible results have been rather difficult to generate. One of the reasons appears to be the plethora of distinct proteins and protein forms that belong to the broader GH-PRL superfamily. The redundancy of GH and PRL proteins for several of their functions and the cross-binding to multiple members of the GH and PRL receptor families increase the difficulty for assessment of the functions of the individual members of these families.

Dissecting the functions of individual GH proteins requires development of appropriate systems to study biological effects and knowledge of the regulatory processes that control the expression of individual genes. The fact that GH genes are expressed in a tissue specific mode may help dissection of their specific functions providing that appropriate systems can be developed for the study of each of these proteins. Significant progress has been achieved using biological systems such as the Nb2 and IM-9 bioassays *(152,308)*, that facilitate the investigation of hormonal effects mediated through lactogenic (PRL-R) or somatogenic (GH-R) receptors. Both systems are extensively used for determining receptor characteristics as well as the study of second messager pathways activated through ligand binding to these receptors. The discovery of many additional receptor forms than the ones expressed in these lymphocyte clones may have somewhat

limited the value of these systems as "screening" methods for the detection of novel GH-like or PRL-like activities, but in contrast has increased their value for the study of the specific effects of proteins that can interact with the particular classes of receptors expressed in Nb2 and IM-9 cells.

B. DETECTION OF GH IN HUMAN LYMPHOCYTES

The first study reported in this thesis was, in fact, based on the use of the Nb2 bioassay as a screening method for the detection of lactogenic hormones, which coupled with the use of monoclonal antibodies indicated the presence of a hGH activity in the conditioned media of sfRamos, an Epstein Barr-negative Burkitt lymphoma cell line. In continuation, I confirmed by all criteria examined (RIA, Western blot, Northern blot, RT-PCR coupled with diagnostic restriction digestion and PCR sequencing), that the hGH-N gene was expressed in this lymphoid cell line. Previously this cell line was reported to secrete a 29K PRL-like peptide, equivalent to 5-10 ng hPRL activity per ml of culture media as estimated by Nb2 assay *(502)*. Human PRL had no effect but anti-hPRL polyclonal antibody inhibited the growth of sfRamos cells. The effect of this antibody was not examined in the Nb2 assay *(502)*.

The data I generated showed that the lactogenic activity present in the conditioned media of these cells (as demonstrated by the mitogenic effect of concentrated conditioned media in the Nb2 assay), was blocked by anti-hGH monoclonal and polyclonal antibodies, but not by anti-hPRL monoclonal or polyclonal antibodies, consistent with a hGH-like rather than hPRL-like lactogenic activity. This was confirmed by RIA that detected hGH but not hPRL immunoreactivity. Immunoprecipitation of concentrated

conditioned medium with polyclonal anti-hGH antibody, followed by Western Blot analysis with a monoclonal anti-hGH antibody, detected a specific 22K band with the same mobility as pituitary hGH. Northern Blot analysis detected a major transcript of 1.0 Kb when a full length hGH cDNA was used as a probe. This transcript corresponds to the predominant 1.0 Kb hGH mRNA in pituitary. A less abundant mRNA, about 1.6 Kb, that can also be detected in human pituitary RNA blots, is observed in sfRamos RNA. It is interesting that this transcript present in very low levels relative to the 1.0 Kb transcript in pituitary RNA, is present at much higher relative levels in sfRamos poly A(+) enriched RNA. Although the 1.6 Kb transcript likely represents a partially processed nuclear transcript the possibility that it represents a hGH related species cannot be excluded. If the 1.6 Kb is indeed a nuclear transcript, differences in the splicing mechanisms between the two tissues could explain why the 1.6 Kb transcript is processed less efficiently in sfRamos cells. Alternatively differences in the stability of this transcript may exist between the two tissues.

At that time, it became apparent that a particularly difficult problem was that all members of the hGH gene family share very high homology and all produce mRNAs of about 1.0 Kb coding for 22K proteins. Although the use of monoclonal anti-hGH antibody in the Western blot indicated the expression of the hGH-N gene product, the Western and Northern blot analysis data could not absolutely determine which member of the hGH/CS gene family was expressed. However, the parallel dilution curve of concentrated conditioned media to the hGH standard curve, in a hGH RIA, and the excellent correlation between the Nb2 bioassay and RIA estimations, did not favor the possibility that the 22K band corresponded to hCS or to the placental hGH-V. Certain anti-hGH antibodies may cross react with hCS although the dilution curve of hCS is not

parallel to the hGH standard curve in a hGH RIA. Moreover, hGH-V was reported to have 20-fold lower activity than hGH in the Nb2 assay, when concentrations were estimated both with a specific hGH ELISA method and densitometric analysis *(492,163)*. In addition, a polyclonal antibody (anti-hGH #1), shown previously to inhibit the action of hGH but not that of the hGH-V or the hCS-A in the Nb2 assay *(174)*, had blocked the effect of concentrated CM from sfRamos cells in the present studies. Thus, these results strongly indicated that sfRamos cells express and release hGH. However, to confirm the expression of hGH in the sfRamos cell line, RT-PCR followed by diagnostic *RsaI* restriction digestion or PCR-DNA sequencing. The restriction digestion pattern and the nucleotide sequence of the RT-PCR-generated 250 bp fragment was identical to those of a hGH-N cDNA, demonstrating conclusively the expression of the hGH-N gene in sfRamos cells.

While these studies were in progress, the production of a 48K and additional GH-like peptides by sfRamos cells was reported *(542)*. A band corresponding to a 22K protein was not detected. A polyclonal antibody against hGH inhibited the spontaneous proliferation of sfRamos cells while hGH alone or in combination with hPRL did not affect the growth of the cells. Several factors might have accounted for the differences between these findings and those reported in our study. These differences include (i) concentration methods for the conditioned media, (ii) denaturing conditions, (iii) antibody type and dilution used for detection in Western blot analysis, as well as (iv) sensitivity and specificity of the methods applied. The combination of immunoprecipitation with polyclonal anti-hGH antibody followed by Western blot with monoclonal anti-hGH antibody increases the sensitivity for detection of hGH about 7-fold compared to the direct application of concentrated conditioned medium to Western blot analysis as

reported by Baglia *et al (542)*. Alternatively the polyclonal antibody we used may have been less capable of immunoprecipitating molecules with lower homology to hGH. The monoclonal antibody used in the Western blot analysis also might not recognize the higher molecular weight peptides. The inhibition of proliferation of sfRamos cells by a polyclonal anti-hGH antibody *(542)* indicates that growth hormone-like peptides as well as the 22K hGH produced by sfRamos cells, may contribute to the autonomous growth of this cell line.

The production of GH by lymphocytes has been studied in normal rat *(543)* as well as in normal *(543,544)* or Epstein-Barr virus (EBV) transformed *(544)* human lymphocytes. Moreover, additional data *(272,273)* support the initial observations although in all cases the characterization of hGH species at the RNA and protein levels was incomplete. The present studies provide direct data for the expression of the hGH-N gene product by a lymphoid cell line of B-lineage grown under serum-free conditions. For the first time the sizes both of the protein and the RNA transcript of GH produced by human lymphocytes have been determined by means of Western and Northern blot analyses. Further, data provided by RT-PCR coupled with restriction digestion and PCR sequencing confirmed the expression of the hGH-N gene. HGH activity in sfRamos conditioned media (not concentrated) ranged between 5-33 pg/ml as estimated by Nb2 or RIA methods. Although there is variation of the values between preparations, the amount secreted (5-33 pg/10^5 cells plated/2-3 day culture) is higher than previously reported for normal or EBV-transformed human lymphocytes under serum growth conditions (0.2-0.6 pg/10^5 cells plated/7 day culture and 0.8-4.8 pg/5 x 10^4 cells plated/7 day culture, respectively) *(544)*.

The clear conclusion of the studies presented in this thesis, is that sfRamos cells express the hGH-N gene and produce a biologically active 22K hGH protein.

C. THE SIGNIFICANCE OF THE DETECTION OF GH IN HUMAN LYMPHOCYTES

I. Possible role of hGH for the survival of sfRamos cells under serum-free conditions

In a very interesting study, Gregory *et al (269)* provided data, linking a germinal center normal B cell population with the cell type susceptible to the development of Burkitt lymphoma. The basis for this association was the presence of a particular pattern of cell specific markers detected in both cell types. This normal germinal center B-cell population appears to be in cell cycle, however, a high rate of apoptotic death accompanies the cycling phenotype. At least two steps would be necessary to transform this normal population to a malignant Burkitt lymphoma cell type. First, the universal, for Burkitt lymphomas, translocations or mutations of the c-myc gene *(545-547)* should occur. This alone, however, would not reduce the apoptotic rate of death of these cells. In fact, it has been shown that, in fibroblasts, c-myc upregulation may result in excessive apoptotic death if sufficient trophic support, by growth factors, can not be maintained *(548)*. Thus, after an event that leads to c-myc deregulation, the actual advance towards a malignant phenotype represents the potential for a second event that would introduce a phenotype not susceptible to apoptotic death. This would be similar to the phenotype of the long-lived pool of B memory cells which, in contrast to the proliferative phenotype

normally enter the G_0 phase. In normal memory cells, this is also accompanied by the loss of germinal center surface markers *(268,269)*. With a more recent study, Gregory *et al (549)* showed that expression of all eight latent EBV antigens in clonal subpopulations of Burkitt lymphoma biopsies was associated with resistance to apoptotic death under growth in conditions of reduced FBS medium content (1% versus 10% regularly) while clonal subpopulations that expressed only the EBV nuclear antigen 1 (EBNA1) were subject to extensive apoptotic death similar to the normal germinal center cells. Thus, it appears that after a second event, which is the EBV infection of germinal center cells with deregulated c-myc, an additional requirement, the expression of all latent EBV antigens, must be met before the demonstration of a fully malignant phenotype.

This analysis emphasizes the importance of the demonstration of autocrine hGH dependence for the serum-free grown sfRamos cells *(542)*. The parental Ramos burkitt lymphoma cell line, an EBV negative cell line, presumably expresses no EBV antigens. A different, but also EBV negative, Burkitt-lymphoma cell line (BL41) examined in the study of Gregory *et al (549)*, demonstrated the phenotype of the EBV positive clonal populations that express EBNA1 antigen only, being susceptible to apoptotic death under low serum conditions. Infection of BL41 with EBV resulted in the BL41-B95 clonal line which is resistant to apoptosis *(549)*. SfRamos cells are able to survive under serum-free conditions, in spite of the absence of EBV infection. Addition of anti hGH antibodies to the culture media, however, resulted in inhibition of thymidine incorporation measured during the last 16 hours of a 96 hour assay period *(542)*. This raises the possibility that a hGH-induced signalling pathway in these cells, overlaps a signalling pathway activated

by one of the EBV latent antigens. The level at which these pathways may overlap could vary from a very early point, such as the expression of a lymphocyte counterpart of an EBV antigen induced by hGH or *vice versa*, to a late point, such as a Jak mediated pathway used by both hGH and an EBV antigen.

Since the support of the survival of sfRamos cells by hGH reflects an effect on a population that, likely, shares several characteristics of the normal germinal center non-memory cell population, this effect may be a more generalized phenomenon that is in agreement with the fundamental actions of hGH on immature or stem cell populations as described by the "dual effector theory" *(310,197)*. Further, the autocrine nature of this action, suggests that additional sources of hGH, apart from the pituitary produced hormone, may contribute to the development and function of the immune system.

II. Tissue-specific expression of hGH proteins

Although the characterization of the lactogenic activity produced by sfRamos did not yield a new gene or a novel lactogenic protein form, the result was equally rewarding, exciting and stimulating. The complete nature of the approach used to demonstrate the expression of hGH in these tumor cells contributes significantly to our knowledge since it clearly demonstrates that sfRamos cells express the hGH-N gene and produce a biologically active 22K hGH protein putting an end to the debate over whether the hGH-N gene can be expressed in lymphoid cells. Further, as discussed in the previous paragraphs, the result links the expression of hGH with the survival of these tumor cells.

In addition to the direct biological importance of these findings, different aspects

of the tissue-specific expression of the hGH/CS gene family, must be reconsidered in light of the demonstration of the hGH-N gene expression in human lymphoid cells. In spite of previous reports for the presence of GH-like molecules in lymphoid cells, it continued to be broadly accepted that the members of the hGH family are expressed exclusively in pituitary (hGH-N) or in placenta (hGH-V and hCS genes). A reason for this consideration, that essentially ignored the data for lymphocyte expression of GH, might be the absence until now, of convincing data regarding the nature of these GH-like substances. The demonstration by the studies presented in this thesis, that hGH-N, and not a hGH-related gene, is expressed in lymphoid cells, however, challenges the view that advocates the exclusive pituitary or placental expression of the hGH/CS family.

Numerous studies have given insight in the mechanism for pituitary expression of hGH, but only few reports and partial explanation for the placenta specific expression of the hGH family has been provided. Pituitary-specific expression is determined by the transcription factor GHF-1/Pit-1 that interacts with elements in the 5' flanking region of the hGH-N gene. Placenta-specific expression is believed to be regulated by sequences in the 3' flanking region of the hCS-B gene where a single DNA element representing a binding site for a transcriptional enhancer factor (TEF-1) was identified.

The expression of hGH in sfRamos lymphocytes, provides evidence for the existence of diverse mechanisms that permit the expression of the hGH family in extrapituitary and extraplacental tissues. Furthermore, it provides a new model for the study of human GH family tissue-specific gene expression that involves mechanisms for both transcriptional activation (hGH-N) and transcriptional blockade (hGH-V and hCS genes).

A question raised during these studies was whether the hGH expression in

sfRamos might represent an ectopic phenomenon only observed in the context of a tumor phenotype. It is known that genomic alterations, often associated with tumors, may activate genes independently of their tissue specific regulatory mechanisms. Inappropriate gene activation might result from the loss or acquisition of regulatory *cis*-elements through rearrangements, insertions or deletions. This does not appear to be the case for the activation of the hGH-N in sfRamos cells as no gross alteration of the hGH gene family locus was detected by Southern analysis.

Thus, the mechanism of expression of the hGH-N gene in these lymphoid cells is of particular interest, particularly because Pit-1/GHF-1 the factor that permits the expression of the hGH-N gene in pituitary *(204-207)* was generally thought to be restricted to pituitary somatotrophs, lactotrophs and thyrotrophs *(70)*. In contrast to this view, however, the detection of Pit-1/GHF-1 RNA in human and rat lymphoid cells by immunocytochemistry, in situ hybridization and RT-PCR has been reported concurrently with, as well as following, this study *(532-534)*. Expression of the hPRL gene, like the hGH-N gene, is dependent on the presence of Pit-1/GHF-1 in the pituitary *(207)*. Ectopic expression of hPRL gene in IM-9-P3 lymphocytes has been described *(277)*. The transcription initiation site used in these cells, is located several Kb upstream of the site employed in lactotrophs and corresponds to the site used for decidual hPRL expression *(493)*. Due to the use of this alternative promoter in the decidua and in IM-9-P3 lymphocytes, transcriptional control is independent of Pit-1/GHF-1 as indicated by the absence of Pit-1 consensus binding sequences from the proximal promoter region within 250 bp upstream of the initiation site *(550)* and the demonstration, by deletion analysis, that two additional Pit-1 sites present in sequences further upstream (nucleotides -2799

to -1792 and -2045 to -2038) have no effect on the lymphocyte expression of hPRL *(551,552)*. Whether the expression of the hGH-N gene in sfRamos cells is controlled by Pit-1 or by other transcription factors, as is the case of the placental members of the family *(78)*, remains to be examined. An interesting possibility is that hGH expression in lymphoid cells represents transcription from an alternative initiation site (cap 2 or cap 3) instead of the initiation site (cap 1) that is dependent on Pit-1 *(553)*. Alternatively, the lymphocyte specific Oct-2 may activate the hGH promoter in lymphoid cells, via binding to the Pit-1 elements of the hGH promoter. Indeed, cross recognition of a hPRL Pit-1 binding site (WT-1P) by Oct-1 and Oct-2 POU-domain proteins and in vitro activation of [3xWT-1P]/[-36/+34]hPRL-luciferase hybrid construct in lymphoid BJA-B cells have been described *(554)*. Finally, it is conceivable, although remote, that an alternative hGH promoter, further upstream, might be used for lymphocyte specific expression of this gene, by analogy to the mechanism described for the decidual, myometrial and lymphocyte activation of hPRL *(493,494,498)*.

III. Re-examining the regulation of the hGH locus

The cloning of the first tissue specific transcription factors underlined the potential for the unravelling of relatively simple models for the tissue specific expression of certain genes. Single factors, like GHF-1/Pit-1, might represent an "ultimate" regulatory point, a single decisive tissue-specific regulatory event that determines gene expression. This perception prevailed in the scientific thinking until the end of the last decade, however, it is now broadly recognized that more complex mechanisms and multiple, non tissue-

specific, regulatory steps may be equally important for exclusive and efficient expression of certain genes in a particular cell type. Additional factors are necessary for regulation of temporary or cell specific gene expression and in many cases combined action of transcription factors with partial tissue specificity confers very tight tissue specific expression. The term "tissue-specific complex" would be rather more appropriate than the term "tissue specific factor" to describe DNA-protein and protein-protein interactions that determine gene expression.

The hGH/hCS gene family is a perfect example of this complexity. Apart from sharing >90% nucleotide sequence homology in their coding and flanking regions, all five genes contain Pit-1 elements in their promoter region but only hGH is expressed in pituitary. Apparently, a restrictive mechanism prevents the promoters of the placental members of this family from being functional in pituitary in spite of the fact that they contain the binding sites for the pituitary-specific transcription factor. Further, we know that despite their structural similarity, common chromosomal location and cellular site of expression (syncytiotrophoblast), the hGH-V and hCS genes are expressed at different levels during pregnancy *(123,78)*, with hCS-A and -B expressed at much higher levels than hGH-V and hCS-L in human placenta.

It would be important to identify DNA elements and protein factors that regulate the tissue-specific and temporal activation (or restriction) of the placental hGH/CS genes and assess their role during placental and/or embryonic development. Given the similarities between the decidual, myometrial and lymphocyte expression of PRL *(493,494,498)*, and the possible hPRL expression in some trophoblasts *(501,286)*, it would be also interesting to determine whether there is any parallelism and similarities between the placental and lymphocyte expression of hGH genes as well as if any of the

placental factors are involved in the regulation of lymphocyte hGH expression.

Although the mechanism of the transcriptional regulation of the hGH family in lymphocytes was an intriguing question, I considered the partially explored [Nachtigal *et al (509)*] idea of a subloci organization within the hGH locus a more promising approach for the identification of "key" regulatory mechanisms for the expression of hGH/CS genes that eventually will also lead to the unravelling of the mechanism of lymphocyte activation of the hGH family. Further placental systems for the study of gene activation have been successfully developed *(338)* and DNA regions important for placental expression have been reported *(507,508)*.

D. DEMONSTRATION OF DISTINCT PATTERNS OF EXPRESSION OF MEMBERS OF THE HGH FAMILY IN PLACENTAL CELL POPULATIONS

The first step and a central point for the analysis of the hGH locus, in terms of sublocus organization, defined as the level of regulatory control exercised from individual hGH/CS genes within the hGH/CS gene locus, would be the ability to demonstrate that particular placental cell populations express distinct members of the hGH family at different levels. Abnormalities of placental development are accompanied by a disruption of the normal pattern of gene expression in this tissue. Human GH/CS expression in hydatidiform moles and choriocarcinomas is greatly reduced compared to the normal tissue.

To distinguish hCS, hGH-V, hCS-L and hGH-N RNA from choriocarcinoma BeWo, JAR and JEG-3 cell lines and samples of three hydatidiform moles (HM-1, -2 and

-3), a RT-PCR method was applied, followed by diagnostic restriction digestion that results in the generation of different size fragments for each of these genes (*Table 7 p. 109*). The procedure was simplified to a single set of primers which were also designed to distinguish processed RNA from any contaminating DNA. This technique provides direct qualitative data for the relative expression of these genes since identical primers are used to generate and detect all five genes of the hGH/CS family and the PCR products predicted and generated are all of the same length (*Table 7 p. 109*). Also, because sizes of specific restriction endonuclease fragments are used to confirm the identity of the hGH/hCS gene product, the exon 3 primer used for RT-PCR could be used as the screening oligonucleotide probe instead of a third (internal) specific oligonucleotide as used in this study (*Figure 17*). A possible limitation is that the selected primers reflect only one region of the hGH/hCS transcripts. However, the pattern of RNA expression indicated for term placenta and BeWo cells is similar to that determined by methods including cDNA cloning, RNA blotting and RT-PCR with alternative primers *(76,123,505)*. Thus, this method of analysis appears representative and a basis for comparison with RNA from other trophoblast and non trophoblastic cells.

Indeed, this study generated evidence for differential expression of the hCS and hGH-V genes in hydatidiform moles and human choriocarcinoma cell lines. The results are summarized in *Table 11*. When compared to the hGH-V:hCS RNA ratio in term placenta (1:23), the hGH-V:hCS ratio is higher in all three choriocarcinoma cell lines, BeWo (1:3.9), JAR (3.5:1) and JEG-3 (1:2.5), examined (*Table 11*). Also, in contrast to the absence or very low levels of hCS-L RNA detected in choriocarcinoma cell lines and two of the hydatidiform mole samples tested (HM-1 and HM-3), the level of hCS-L

RNA in a third hydatidiform mole sample (HM-2) was substantially higher than hGH-V (*Table 11*).

Table 11. **Levels of hCS, hGH-V, hCS-L and hGH-N RNA as a percentage of total hGH/hCS RNA in trophoblast samples**

RNA	Placenta (n=3)	HM-1	HM-2	HM-3	BeWo (n=4)	JAR (n=2)	JEG-3 (n=4)
hCS (A and B)	95.0 ± 0.5	99.6	87.1	99.1	79.5 ± 2.5	22.1 ± 5.4	71.1 ± 3.3
hGH-V	4.2 ± 0.5	<1	1.7	<1	20.5 ± 2.5	77.9 ± 5.4	28.9 ± 3.3
hCS-L	<1	<1	11.2	<1	ND	ND	ND
hGH-N	ND	ND	ND	ND	ND	ND	ND

Results are expressed as mean plus or minus standard error of the mean. ND: not detectable after 40 cycles of amplification.

These findings suggest that neoplastic trophoblasts have either a deregulated pattern of placental hGH/hCS gene expression or they reflect differences of expression that accompany the process of differentiation of particular cell subpopulations.

Differences in mRNA expression of the hGH/CS genes could be either due to differential transcriptional activity, RNA processing or mRNA stability of the corresponding transcripts. Differences in RNA processing and mRNA stability however, are rather unlikely to account for extreme differences between the expression of a gene in two cell subpopulations.

The pattern of hGH/CS expression in JAR cells clearly reflects an inactive state of the hCS genes when compared to the normal placental syncytiotrophoblasts. In contrast, the hGH-V gene appears active (3.5-fold higher levels than hCS) in JAR cells, probably at levels not more than one order of magnitude different than the activity of this gene in placental syncytiotrophoblasts. Differences in RNA processing and mRNA stability might account for moderate differences in hGH/CS gene expression between the choriocarcinoma cell lines examined in the present studies. Considering, however, that

hCS expression is 23-fold higher than hGH-V expression in term placenta, differential RNA stability would be unlikely to account for the inactivity of the hCS genes in JAR cells when compared to normal placental syncytiotrophoblast. Therefore, though the possibility of involvement of RNA processing and mRNA stability mechanisms cannot be excluded, the differences in hCS/GH-V expression observed between normal and abnormal trophoblasts likely represent differences in the transcriptional activities of individual hGH/CS genes in distinct syncytiotrophoblast subpopulations and presumably reflect involvement of restrictive or activating transcriptional mechanisms. JAR cells represent an example of a trophoblast subpopulation where the hCS genes are drastically repressed, when compared to the differentiated syncytiotrophoblast, while the hGH-V gene is active although not at a fully competent state.

An assessment of hGH-V RNA levels during pregnancy indicates an increase by the 20th week of gestation *(123)*. This coincides with the period in which the villous cytotrophoblasts decrease in number as they lose the capacity to divide and commit to fuse and form syncytial structures such that few cytotrophoblasts exist at term *(336)*. This is consistent with the observation that in BeWo cells treated with methotrexate, and believed to represent a transitional stage between cytotrophoblast- and syncytiotrophoblast-like cells, there is an increase in the ratio of hGH-V versus hCS RNA expression *(496, see Table 12 p.175)*. The JAR cells show intrinsically much higher hGH-V than hCS RNA levels without methotrexate treatment and, thus, might represent transitional trophoblasts or a trophoblast population that is committed to the specific expression of the hGH-V gene. These cells do not differentiate further into villous syncytial structures which are considered essential for efficient hCS expression *(365,371)*. Regardless of whether a transitional stage in syncytiotrophoblast

differentiation is accompanied by an increased hGH-V to hCS ratio, hGH-V gene activation in JAR cells occurs in spite of the immaturity of the transcription machinery that fails to commit these presumably poorly differentiated syncytiotrophoblasts to the expression of high levels of hCS.

Thus, these studies support the hypothesis that hGH-V and hCS expression are not "coupled"; that is, they are not regulated by the same factors as their similar developmental and tissue-specific expression suggests (*123,125*). A possibility would be that a common regulatory element, like the placenta-specific enhancer region located 2 kilobases (Kb) downstream of the hCS-B gene (*507*), is responsible for the expression of the hGH-V as well as the hCS genes. If this were true, differential levels of hCS versus hGH-V gene expression would be explained by a "position" effect as the hGH-V promoter is placed about 12 kb upstream of the hCS-B enhancer *(507)* and 10.5 kb downstream of a highly homologous sequence 3' of the hCS-A gene *(76)*. However, the low level of hCS versus high levels of hGH-V RNA in JAR cells suggests that there is distinct transcription machinery for the activation of hGH-V that may induce this gene in the absence of further differentiation events that are required for the efficient expression of the hCS genes *(365)*. Alternatively, distinct subpopulations of intermediate trophoblasts or syncytiotrophoblasts are responsible for the expression of these genes. The latter is supported by the fact that, *in situ* hybridization analysis of normal placenta has revealed a uniform syncytiotrophoblast distribution of hCS mRNA, but a focal distribution of hGH-V mRNA that was detected in only a few syncytiotrophoblasts *(345)*.

I. Expression patterns of hCS/GH-V versus hCGα/CGß and the differentiation state of trophoblasts in human placenta and choriocarcinoma cells

Human CS/GH-V synthesis is considered, by current criteria, the most reliable hormonal marker of terminal syncytiotrophoblast differentiation. Impaired hCS/GH-V synthesis, at levels several hundred-fold lower than in normal term placenta, is observed in undifferentiated trophoblasts of placental abnormalities such as hydatidiform moles and choriocarcinomas *(81,364-366,371,373-375,505)*.

Total hCS/GH-V mRNA expression, as determined by Northern analysis using 100 μg total RNA, was more than 10-fold higher in BeWo than in JAR cells while it was undetectable in JEG-3 cells *(505)*. The RT-PCR analysis, presented here, revealed a similar pattern of expression between JEG-3 and BeWo cells (hCS:GH-V ratios 2.5:1 and 3,9:1 respectively) in spite of their substantial difference (over 100-fold) in total hCS/GH-V mRNA levels. Thus, although the transcriptional machinery of the clonal JEG-3 cells appears more immature than the one of BeWo cells, the pattern of expression rather reflects a common differentiation descent from trophoblasts with similar hormone producing potential.

In contrast, JAR cells that express detectable hCS/GH-V mRNA levels, but at least 10-fold lower than BeWo cells *(505)*, may represent a trophoblast with a distinct differentiation descend. Although JAR produce hGH-V versus hCS at a ratio 3.5:1, hCS mRNA levels in JAR are likely higher than hCS levels in JEG-3, since total hCS/GH-V mRNA was undetectable in JEG-3 cells by Northern analysis. Therefore, in terms of hCS production, the differentiation state of JAR cells, on average, may be comparable or slightly more advanced than the differentiation state of JEG-3 cells. By the same criteria,

in terms of hGH-V production JAR cells are comparable or less differentiated than BeWo cells, however their hormone producing potential appears different.

Since both cell lines, BeWo and JEG-3 represent heterogenous populations it is possible that a JAR subpopulation reflects a differentiation state similar to that of the clonal JEG-3 cells, while another subpopulation represents a differentiation state compatible with hGH-V expression. This is supported by the fact that in normal placenta a focal pattern of hGH-V expression has been observed and only few syncytiotrophoblasts express this gene *(345)*, which explains, at least in part, the much lower levels of hGH-V compared to hCS. In fact multiple levels of differentiation have been recognized in JAR populations with only 2% representing a differentiated large multinucleated form with high hCG production *(555)*. It is not known, however, whether these multinucleated cells produce either or both hGH-V and hCS. In conclusion, if hCS/GH-V mRNA production reflects reliably the differentiation state of these choriocarcinoma cell lines, then BeWo cells must represent a range of post-intermediate trophoblast/early trophoblast cell subpopulations, some of which have acquired the ability to express basal levels of the hCS and hGH-V genes. In contrast, JAR and JEG-3 cells appear less capable for hCS production, however, the former appear able to express hGH-V.

Interestingly, in spite of their common severe inadequacy in hCS production, JAR and JEG-3 cells appear morphologically different. JEG-3 resemble the morphology of BeWo cells, growing as a monolayer of multinucleated cells, however JAR cells show a multilayer growth with formation of cellular clusters or "piles". These features may reflect a phenotype similar to that of non-villus invasive migratory trophoblastic forms (interstitial or stromal trophoblasts) which tend to form trophoblasts columns that originate from the tips of the anchoring villi to invade the decidua and the myometrium.

These cells have also the ability to fuse and form syncytial placental bed giant cells *(346,385)*. By analogy to the villus mononucleated and multinucleated intermediate trophoblasts *(364,556)*, columnar trophoblastic populations include mainly mononucleated intermediate forms while within the decidual tissue both mononucleated and multinucleated intermediate forms may be present *(385)*.

Whether subpopulations of JAR may include columnar or decidual type trophoblast intermediate forms *(385)* that have acquired the ability to express the hGH-V gene is not known. However the idea of hGH-V gene expression in columnar invasive trophoblasts is attractive, particularly since no function has been attributed to the presumably transmembrane hGH-V2 protein which is speculated to play some role in cell-cell communication and cellular interactions *(103,124,125)*.

If, indeed, these choriocarcinoma cell lines represent trophoblasts at different differentiation states then this should be also reflected by differences in the expression of additional cytotrophoblast and syncytiotrophoblast markers, particularly human chorionic gonadotropin (hCG). To examine whether the hCS/GH-V expression patterns observed in normal placenta and choriocarcinoma cells are correlated with the variation of hCG subunit mRNA and/or protein values, a comparison analysis was made for the hCGα/hCGβ expression pattern in normal placenta tissue, placental cultures or choriocarcinoma cell lines, in the presence or absence of agents that induce trophoblast differentiation such as methotrexate (MTX) and cAMP analogues[16] *(Table 12)*. *Table 12*,

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[16] Cyclic AMP causes cytotrophoblasts to round up and promote cytoplasmic differentiation (increasing the density of the ER, the prominence of Golgi system and mitochondria) but does not influence syncytium formation. These changes appear similar to the changes present in intermediate cytotrophoblastic forms *(338)*.

Table 12. Comparison of hCS/GH-V versus hCGα/hCGβ expression pattern in normal and abnormal trophoblasts in the presence or absence of differentiating agents.

CELLS/ RATIO→ ↓	hCS:hGH-V (mRNA)	hCGα:hCGβ	ASSAY	Refs
Placenta (early)	ND	2.0:1 1.7:1	mRNA PROTEIN	559 364
Placenta (term)	23:1*	12:1 (hCGα+hCGβ: ↓)	mRNA & PROTEIN	364 559
Placental cells (culture, term)	ND	14:1	PROTEIN	556
Placenta+ cAMP (culture, term)	ND	3:1 (hCGβ ↑)	PROTEIN	556
Placenta + MTX (org. cult. early)	ND	hCG x 1	PROTEIN	560
BeWo	3.9:1*	hCGα<hCGβ 89:1	PROTEIN	558 557
BeWo + MTX	x 0.25 (hGH-V:x 0.2 hCS: x 0.05)	x 0.4 (hCGαx 3 hCGβ x 7)	mRNA	496
BeWo + cAMP	1:1 (hGH-V: ↑)	17:1 (hCGαx 3.7, hCGβx18.9)	mRNA (hCS/GH-V) PROTEIN (hCG)	561 557
JEG-3	2.5:1*	1:1	mRNA	562
JEG-3 + MTX	ND (hCS+GH-V:x1)	x 15-20 (hCGβ x 1) x5-21 (hCGα x 100-170 hCGβx8-22)	mRNA	375 497
JEG-3 + cAMP	ND	x 0.5 (hCGα x 25, hCGβ x 52) x 0.8 (hCGα x36, hCGβ x 43)	mRNA PROTEIN	563
JAR	1:3.5*	2:1	PROTEIN	556 557
JAR + MTX	ND	ND (hCGα x 4) (hCG x 8)	mRNA PROTEIN	535 560
JAR + cAMP/ 8-Br-AMP	ND	2:1 (hCGα+hCGβ: x 3) 1:1 (hCGα x 1.5, hCGβ x 3.4)	PROTEIN	364 556 557

* Results generated by RT-PCR in the present study.

includes patterns of hCGα/hCGß expression in various trophoblasts as determined during previous assessments *(496,353,364,375,557-559,560)* as well as the patterns of hCS/GH-V expression revealed by RT-PCR analysis.

It is suggested by this analysis that a more advanced trophoblast differentiation phenotype may be associated with a higher response of the hCGß subunit mRNA and protein to cAMP analogs. 8-Br-adenosine treatment increased the percentage of the hCG producing JAR cells (4% in controls) by 2- to 4-fold, however, the ratio of hCGα : hCGß (2:1) was unaffected after 8-Br-adenosine treatment *(556)*; a reduction, however, of the hCGα:hCGß ratio from 2:1 to 1:1 after cAMP treatment has also been reported *(557)*. In contrast in normal trophoblasts hCGα:hCGß decreased from 14:1 in control to 3:1 after treatment due to enhanced hCGß production *(556)*. 8-Br-adenosine was considered to stimulate hCG production in JAR by promoting differentiation and exit from the cell cycle of a population of dividing cells *(556)*. However the greater effect of 8-br-adenosine on hCGß synthesis in mitotically inactive cytotrophoblasts from term placenta indicates that the effect on normal trophoblasts involves pathways that mature at a later differentiation state *(556)*. By similarity to JAR cells *(556)*, JEG-3 cells respond to cAMP with only marginally greater effect on hCGß synthesis: hCGß synthesis hCGα was about 2-fold higher at the mRNA level and 1.2-fold at the protein level *(563)*; hCGα:hCGß ratio has been reported to be roughly 1:1 under control conditions *(562)*.

Normal term trophoblasts in culture responded with a 4.7-fold greater effect on hCGß than hCGα synthesis *(556)* compared with a 0 to 2-fold greater effect on hCGß observed in JEG-3 and JAR choriocarcinoma cells *(557,563)*. Nevertheless, by similarity to the response of normal term trophoblasts *(556)*, BeWo cells responded to cAMP with

a 5-fold greater effect on hCGß compared to hCGα synthesis and secretion *(557)*[17].

In Bewo cells treated with methotrexate (MTX) again a greater effect was observed on hCGß which was increased by 7-fold compared to 3-fold of hCGα *(496)*. In contrast in JEG-3 cells, there was a 15 to 20-fold increase in hCGα steady state mRNA level while hCGß mRNA was constant *(375)*. Nuclear run on experiments showed no difference in transcriptional activity between the two subunits *(375)*. These, MTX-treated, JEG-3 cells have lost their proliferating potential, appear to have a complex reticulated cytoplasm and, bearing such characteristics, were characterized as trophoblasts at a "intermediate" (transitional) state towards final syncytiotrophoblast differentiation *(371)*.

This analysis suggests that there may be a distinct pattern for the response of the hCGß mRNA and protein levels, to differentiation agents (cAMP and MTX) that depends on the differentiation state of certain trophoblast populations. This pattern appears related to the hCS expression pattern of these populations, with higher hCS expression being coincident with a greater hCGß response to either cAMP or MTX treatment. MTX treatment, however, does not appear to affect hCG expression in early placenta organ culture *(560)*, likely reflecting the inability of MTX to alter the characteristics of differentiated placental trophoblasts with an ultimately maximized hCGß synthetic potential which is not subject to further increase. This is supported by the fact that after the 10th week both hCG subunits but particularly hCGß decrease resulting in lower hCG

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[17] Contradictory results have been generated for the relative levels of hCGα versus hCGß subunits in BeWo cells. Excess of the hCGß subunit (and not excess hCGα) has been reported both extra- and intracellularly in *BeWo* cells *(558)* in the absence of any stimulation. However, an earlier study *(557)* did not produce the same result, but in contrast revealed a hCGα:hCGß ratio of 89:1. Since the production of hCG in BeWo and JAR cells is comparable [BeWo: 3414; JAR: 2373; JEG-3: 1494 mIU/10^6/48 hours *(338)*], and hCG mRNA and protein levels vary in parallel *(364,559)*, any differences in hCGα:hCGß ratio rather reflect differences in the mRNA levels of hCGα between these two cell lines.

secretion and a hCGα:hCGß ratio that increases from 2:1 to 12:1 *(364,559)*.

It would be interesting to apply the RT-PCR method to study the variation of the hCS/GH-V ratio in response to trophoblast differentiation agents. Similarly, examining by RT-PCR, the relative levels of different hCGß subunits products of distinct genes present within the hCGß/hLHß gene cluster *(559)* may reveal informative patterns of expression and response to differentiating agents.

II. Relating abnormal gene expression and phenotype: diagnostic and prognostic value of irregular patterns of hGH/CS gene expression, for hydatidiform moles (HMs) and choriocarcinomas.

Summarizing, the RT-PCR method was used to define which of the hGH/hCS family members are expressed in normal and abnormal trophoblasts. The expression of individual members of the hGH/hCS gene family was examined in normal placenta, hydatidiform moles (HMs) and choriocarcinoma cell lines and revealing a distinct pattern of expression of hGH family members between different cells of placental origin. It was shown that: (1) hGH-V mRNA is present in hydatidiform moles and choriocarcinoma cells (2) the pattern of expression of hCS/hGH-V in choriocarcinoma cells is different from both normal term placenta and hydatidiform moles with the ratio of hGH-V versus hCS being much higher in choriocarcinoma cells (3) hCS-L mRNA was present in hydatidiform moles at variable levels but was not detected in choriocarcinoma cells.

These results suggest that the hGH-V and hCS-L genes are controlled by regulatory mechanisms distinct from those controlling the expression of the hCS-A and hCS-B genes and raise questions about their possible involvement in the pathology of

placental abnormalities.

Further, apart from raising the possibility of a putative hCS/GH-V involvement in trophoblast pathogenesis, a more direct benefit of the present studies may lie in the potential use of detecting distinct hCS/GH-V expression patterns in the differential diagnosis of placental abnormalities and the prognosis of placental tumors. Recognition of distinct subtypes of a pathological entity is often difficult and in many cases determines the therapeutic action taken for its treatment.

Two, macroscopically, microscopically, and genetically, distinct types (described in the **INTRODUCTION**), of non-invasive HM exist: the **complete** and the **partial** *(376,377,379-384)*. Usually, non-invasive HMs require only surgical treatment, however the finding of metastatic tissue at the time of the surgery or, after surgery, during a follow-up search that normally takes place when hCG levels remain elevated, requires chemotherapy *(564)*. Chemotherapeutic agents, such as methotrexate, have been used successfully for most of these cases. However, not all cases respond well to this type of treatment *(564,565)*. In these cases the early identification of a HM subtype that is prone to metastasis and/or poor response to chemotherapy could be beneficial. Further, it is known that the risk of choriocarcinoma for women with a diagnosis of a HM is up to 4000 times higher (2-3% of all molar pregnancies) than for normal women, and that 50% of the choriocarcinomas follow a history of HM in the same or a previous pregnancy *(383,385)*. As is also the case with metastatic HMs, for the majority of choriocarcinomas chemotherapy is a successful treatment with a cure rate at early stages of about 95% *(564)*. However, it would be of benefit to recognize the types of HM that are associated with later development of choriocarcinomas, particularly the ones that do not respond

well to chemotherapy. Similarly it would be of enormous benefit if we could identify at the time of diagnosis those choriocarcinomas that even if they are contained within the uterus, will respond poorly to single-agent chemotherapy requiring a combination chemotherapy *(564)*.

Due to the fact that the differentiation of trophoblasts results in reduced proliferative potential and highly tissue specific gene expression, with the expression of the hGH/CS family being the ultimate marker for terminal differentiation *(364)*, certain patterns of hGH/CS expression may reflect a particular differentiation state that may be linked to a higher or lower tendency for malignant behaviour. Therefore, determining the pattern of hGH/CS expression in a large number of HMs and choriocarcinomas may link specific patterns of hGH/CS expression with either benign or malignant behaviour. This may lead to the development of relatively inexpensive assays with diagnostic and/or prognostic value for the evolution of these tumors. The identification of tumors at high risk for malignant behaviour will allow the administration of a more aggressive therapy much earlier in the progression of these abnormalities, increasing the chances for cure and survival.

E. AIMING AT THE IDENTIFICATION OF REGULATORY EVENTS THAT DETERMINE TIME AND CELL SPECIFIC EXPRESSION OF MEMBERS OF THE HGH GENE FAMILY

I. Hypothesis and objectives

An important conclusion from the RT-PCR analysis in cells of placental origin is that the expression of hCS (hCS-A/B) is severely impaired while expression of hGH-V or hCS-L may be less affected in some placental abnormalities. Since hCS represents a major placenta product it is logical to hypothesize that factors absent or defective in placental abnormalities control hCS-A or hCS-B gene expression. With this prospect studies on the placenta-specific expression of the hCS-B gene and specifically on the identification and characterization of 3' flanking DNA elements of this gene, were undertaken.

Tissue specific gene expression determines differentiation and functional competence of a particular tissue cell population. A 1022 bp 3' flanking sequence of the human chorionic somatomammotropin (hCS-B) gene was shown to direct placental specific expression of this gene. A single DNA-protein interaction involving a 22 bp element and a protein factor termed TEF-1 was demonstrated in this region by DNAase I protection experiments. Work in our laboratory showed, however, that a synthetic 22 bp element was neither as potent nor as specific an enhancer as was the full length fragment *(497 and Figure 23B)*.

The **hypothesis** that emerged was that additional elements within the 1022 bp fragment confer tight control over the tissue specific activity of this enhancer region. The

objectives of these studies were a detailed characterization of the 3' enhancer region and the identification of the DNA elements and transcription factors that interact with these DNA elements and determine placenta-specific expression.

II. Evaluation of the approach and methods used for *in vitro* assessment of DNA regulatory elements

a. The use of malignant cell lines for the study of normal regulatory pathways

Although it seems "paradoxical" to study normal regulatory pathways using tumor cell lines the information acquired by these studies is invaluable. Transcription factors involved in activation of the hCS-A/B genes may be present at much lower levels or be mutated in tumor cells. However, the systems used to analyze the functional significance of DNA regulatory sequences are based on the comparison of the effects of such sequences on the expression of highly sensitive reporter gene constructs with the effects of non related or modified sequences on the expression of the same reporter genes. In this way even the effects of underexpressed or functionally impaired transcription factors can be assessed based on the assumption that some function will be maintained. A restriction for the use of these systems is that the reporter genes have to be expressed at relatively high levels in the presence of positive regulatory sequences so as to allow dissection of the various components of the regulatory mechanisms.

b. ***Does chromatin assembly and nuclear matrix association occur during in vitro transient plasmid DNA transfer?***

A serious concern about the validity of the information generated by *in vitro* plasmid DNA transfer focuses on the presumed inability of a transiently transfected reporter construct to be integrated in chromatin structures that reliably reflect the chromatin profile of an endogenous gene, if associated with chromatin at all.

In general, investigators that use *in vitro* transient gene transfer systems, in their analyses, consider the transfected plasmid DNA essentially free of histone association. Several pieces of evidence however suggest that there is a certain degree of association of transferred plasmid DNA with histones and nuclear matrix proteins.

Jeong and Stein (*566*) reported that nucleosome like structures can be formed on transfected DNA and nucleosome ladders could be produced after micrococcal nuclease digestion. These ladders, however, may differ in some of their characteristics, such as the size of the generated fragments, from typical ladders generated by digestion of endogenous genes. Upon digestion of the nuclei about 10% of transfected plasmid DNA was associated with soluble chromatin fragments and generated typical nucleosome ladders. However, 90% of the chromatin fragments that contained transferred plasmid DNA sequences were not soluble at low ionic strength implying association with nuclear structures (such as the nuclear matrix or the nuclear pore complex) or nuclear compartmentalization which modified the chromatin assembly process. In an earlier study, Marini and Benbow *(567)* had generated similar results. They showed that after microinjection of plasmid DNA into *Xenopus laevis* embryos, only 14% of the **circular**

plasmids replicated and 1% underwent more than one round of replication from the fertilization to the blastula stage. In addition, regardless of their replication profile the majority of the plasmids became resistant to micrococcal nuclease cleavage likely due to sequestration into pseudonuclear compartments rather than nucleosome assembly. Interestingly, in contrast to the circular templates, **concatamerized linear** plasmids underwent at least two rounds of replication and showed micrococcal nuclease patterns similar to endogenous chromatin. As was the case for Jeong and Stein *(566)*, they suggested that circular plasmids were mainly associated with the nuclear matrix or the nuclear envelope.

Additional studies have provided insight into the possible dependence of the transcriptional activity of certain plasmid templates on chromatin assembly and association with nuclear matrix. In an excellent study, Jimenez-Garcia *et al (16)* showed that following transient transfection, a focal nuclear localization of actively transcribed (single strand) plasmid DNA, as well as synthesized RNA, was observed. This focal distribution, followed the selective "migration" of transcription and RNA splicing factors to these nuclear regions. The lack of a diffuse pattern for the transcribed DNA suggests "docking" to nuclear structures, likely components of the nuclear matrix. However, it is unknown whether this focal distribution could be dependent on certain DNA sequences, that bearing characteristics usually found in matrix attachment regions (MARs), may be necessary to mediate attachment to the nuclear matrix during transient transfections. If this were true, the nuclear matrix association and focal distribution of a transiently transfected plasmid might be a sequence specific rather than a universal phenomenon. Further, Shild *et al (568)*, in an *in vitro* chromatin reconstitution system, showed the importance of nucleosome positioning for the potentiation of the

transcriptional activity of a plasmid DNA template through the interaction of distant (~200 bp) DNA elements that were brought into proximity through nucleosomal packaging.

If, indeed, association of transferred plasmid DNA with the nuclear matrix and nucleosomal packaging play a significant role for its transcriptional activity, then the effectiveness of transient transfections may be dependent on a putative plasmid-matrix interaction and nucleosomal packaging that may occur during cell replication. This would imply that the rate of DNA replication (and the accompanying nucleosomal packaging) during mitosis, rather than the amount of the uptaken plasmid DNA may be more important for determining the levels of reporter gene expression. Indeed the dependence of transcriptional activation of transferred Xenopus *laevis* ß-globin gene constructs on DNA replication was demonstrated by the inability of non replicating plasmids to be transcribed *(569)*. Although non-replicating plasmids can be associated with nucleosomes, it is possible that in some cases accurate nucleosome positioning requires the expression of factors associated with particular stages of the cell cycle or depends on competition kinetics between transcription factors and histones that become favourable during replication. The dependence of transcriptional activation on DNA replication is of importance in cases of the pre-emptive competition model for gene activation *(60)* but not in cases of the dynamic competition model. These two models (described in the **INTRODUCTION**) differ in the ability of transcriptional activators to displace nucleosomes once these are packaged with the DNA but in either case it appears that very complex DNA regions, namely enhancers or locus control regions (LCRs) are necessary for transcriptional activation, since as suggested by Wolffe *(570)* and

Felsenfeld *(60)*, respectively, they "play a primary role in entering into cooperative binding that keep the promoter free of histones".

Although the above data suggest the implication of nucleosomal and nuclear matrix components in the transcriptional activation of transiently transfected plasmid DNA, other studies advocate the inability of this "reconstitution" to reflect accurately the endogenous chromatin configuration. Archer *et al (571)*, showed that although during stable transfections of the MMTV promoter, NF-1 requires a glucocorticoid receptor (GR) mediated disruption of a positioned nucleosome before it can bind its recognition sequence and activate transcription, during transient transfections, the GR function is unnecessary, as NF-1 can access its binding site, likely, due to inefficient nucleosome assembly.

It is conceivable that individual plasmids exhibit distinct behaviours during transient transfections depending mainly on the mechanism that determines their transcriptional activation. This may prove to represent a rather unexplored route for the investigation of the mechanisms that regulate gene expression. Combination of information on the transcriptional activity of a transferred plasmid with information about its nuclear distribution, nucleosomal packaging, association with the nuclear matrix or dependence on DNA replication may offer new insights into the functional significance of DNA regulatory sequences, especially in the context of their interaction with general transcription factors that may control regulatory events such as chromatin assembly and association with the nuclear matrix.

III. Identification of new regulatory sequences within the hCS-B enhancer

Using the heterologous system employed by Walker *et al (507)* to identify the TEF-1 site in the hCS-B enhancer and gel mobility shift as a more sensitive assay for DNA-protein interactions, I identified a functional DNA region within 120 bp upstream of the TEF-1 site. Although distinct, an interaction is suggested between this upstream region and TEF-1, and sequences within this upstream region contain both repressor (RF-1) and derepressor (DF-1) elements. Efficient placental hCS-B enhancer activity is dependent on the presence and function of an intact DF-1 site, which relieves the effect of a potent repressor mechanism that is otherwise able to eliminate enhancer activity in placental cells.

My studies show that the 1-241 fragment of the 1022 bp hCS-B 3'-flanking sequences characterized as an enhancer by Rogers *et al (506)*, maintains the potency and placental specificity of the enhancing activity of the full length 1022 bp sequence. Both the 241 and 1022 bp fragments contain the 22 bp TEF-1 site found in the hCS-A as well as the hCS-B 3'-flanking DNA *(76,506,507)*. Examination by gel mobility shift assay reveals tissue-specific DNA-protein interactions between the 1-80 bp region of the 1022 bp fragment, which is distinct from the 22 bp TEF-1 element at nts 117-139, and transcription factors present in placental or non placental cervical cells.

Furthermore, disruption of DF-1 and RF-1 sequences in the 1-80 bp region can modify the function of the 241 bp enhancer fragment in placental and non placental cells after gene transfer. Mutation of the DF-1 site at nts 63-67 modifies a placental protein-DNA interaction and eliminates the enhancer activity in JEG-3 cells as well as the small enhancing activity of the 241 bp fragment in HeLa cells. However, the DF-1

oligonucleotide had no intrinsic enhancer activity when tested independently from sequences of the 241 bp enhancer fragment. By contrast, modification of the RF-1 region at nts 24-28 increased the enhancer activity of the 241 bp fragment in JEG-3 cells and HeLa cells and a RF-1 oligonucleotide repressed the activities of the TEF-1 and SV40 enhancers in JEG-3 cells.

The absence of a statistically significant effect of a RF-1 mutation in BeWo cells is consistent with the results obtained by Jiang and Eberhardt *(541)* that showed no effect by the mutation of RF-1 in the 241 bp enhancer on CS promoter activity in BeWo cells and would suggest that the effect of RF-1 is fully neutralized by DF-1 in placental cells. It is conceivable that a difference may exist between the ability of a DF-1 mechanism to neutralize repression in BeWo versus JEG-3 cells due to the fact that these two cell lines represent distinct differentiation states and competence in hCS production. This raises the possibility that when a functional DF-1 element is present, the RF-1 repressing activity on enhancer sequences present within the 241 bp fragment is subject to placenta specific derepression, dependent on the differentiation state of syncytiotrophoblastic populations. It is noteworthy, however, that in non corrected CAT assays (not normalized against control luciferase activity) mutation of RF-1 did not alter the effect of the 241 bp fragment on the SV40 promoter function in both JEG-3 and BeWo cells but resulted in enhancing activity of the otherwise inactive 241 bp fragment in HeLa cells *(572)*.

In conclusion, these results suggest that the 241 bp sequences that flank the 22 bp TEF-1 enhancer element contain: (i) one (RF-1) or more negative regulatory elements that are able to diminish TEF-1 activity and (ii) a regulatory (DF-1) element, that neutralizes this repressor mechanism and permits full hCS-B enhancer function in placental cells. The combination of these two (or more) regulatory elements may permit

placental specific enhancing activity of the 22 bp TEF-1 element and limit this activity in cervical carcinoma cells.

Mutation of the DF-1 site blocks derepression and results in a loss of enhancer activity by the 241 bp fragment. It is characteristic that the remaining activity is 5.3-fold lower than the enhancing activity of TEF-1 alone indicating that a potent repressor mechanism inhibits the function of TEF-1 in the absence of an intact DF-1 site. It is possible that DF-1 may exert its effect in placental cells by neutralising the effects of repressing factors on TEF-1 activity, and stabilizing formation of an active complex involving promoter elements. The Sp1 site in the promoter region of the hCS-B gene was shown to be important for efficient hCS-B promoter activity in JEG-3 cells and, thus, placenta-specific expression *(78,508)*.

An interaction between RF-1 and DF-1 is indicated by gel mobility shift competition experiments. The possibility that the interaction between DF-1 and RF-1 is tissue-specific is raised by the observation that the DF-1 oligonucleotide competes effectively for RF-1 complexes in the presence of placental but not non placental nuclear proteins. Further, deletion analysis supports the idea of a negative regulatory element (such as the RF-1 site) that is neutralized by the action of a neighbouring (DF-1) element. A fragment spanning nts 103 to 241 of the 241 bp fragment, that excludes DF-1 as well as RF-1 sites, retained 95% of the enhancing activity of the 1022 bp sequence (*not shown*) in agreement with the value (93%) reported previously *(507)*. Removal of the DF-1 site should result in loss of stimulating activity if sufficient information required for repression was present within the 138 bp (nts 103-241) originally characterised as the enhancer region by Walker *et al (507)*.

Additional data generated during the initial analysis of the hCS-B gene showed

that a 134 bp fragment (nts 1-134) that includes RF-1 and DF-1 but not an intact TEF-1 element retained only 2.4% of the enhancing activity of the 1022 bp sequence *(507)*. This might suggest that no enhancer element is present within this fragment which is also supported by data demonstrating the absence of an intrinsic enhancer activity within the DF-1 oligonucleotide sequences when used alone. However, the results of Jacquemin *et al (540)* point at the possibility that DF-1 combined with additional elements within the 22-104 region may have enhancer activity that is repressed by one or more elements intact within the 1-134 bp region but absent or impaired, due to partial deletion, within the 22-104 region.

In summary, a repressor mechanism has been identified within the 241 bp hCS-B enhancer region. This repressor mechanism inhibits TEF-1 enhancer activity, and one of its components (RF-1 site) is found upstream of the TEF-1 site. A DNA region (DF-1 site) with derepressing activity that is required for efficient 241 bp hCS-B enhancer activity has also been identified. Disruption of the DF-1 region eliminates enhancer function in placental cells. This region is found adjacent to the RF-1 region, and gel mobility shift competition analysis suggests that RF-1 and DF-1 sites may compete for common protein factors or participate in the formation of a common complex. These results indicate that the TEF-1 regulatory region is necessary, but not sufficient for hCS-B enhancer function.

IV. Interaction of the new sequences with the major positive regulatory element (TEF-1) of the hCS-B enhancer

Since TEF-1 is necessary for hCS-B enhancer function, it is likely that the new

elements participate in a TEF-1 mediated mechanism. Indeed, pre-incubation of JEG-3 cell nuclear extract with the 22 bp TEF-1 element reduced the formation of the major complex on the 1-80 bp region, suggesting that the TEF-1 element may compete, directly or indirectly, for some of the factors that interact with the newly identified sequences. However, although TEF-1 factor(s) may participate in complexes formed on elements within the 80 bp region, this does not require a direct protein-protein interaction with DF-1 or RF-1. It is possible that the TEF-1 element could compete for an accessory factor common to complexes formed on the TEF-1 element and 1-80 bp region; a TEF-1 accessory factor or co-activating factor has been described *(573)*. Neither a direct nor an indirect interaction is inconsistent with the weak or no competition for complexes generated by DF-1 or RF-1 labelled oligonucleotides by the TEF-1 element. Unlike the complex formed on the full length 1-80 bp region which includes both DF-1 and RF-1 sites, TEF-1 or the accessory factor might be absent from complexes formed on DF-1 or RF-1 oligonucleotides when these are used alone. The data are consistent with this possibility since mutation of the DF-1 site within the 1-80 bp region inhibits the formation of a complex with the same mobility as that competed by the TEF-1 element. Presumably, this mutation creates a 1-80 bp region with an intact RF-1 but an impaired DF-1 site, that is less effective in supporting the incorporation of either the TEF-1 or the accessory factor into the complex formed. Alternatively, the failure to see little or any competition of DF-1 or RF-1 complexes with the TEF-1 element could reflect the incorporation of the competitor DNA into the complex on the labelled DNA with little change in gel mobility and reflect a limitation of the competition gel mobility assay to assess protein-protein interactions. Confirmation of protein-protein interactions would be resolved by the cloning and expression of the transcription factors involved.

V. Paradigms of repressor-derepressor systems involved in regulation of promoter and enhancer activities

The presence, in the regulatory regions of a gene, of repressors that limit transcriptional activity in non-expressing tissues is a rather common phenomenon. Two silencer elements, about 300 and 500 bp upstream of the mRNA start site, have negative regulatory effects on the rat GH promoter and result in the decrease of its transcriptional activity in non pituitary cells. However, no effect is observed in pituitary cells *(574,575)*. The most proximal silencer (-309 to -266) has been reported to bind a member of the NF-1 transcription factor family *(576,577)*. Similarly, two adjacent regulatory elements, termed elements I and II, described during an early study on the hGH promoter *(232)* result in repression of a minimal hGH promoter (-100hGHp) activity due to repression on the activating element II [nucleotides -275 to -257, similar to adenovirus major late transcription factor (MLTF) or upstream stimulatory factor (USF)] by the repressing element I (single strand DNA binding factor, upper strand: nucleotides -308 to -281, lower strand: -279 to -250). This was observed both in extrapituitary and pituitary cells, however in a previous study Cattini *et al (203)* had shown that deletions of these elements did not affect hGH promoter activity in pituitary cells. This suggested that the tissue-specific control elements in the promoter region of hGH that were first identified in that study (further characterization of these elements and the corresponding regions of the rat GH promoter lead to the identification of Pit-1/GHF-1) or additional upstream elements, presumably neutralize the element I-mediated repression in pituitary cells *(232,203)*.

A repressor element was also described in the promoter region of the rat prolactin (rPRL) gene and localized between nts -127 and -116 (5'-GATGTTTAAAATT-3') *(578)*. This region shares similarities with the 3' extended half of the DF-1 region (5'-ctagGATGTTTtctAAA-3') which is essentially the part of DF-1 mutated by Jiang and Eberhardt *(541)* in their analysis. A mutation (5'-GATGTTgtcgAc-3') introduced in this region of the rPRL gene resulted in the relief of repression in non pituitary cells *(578)*. In contrast, a 25% reduction of expression was obtained after transfection of pituitary GH4 cells. Similar results to the ones obtained in pituitary cells were generated by a clustered point mutation analysis *(579)*. In an independent study, of the same rPRL DNA region, a similar mutation (5'-GATGgggtaccc-3') did not result in an increase of expression in non pituitary Rat-1 cells co-transfected with Pit-1, but rather in a small decrease. These data and the results from a deletional analysis by Jackson *et al (578)* indicated that the repressor element was active in non pituitary cells but not in pituitary cells unless certain upstream cell-specific elements were deleted. This, as well as the studies of Peritz *et al (232)* and Cattini *et al (203)*, represent examples of pituitary derepression of a negative regulatory mechanism that is similar to the one described here for DF-1 in placental cells.

Further, an additional element was identified in the interfootprint region between footprints I and II of the rPRL promoter *(578)*. The activity of the promoter was dependent on the presence of this region and two distinct interactions were demonstrated by gel mobility shift assays. Low affinity or specificity of these interactions, under certain experimental conditions, has presumably resulted in the absence of DNAase I protected regions by footprinting experiments. Similarly, during an early footprinting

analysis (*not shown*), no nuclease protection over the DF-1 region was detected, in agreement with previous results by Walker *et al (507)*, that only detected protection over the TEF-1 region. The identification of functionally significant DNA elements by gel mobility shift assays and mutational analysis in the present study and that of Jackson *et al (578)*, despite the absence of readily detectable DNA-protein interactions by footprinting analysis, suggests the presence of classes of transcription factors that, under certain experimental conditions, demonstrate unstable DNA-binding profiles. Since three distinct patterns of DNAase I protection were detected over DF-1, by Jiang and Eberhardt *(541)*, Jacquemin *et al (540)* and the present analysis, and three distinct DNA elements may be present within the DF-1 region (PSF-B1$^{7/8}$, ets$^{7/8}$ and Oct$^{8/8}$, *see Figures 37 and 38 and p. 151 and 152 in* **RESULTS**), it is likely, that multiple factors and cooperative interactions are responsible for complex formation in this region. This would make the presence of several factors crucial for the detection of DNAase I protection and thus more sensitive to the variation of the concentration and the functional integrity of these factors. This could explain the discrepancy between the results of our initial analysis as well as the analysis of Walker *et al (507)* and the analyses presented in Jiang and Eberhardt *(541)*, Jacquemin *et al (540)*, as well as in this thesis *(Figure 38 p. 152)*.

VI. Similarities of DF-1 and RF-1 with characterized DNA elements

A search for the RF-1 region (5'-TGGTGTGG-3') revealed similarity to the TEF-2 site (5'-gGGTGTGG-3') present within the SV40 enhancer. TEF-1 and TEF-2 cooperate in enhancer activity although they bind independently to their motifs *(536)*.

This suggests the existence of a mechanism that uses TEF-1 and TEF-2 in an indirect mode of interaction similar to the mode of interaction described in this thesis for TEF-1 and RF-1. Although RF-1 appears to have a negative effect on TEF-1, this is not inconsistent with the fact that TEF-1 and TEF-2 cooperate in SV40 enhancer activity. Additional elements within the SV40 and hCS-B enhancers could modify the function of the RF-1 and TEF-2 sites even if these recognized the same protein factor(s). It is interesting, that the PSF-A site, which also has similarity with the TEF-2 site (5'-tGGTGTtGG-3' versus 5'-GGGTGT:GG-3'), represses in a pituitary specific mode the hCS promoter in the presence of the hCS-B enhancer *(509)*.

A search for the DF-1 region (5'-TAGGATGT-3') located an element with very similar sequences (5'-gAGGATGT-3') in the 3' enhancer of the T-cell receptor gene (*Figure 39*). Like the sequences adjacent to the PSF-B site and upstream of all the

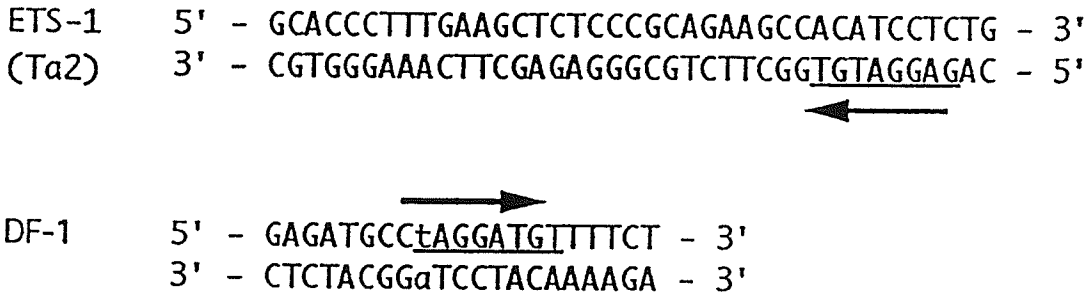


FIGURE 39: Comparison of the DF-1 site (5'-TAGGATGT-3') to an ets-1 element (5'-gAGGATGT-3') in the 3' enhancer of the T-cell receptor gene

placental members of the growth hormone gene family on chromosome 17, it is in the reverse orientation relative to the DF-1 region. This element represents a binding site for the proto-oncogene ets-1 which is expressed in lymphoid tissues and codes for a nuclear

protein involved in transcriptional activation *(580-582)*. Mutation by 5 bp substitutions of the core of the ets-1 binding site (5'-ctcctTGT-3') resulted in a 95% loss of enhancer activity with a SV40 promoter system *(580)*. Mutation of the DF-1 site in essentially the same core sequences (5'-TttacgGT-3') resulted in a 97.5% loss of hCS-B enhancer activity using the same SV40 promoter system in the present studies. It is interesting, that although Ets-1 may be restricted to lymphoid cells, a relatively abundant Ets binding activity was described in trophoblasts *(583)* and must, presumably, be attributed to a ubiquitous or a trophoblast member of the Ets family.

VII. The ets family of transcription factors

The ets family of proto-oncogene proteins [consensus binding sequence $^{G}/_{C}{}^{A}/_{C}GGA^{A}/_{T}G^{C}/_{T}$ *(584,585)*] is involved in transcriptional activation of various genes *(580-583,585-590)*. Transcriptional activation of ets regulated genes is depended on functional ets elements located in either promoter sequences, such as the Moloney murine sarcoma virus long terminal repeat (MMSV-LTR) that contains the ets-related PU.1 binding site, or enhancer regions, such as the 3' flanking T cell-specific enhancer of the T cell receptor α gene that contains an ets-1 site *(580-583)*.

Ets genes are implicated in the formation of organs, such as the lungs *(591)* that require extensive tissue remodelling during their development that results in a complex histological and anatomical structure. The proto-oncogenes c-ets-1 and -2 have been shown to activate transcription of the stromelysin and collagenase genes *(589,590,592)* which are highly expressed in transformed cells and tumors *(592-597)*. This gene codes

for one of the matrix metalloproteinases that degrade extracellular matrix and basement membrane components and are involved in invasion and metastasis of tumor cells *(597-601)*.

Extensive uterine remodelling during the implantation process, the invasion by trophoblasts and the subsequent growth of the placenta essentially reflects "pseudomalignant" phenotypes where invasion and "metastasis" occur in a programmed and controlled mode. It is conceivable that ets proteins expressed in the cytotrophoblasts initiate a series of morphological and biochemical changes in these cells that differentiate and develop their hormone producing potential. In this context ets proteins might be responsible for a placenta specific relief of the suppression exercised on the TEF-1 enhancing action and induction of high levels of hCS expression in the mature syncytiotrophoblast.

VIII. Presence of ets and TEF-2 like sequences in regulatory regions of placentally and pituitary expressed genes

If ets proteins have, indeed, a role on placental developmental processes as well as on placenta- versus pituitary-specific expression of the hGH/CS gene family, ets elements should be present in the regulatory regions of other placentally and/or pituitary expressed genes, as well.

To examine if this were the case a manual (non computerized) search, for putative ets and TEF-2 binding sites within regulatory regions of placentally and pituitary expressed genes from various species, was conducted. The results of this search, that include putative binding sites with a nucleotide similarity to the ets consensus binding site

[$^{C}/_{G}{}^{A}/_{C}$GGA$^{T}/_{A}$G$^{T}/_{C}$ *(584,585)*] (or to the SV40 TEF-2 site) of 6 out of 8 or better, are presented in *Tables 13-15*. Although there is some redundancy even within the ets core ($^{C}/_{A}$GGA), only sites with an AGGA ets core, identical to the one present in the DF-1/ets[7/8] site, were sought during this search.

a. Human GH-V/CS P-sequences and hCS-A promoter

The hGH-V/CS P-sequences were the first to be examined due to their involvement in pituitary repression of hGH/CS genes. As mentioned before, RF-1 mutation interrupts a TEF-2-like sequence (tGGTGTGG, mutated to tacgtcGG) that differs only in one base pair from the TEF-2 (GT-IC) motif (GGGTGTGG) of the SV40 enhancer. The TEF-2 element may be related to the AP3 transcription factor complex that has been shown to bind to the GT-IC motif *(602-604)*. Additionally the same mutation disrupts a PSF-A like sequence (TGGTGT:GG::ACggCAA). PSF-A (TGGTGTTGGCAACCAA) contains a 9 bp sequence that differs in two nucleotides (a substitution and an insertion) from the TEF-2 element (tGGTGTtGG) and is placed in a 3' to 5' orientation 2 Kb upstream of the promoters of all the placental members of the hGH gene family. Pituitary specific repression of the hCS-A promoter has been shown to be mediated by PSF-A *(509)*. Two additional TEF-2 elements are located within 150 bp upstream of PSF-A. The first (GGGTGcTGG) is located approximately 130 nts upstream of PSF-A and the second (GGGTGTGG) is overlapping with two tandem repeats GGGTGT-GGGTGT that are located at the upstream border of the PSF-A DNAase I protected region and 14 nucleotides downstream of a second DNAase I protected region termed PSF-B, also involved in pituitary specific repression of the hCS-

A promoter.

DF-1 mutation disrupts an ets-like (single nucleotide insertion) element (CtAGGATGT mutated to CtttacgGT) part of an 11 bp sequence (GCCtAGGATGT) that is also present in a 3' to 5' orientation on the downstream border of the PSF-B DNAase I protected region within 50 nucleotides upstream of the PSF-A element. Interestingly a a sequence similar (7/8 nucleotides identical) but clearly distinct from ets (GAG:ATGCC, lacks the $^{A}/_{C}$GGA ets core) lies 5' next to the DF-1 element. A very similar 8 bp sequence with one additional nucleotide insertion (GAG:ATGgC, hereafter PSF-B1) is present within the DNAase I protected PSF-B region. Mutation of PSF-B1 together with additional 10 bp of immediate downstream sequences abolishes pituitary specific repression by the PSF-B element. The PSF-B1-like element immediately upstream of DF-1/ets$^{7/8}$ could be an additional candidate (apart from RF-1) for the JEG-3 (and possibly HeLa) repressor activity present within the 103 bp region of the 241 bp fragment that does not include TEF-1. The removal of a G from the core of the ets binding site in PSF-B1 and the PSF-B1-like sequence in the DF-1 region, suggests that a different class of transcription factors recognizes these motifs since the $^{A}/_{C}$GGA core rather represents a requirement for ets binding *(584,585)*. In fact, PSF-B1 has extensive similarity (8 out of 10) with the NF-GMa binding site (GAGATtGCAt) that is involved in cytokine gene activation *(584)*. The PSF-B1-like sequence of the DF-1 region (GAGATtgCat), however, has reduced similarity (6 out of 10) with NF-GMa.

Two additional sequences containing core ets-like sequences are present within 100 bp upstream of the PSF-B element (CAGGA:GT and aAGGA:GC) in various (see *Table 13*) members of the placental members of the hGH gene family.

b. Placentally expressed genes of various species

Promoter and upstream regulatory sequences from seven genes expressed in either the placenta or decidua of different species [mouse PL-I, mouse proliferin 3 (PLF3), rat PLP-A, monkey GH-V, human decidual PRL, human pro-GnRH and human CGß5

Table 13. Distribution of putative ets and TEF-2 sites in regulatory regions of the hGH/CS family.

HUMAN hGH/hCS FAMILY			
GENE	BINDING SITE	REGION AND NUCLEOTIDE POSITION	SEQUENCE
hCS-A	ets$^{7/8}$ DF-1$^{7/9}$ (ets$^{6/8}$)	PROM. -133/-126 -2/+7	GAGGA:GC CTAGGATcc
ALL but hGH-V	ets$^{6/8}$	P-SEQ. +110/+116	aAGGA:GC
ALL but hCS-L	ets$^{6/8}$	+92/+98	CAGGAA:T
hCS-L	ets$^{7/8}$		CAGGA:GT
ALL	DF-1$^{7/8}$ (ets$^{6/8}$, no AGGA core)	+141/+148	GAGATGgC GAG:ATGg
ALL	DF-1$^{11/11}$	+174/+164	GCCTAGGATGT
hCS-L hGH-V	TEF-2$^{8/9}$	+69/+78	GGGTGcTGG
hCS-A	TEF-2$^{7/9}$		GGGTGcTaG
hCS-B	TEF-2$^{7/9}$ RF-1$^{8/9}$		TGGTGcTGG
ALL	TEF-2$^{8/8}$	+180/+188	GGGTGTGG

subunit gene *(430,550-552,559,605-613]* were analyzed for presence of putative ets and TEF-2 binding sites.

A very interesting pattern of distribution of ets sequences exists within DNAase I protected sequences that overlap with a cyclic AMP responsive region (-311 to -202) in the upstream promoter sequences of the hCGß5 gene *(605-607,559)*. These sequences [GAGGCTTCGGCCCCGTGGGCAGGACACACCTCCTGCGGGCCTATTC, nucleotides -321 to -276; and TTTCCGGGGACCGCTCCGGGCATCCTGGCTTGAGG-GTAGAGTGGG, nucleotides -243 to -199) contain an $ets^{8/8}$ (nucleotides -223 to -216), and an $ets^{7/8}$ (nucleotides -294 to -287) binding sites (underlined) both in a 3' to 5' direction. Additionally a sequence immediately upstream of $ets^{7/8}$ (CAGGAcaC, nucleotides -302 to -295, in bold), and two sequences in regions not protected from DNAase I digestion (GAGGAcaT, nucleotides -323 to -330 and tAGGAAcC, nucleotides -187 to -194), represent $ets^{6/8}$ sites.

The -223 to -216 $ets^{8/8}$ element is placed between the cores of two of the four trophoblast specific (TSE) elements (gCTCCGGG, nucleotides -230 to -223 and CTTGAGGG, nucleotides 214 to -207, TSE core consensus, CCNNNGGG) present within DNAase I protected regions in the hCGß5 promoter, while the neighbouring -302 to -295 $ets^{7/8}$ and -287 to -294 $ets^{6/8}$ are positioned between and partially overlap with the cores (CCCGTGGG, nucleotides -310 to -303 and CCTGCGGG, nucleotides -290 to -283) of the remaining two TSE elements.

Interestingly the -187 to -194 and -323 to -330 $ets^{6/8}$ sites overlap with an additional TSE core sequence (CCTAAGGG, nucleotides -190 to -182) and a GATA element (AGATAA, nucleotides -335 to -330; GATA consensus WGATAR), respectively,

present within regions not protected from DNAase I digestion. These elements are presumably, not able to form stable protein complexes in spite of their similarities to the GATA and TSE elements present in the hCGα gene (-136 to -131 and -152 to -145, respectively), which do not overlap with putative ets binding sites and are able to interact with proteins *(605)*.

A similar close co-existence of ets and TSE elements is observed within 35 nucleotides in the rat PLP-A promoter region. An ets$^{8/8}$ sequence, a DF-1$^{7/8}$, two ets-related PEA3$^{6/6}$ elements (AGGAAG) and an ets$^{6/8}$ sequence are present within 0.5 Kb of rPLPA promoter sequences *(613)*. Interestingly, the ets$^{6/8}$ sequence (CAGGActC, nucleotides -116 to -109), lies next to a TSE core sequence (CCcGTGGG, nucleotides -107 to -100) that also represents a putative AP-2 site). Further, among the six TEF-2-like sites that are present within upstream rPLPA promoter sequences, one TEF$^{7/8}$ site (GGGTGTaG, nucleotides -128 to -121) overlaps with a second TSE core sequence (CCTTAGGG, nucleotides -134 to -126).

Finally, in a recent report *(610)*, 491 bp of upstream promoter sequences, were shown to confer cAMP responsiveness to the monkey GH-V gene. This region contains four putative ets elements: an elf-1$^{9/11}$ [AGGAGGAAAgg, nucleotides -382 to -372; elf-1: AGGAGGAAAAA *(587)*], two ets$^{7/8}$ (CAGGATtC, nucleotides -472 to -479; and CAGGATcC, nucleotides +1 to +8, overlaps the transcription initiation site) and an ets$^{6/8}$ [GAGGAgcT, nucleotides -133 to -126, overlaps with the distal Pit-1/GHF-1 site that is also the region equivalent to the Sp1 site of the hCS promoter *(508,611)*] that lays immediately downstream of a RF-1$^{7/8}$ sequence (T:GTGTGG, nucleotides -340 to -334).

The presence of ets sites in regulatory regions of the hCGß5 and monkey GH-V

genes that confer cAMP responsiveness, is consistent with a putative role of ets in cAMP-mediated pathways. Recently, a new ets cDNA (TH-ets), was obtained by screening an ES embryonic tumor cDNA library with a yeast two-hybrid system, in an attempt to clone transcription factors interacting with CBP (CREB Binding Protein, binds the PKA phosphorylated form of CREB) a likely cofactor for CREB *(608)*. Our published *(609)*, as well as yet unpublished data *(561)* suggest that apart from the activation of hCG by cAMP both the CS and hGH-V genes are also regulated by cAMP.

A list of ets and TEF-2 sites found in the regulatory regions of the various placentally expressed genes are included in *Table 14* .

Table 14. Putative ets and TEF-2 sites present in regulatory regions of placentally expressed genes from various species

PLACENTAL EXPRESSION			
GENE	BINDING SITE	REGION AND NUCLEOTIDE POSITION	SEQUENCE
mPL-1	ets$^{8/8}$ PEA3$^{6/6}$ ets$^{6/8}$ TEF-2$^{7/8}$	PROM. -128/-121 -108/-102 -259/-265 -52/-45	GAGGATGC -AGGAAG- CAGGA:Ga GGGTtTGG
mPLF3	ets$^{7/8}$ ets$^{6/8}$ TEF-2$^{7/8}$ RF-1$^{7/8}$ (TEF-2$^{6/8}$)	PROM. -271/-264 -63/-70 -72/-79 -638/-631 -476/-469 -926/-919	GAGGAAGa CAGGAAGa aAGGAcGT CAGGAgaT GGGTGTtG TGGTtTGG

PLACENTAL EXPRESSION			
rPLP-A	ets$^{7/8}$ ets$^{6/8}$ PEA3$^{6/6}$ DF-1$^{7/8}$ (ets$^{6/8}$) TEF-2$^{7/8}$ RF-1$^{7/8}$ (TEF-2$^{6/8}$)	PROM. -377/-384 -116/-109 -543/-538 -27/-19 -129/-122 -925/-919 -954/-948 -962/-955 -151/-144 -43/-36	CAGGAAtC CAGGActC -AGGAAG- TAGGAgGT GGGTGTaG TGG:GTGG TGG:GTGG TGGTGgGG TGGTGTtG TGGTGTGa
hGnRH	PEA3$^{6/6}$ (GA-BP$^{5/5}$) GA-BP$^{5/5}$ RF-1$^{7/8}$ (TEF-2$^{6/8}$)	PROM. -450/-445 (PLAC.) -491/-487 -597/-590,	-AGGAAG- --GGAAG- TGGTGgGG
hPRL	ets$^{8/8}$ ets$^{7/8}$ ets$^{6/8}$ DF-1$^{7/8}$ (ets$^{6/8}$)	PROM. -76/-69 (DEC.) -12/-19 -1354/-1347 -195/-203 -175/-182 -626/-633	CAGGAAGT GAGGAtTC CAGGATGg CAGGAgaC TAGGAgGT TAGGATaT
monkGH-V	elf-1$^{9/11}$ ets$^{7/8}$ ets$^{6/8}$ RF-1$^{7/8}$	PROM. -382/-372 -472/-479 +1/+8 -133/-126 -340/-334	AGGAGGAAAgg CAGGATtC CAGGATcC GAGGAgcT T:GTGTGG
hCGß5	ets$^{8/8}$ ets$^{7/8}$ ets$^{6/8}$	PROM. -216/-223 -287/-294 -302/-295 -323/-330 -187/-194	CAGGATGC CAGGAgGC CAGGAcaC GAGGAcaT tAGGAAcC

c. *Pituitary expressed genes from various species*

The human, equine, murine, rat and bovine CGα subunit gene promoters, the rat prolactin promoter and the salmon CG IIß subunit gene promoter, were analyzed for the presence of ets and TEF-2 sequences. The human and equine CGα genes are expressed both in placenta and pituitary, however, the bovine, rat and murine CGα genes are expressed only in pituitary. The salmon gonadotropin IIß subunit gene is expressed in pituitary cells distinct from the cells expressing the salmon gonadotropin I gene *(242,605,614-616)*.

An ets$^{8/8}$ element (CAGGATGT, nucleotides -286 to -279, numbers correspond to human sequences), is conserved between the promoter regions of the human, equine, murine and bovine CGα genes but is not present in the rat CGα gene *(605,614)*. An ets$^{6/8}$ sequence (GAGGAcgT, nucleotides -16 to -9, numbers correspond to human sequences) is conserved between the human and bovine CGα genes but is replaced by an ets$^{8/9}$ sequence (GAGGAcAGT, bears a single nucleotide insertion compared to a consensus ets$^{8/8}$ element) in the equine CGα gene. In contrast it is absent from the rat and murine CGα genes, however, a very similar ets$^{7/9}$ sequence is present in a different region of the rat CGα gene (aAGGAcAGC, nucleotides -134 to -142).

A number of ets related sequences are also present in the 5' flanking region of the salmon gonadotropin ß II gene *(615,616)*. One ets consensus sequence and eight ets$^{7/8}$ consensus site are present within 3.4 kb of 5' flanking sequences. Three of the putative ets$^{7/8}$ elements contain GA-BP$^{5/5}$ binding sites (GGAAG) for the GA binding protein (GA-BP). An additional GA-BP$^{5/5}$ site is also present within the same 3.4 kb sequences.

Two sequences appear particularly interesting. The first spans 14 nucleotides,

represents a DF-1[11/14] region (CaAGGAaGTcTTCT, nucleotides -2333 to -2320) and flanks an ets[7/8] site (aAGGAAGT) which contains a PEA3[6/6]/GA-BP[5/5] site (underlined). The second is a region termed the **GTH box** (sequence highly homologous to sequences present in the 5' regions of the FSH ß and LH ß mammalian genes) and shares a 18/25 nucleotide similarity with a region that flanks the DF-1 site in the 241 bp CS-A enhancer and 17/25 with the homologous region of the CS-B gene. Interestingly the **GTH box** bears the same substitution that the DF-1 site of the CS-A enhancer bears relative to the CS-B enhancer (TAGGgTGT instead of TAGGATGT). Further, it is noteworthy that the proximal 5' sequences (-95 to -35) of the salmon gonadotropin IIß gene contain two silencer elements. The most proximal element contains an ets[7/8] site in a 3' to 5' orientation just 5 nucleotides upstream of the TATAA box. A 13 nucleotide region that contains and flanks the ets[7/8] site is similar to a silencer element in the chicken lysozyme gene (*615,617*).

Table 15 contains a list of putative ets and TEF-2 sites present in the regulatory regions of these pituitary expressed genes *(242,605,614-616)*.

d. Putative ets elements in the regulatory regions of placentally expressed genes are conserved across species

It is evident from the above genomic DNA sequence analysis (*Tables 13-15*), that a widely spread occurrence of putative ets elements is conserved across species. Most of these sequences have passed unnoticed until today and thus no functions have been ascribed to these putative cis-elements. However, it appears that the presence of ets

sequences within all placentally expressed genes examined, far exceeds that predicted by a random distribution. This suggests a conservation of these DNA sequences through evolution and leads to the hypothesis that ets elements and their corresponding transcription factors, are functionally significant for placenta-specific gene expression.

Table 15. Putative ets and TEF-2 sites present in regulatory regions of pituitary expressed genes of various species.

PITUITARY EXPRESSION			
GENE	BINDING SITE	REGION AND NUCLEOTIDE POSITION	SEQUENCE
rPRL	$ets^{8/8}$ $ets^{7/8}$ $PEA3^{6/6}$	PROM. -71/-64 -157/-164 -178/-185	GAGGATGC CAGGAAGa -AGGAAG-
$CG\alpha^{h,c,mu,b}$ $CG\alpha^{h,b}$ $CG\alpha^{c}$ $CG\alpha^{r}$	$ets^{8/8}$ $ets^{6/8}$ $ets^{8/9}$ $ets^{7/9}$	PROM. -286/-279 -16/-9 -134/-142	CAGGATGT GAGGAcgT GAGGAcAGT aAGGAcAGC
Salmon CG IIß	$ets^{8/8}$ $ets^{7/8}$ ($PEA3^{6/6}$: underlined) $ets^{6/8}$ $GA\text{-}BP^{5/5}$ $DF\text{-}1^{8/8}$ (hCS-A) $DF\text{-}1^{7/8}$ (hCS-B)	PROM. -153/-146 -3231/-3224 -3184/-3177 -2332/-2325 -2114/-2121 -1804/-1811 -757/-750 -307/-314 -35/-42 -2147/-2154 -2102/-2109 -653/-646 -156/-149 -2120/-2124 -234/-227	GAGGAAGT GAGGAAGa GAGGATtT aAGGAAGT CAGGAATg CAGGAgGT GAGGAAtT CAGGAAGg GAGGAgGC CAGGATaa GAGGATca aAGGAAaC CAGGAgGa --GGAAG- TAGGGTGT TAGGgTGT
hGH	$ets^{7/8}$ $DF\text{-}1^{7/9}$ ($ets^{6/8}$)	PROM. -133/-126 -2/+7	GAGGA:GC CaAGGATcc

IX. Paradigms for ets dependence of the promoter activity of placentally expressed genes

To further support the above hypothesis, a search (MEDLINE, SilverPlatter 3.11) for reports that have established the contribution of ets elements to the regulation of placentally expressed genes, was conducted.

Two reports that suggest the functional significance of ets sequences for placenta-specific expression of the FBP (Folate Binding Protein) and F-type of the PFK-2/FBPase-2 (6-phosphofructo-2-kinase/fructose-2,6-bis-phosphatase) genes, have appeared to date *(618,619)*. In both cases GGAAG motifs, which represent binding sites for the GA-binding protein, a member of the ets family, were found in the promoter regions of the FBP and PFK-2/FBPase-2 genes.

Interestingly both genes are presumably dependent on Sp1 mediated initiation of transcription, since no TATA boxes were found in their promoter regions. The structural interaction of the Sp1 and GGAAG sites in the promoter of the FBP gene with Sp1 and GA-BP was demonstrated by gel shift and supershift mobility assays while the functional significance of the Sp1/ets region was shown by the ability of this region to direct CAT expression in 3T3 mouse cells. No mutational analysis, however, was used to demonstrate the functional importance of these sites independent of each other. In contrast, mutagenesis demonstrated that of one of the ets sites (shown to be a binding site for the rat GA-BP) in the F-type promoter of the PFK-2/FBPase-2 gene was mainly responsible for transcriptional activity. It is noteworthy that the hCS promoter activity is also dependent on Sp1 supporting the possibility that DF-1 may be involved in enhancing Sp1 mediated transcription through interactions with other CS-B enhancer

binding factors like RF-1/TEF-2 and TEF-1.

An additional gene, L-plastin, that is normally expressed at high levels in hematopoietic cell types, but is expressed ectopically in 79% of cancers of the female reproductive tract including placental tumors, was also reported to contain 4 potential ets elements as well as an Sp1 element in its 5' flanking region *(620)*.

X. Modified DF-1 sites are present in the non functional hCS-A and hCS-L enhancer regions.

A comparison of the 241 bp hCS-B enhancer region with the equivalent region of the hCS-A sequences revealed identity in 235 out of 241 nts. Although the 22 bp TEF-1 protected region is conserved between the two genes, the DF-1 region in the hCS-A sequences bares one substitution (5'-CTAGGgTGT-3') and the region between the RF-1 and DF-1 sites (termed AF-1, *for a more detailed description see p. 216 in* **PROSPECTS**) shows one substitution and one deletion. The other three substitutions occur at nucleotides 86, 150, and 202. It is noteworthy that Saunders's group *(506,507)* first reported that the hCS-A "enhancer" region, when included in a 5.7 Kb fragment that flanks the hCS-A gene, was inactive in JEG-3 cells after gene transfer suggesting the importance of those modifications for hCS enhancer function. Also Jacquemin *et al (540)*, were driven to the same conclusion after examining the respective regions of the hCS-A and hCS-L genes that corresponded to the hCS-B enhancer region they used in their studies [-156-(+)145]. It is possible that the increased complexity in the control of the hCS enhancer regions and the competition between positive regulatory elements may

contribute significantly to the developmental control of gene expression of the hCS-A and hCS-B genes. These genes are expressed at different levels during different stages of placenta development throughout pregnancy *(123)* suggesting that the hCS enhancer sequences may have the flexibility to mediate the effects of a variety of positive or negative regulatory mechanisms.

It is possible, however, that a "salvage" of hCS-A enhancer function *in vivo* occurs through interactions with additional sequences in the hCS-A gene, such as promoter sequences or possibly, the highly similar PSF-A and PSF-B regions. Assuming that the nucleotide substitutions over the DF-1/ets (also over the AF-1 region as suggested by preliminary functional analysis by mutagenesis of the AF-1 region, *see p. 216 in* **PROSPECTS**) knock out hCS-A enhancer function due to failure to derepress, an attractive alternative possibility is that, repressor sequences within the hCS-A enhancer region become differentially methylated and thus inactivated at some time during embryonic or placental development.

F. SUMMARY AND SIGNIFICANCE OF THIS PH.D RESEARCH

The studies presented in this thesis cover a broad area of the current endocrinological research on the hGH/hCS family of genes.

The various protein gene products of the hGH/hCS and hPRL family of genes have common but also different properties which have helped in the assessment of the function of different protein domains and their interactions with the corresponding receptors of the cytokine receptor superfamily. Additionally, in spite of their great similarity hGH/hCS genes are expressed in different tissues providing a model system

for the study of tissue specific gene expression.

Members of the hGH/hCS family have been implicated in a variety of biological effects ranging from cell proliferation to tissue specific differentiation processes. Human GH has been hypothesized, and in certain cases shown, to be a "priming" factor for cells before they are able to respond to a growth stimulus (i.e. a growth factor). Similarly hCS has been implicated in fetal growth and other functions during pregnancy. Abnormal cell growth has been shown in few cases to be supported by the presence of hGH/hCS/hPRL although the production of these proteins by tumor cells was not rigorously established. Certain abnormal situations appeared to be accompanied with abnormal expression of hGH/hCS.

Starting from the biological effects of the conditioned medium of serum free grown human Burkitt tumor lymphocytes on Nb2 cells, I demonstrated with a series of studies, and in contrast to the preexisting literature, that biologically active hGH and not hPRL is likely to be the autocrine activity produced by these cells. With this, I showed that the hGH/hCS family is transcriptionally active in an extrapituitary and extraplacenta tissue in spite of the probable absence of "required" permissive transcription factors for the expression of these genes.

Further, I showed that a variety of regulatory mechanisms must be responsible for the expression of individual "placental" members of the hGH/hCS family. Although only one placenta specific DNA regulatory enhancer region (3' of the hCS-B gene) and one transcription factor (TEF-1) had been identified at that time to regulate placental expression in the hGH/hCS locus, I demonstrated that the relative levels of expression of hCS versus hGH-V genes are greatly different between various normal or abnormal cell populations of placental origin. The demonstration of differential expression in these

cell populations raised questions about the possible involvement of the hGH/hCS genes in placental abnormalities. It also indicated that a single regulatory event (i.e. the TEF-1 interaction with the hCS-B enhancer region) could not be responsible for the regulation of all the placental genes of this family.

Additionally, because of the fact that hCS expression in aberrations of placental development such as hydatidiform moles and choriocarcinomas is greatly reduced relative to the normal tissue, certain transcription factors that contribute to hCS expression may be, functionally impaired (due to low levels of expression or mutations) in these pathological situations. Thus, transcription factors involved in the tissue specific expression of the hCS genes may be also involved in the pathogenesis of certain placental abnormalities. It is conceivable that either the hGH/hCS proteins or the transcription factors regulating their expression may have direct contribution to the pathophysiological changes that lead to impaired placental development, such as in cases of spontaneous abortions or preeclampsia, or tumor formation and preservation.

This general hypothesis postulates that studies on the characterization of the regulatory sequences of hCS genes are going to identify DNA elements and factors that determine not only placenta specific expression, but placental growth, development and differentiation, as well. This led to the designing of detailed studies on the characterization of the hCS-B enhancer region, in an effort to identify new DNA regulatory elements, involved in the placenta-specific expression of this gene. The choice of this region was suggested by the intriguing observation that although TEF-1 was rather necessary for placenta specific expression, it did not demonstrate a placenta-specific DNA binding profile.

My studies led to the identification of two new DNA sequences with tissue-

specific gel mobility shift profiles and the unravelling of a complex repression-derepression mechanism that may explain why the hCS-B enhancer is only functional in placental cells. With these experiments I have generated data that indicate the functional significance of the DF-1 and RF-1 sites and their structural interactions with placental, pituitary and/or cervical nuclear proteins, as well as suggest interactions between DF-1 and RF-1 protein complexes. Additionally gel mobility shift competition experiments and mutational analysis suggest interactions between the DF-1 and TEF-1 elements. This is significant since although TEF-1 confers neither full nor tight tissue-specific enhancer activity, its interactions with factors that demonstrate a degree of tissue specificity, like DF-1 and/or RF-1, may secure placenta-specific enhancing activity.

An exciting finding of these studies, that satisfies the predictions deriving from the above general hypothesis, was the extensive similarity of the DF-1 binding site with the binding sites for a family of transcription factors. DF-1 had a 7/8 nucleotide identity with a binding site for the proto-oncogene ets-1. RF-1 had also a 7/8 nucleotide identity with a binding site for the TEF-2 factor. Ets-1, as well as other members of the ets family, appears to be an important gene for both normal developmental processes and malignant transformation, while TEF-2 has been shown to act synergistically with TEF-1, in the context of the SV40 enhancer, to regulate the transcriptional activity of the SV40 promoter.

The identification of RF-1 and DF-1 and their association with TEF-2 and Ets proteins, points at the possible involvement of a proto-oncogene in the expression of hCS. This may explain the remarkable decline in hCS levels in the case of placental abnormalities, if ets proteins prove to be functionally impaired in these pathological conditions. Purification and cloning of DF-1 and TEF-2 will allow to assess directly the

protein-protein interactions, tissue distribution and developmental pattern of expression as well as the possible effects of these factors on development or other cellular processes.

The placenta is a tissue with multipotent ability for gene expression. Failure in placental development or inability to express its products will result in embryonic death or severe congenital abnormalities. Because of the close connection between tissue specific gene expression and tissue phenotype, tissue specific transcription factors, like DF-1, are possibly involved in the pathology of placenta abnormalities such as hydatidiform moles and choriocarcinomas in which the normal biochemical and structural phenotype is altered. Uncovering the complexity of regulatory regions, identification of the synergistic or antagonistic interactions between positive and negative regulatory elements and cloning of factors that rule placental function and specific gene expression in this tissue would create a number of possibilities for new diagnostic tools and therapeutic agents for problematic pregnancies and/or congenital abnormalities.

G. PROSPECTS

A number of questions generated by these studies will provide an axis for future work in this field.

I. DNA sequence requirements for the repressor mechanism identified within the hCS-B enhancer

The precise sequence requirements and protein factors associated with the repressor activity present in this 240 bp fragment remain to be resolved.

In a preliminary analysis for additional sequences within the 241 bp enhancer, competition experiments show that 62 bp fragments spanning regions RF-1 and DF-1 could inhibit the major complex formation by JEG-3 nuclear extracts regardless of the presence of either one or both RF-1 and DF-1 mutations *(621)*. This indicates that additional sequences within the 62 bp fragment other than the mutated elements are also involved in direct DNA-protein interactions absolutely essential for complex formation. Stable and functional complex formation, however, requires also wild type DF-1 sequences as indicated by gel mobility shift assays using DF-1 mutated labelled DNA fragments and the functional analysis. Further, a RF-1 mutation does not restore the enhancer activity of the DF-1 mutant 241 enhancer fragment *(621)*, which in combination with the deletional analysis of Jacquemin *et al (540)* and Jiang and Eberhardt *(541)* suggest the presence of a complementary repressor mechanism.

An enhancing activity, equivalent to TEF-1 enhancing activity when alone, is present within 22-102. This activity is inhibited by sequences overlapping or within 1-22 and/or 102-134 since the 1-134 fragment maintains 7-fold lower enhancing activity (2.4% of the enhancing activity of the full length 241 bp enhancer) than the 22-104 fragment (17% of the enhancing activity of the 241 bp enhancer). Repressor activity may be also present between nucleotides 57-103 as suggested by the comparison of the relative, to the 1-241 enhancer, activity of nucleotides 57-242 [22.5% *(541)*] to the relative activity of nucleotides 103-241 [93-95% *(507,539)*] providing that the difference in enhancing activity between the two fragments is not due to promoter specific interactions.

The identified footprints over the 57-103 region *(Figure 37 and 38 p. 151-152)* suggest multiple DNA-protein interactions (*see p. 152-153 of* **RESULTS**). Jiang and

Eberhardt *(541)*, detected a protected region extending from nucleotides 67-83 while Jacquemin et al *(540)* showed a more extended protected region from nucleotides 49-87. Our analysis showed two bordering footprints from nucleotides 42-60 and 62-75. It is conceivable that the repressor and the derepressor sequences are overlapping extensively and the regulation of the function of this region involves mutually exclusive binding of certain transcription factors or modification of pre-existing complexes with the binding of additional factors that change the functional conformation of a pre-complex. It is also possible that no distinct repressor sequence other than RF-1 is present in this regulatory region. In this case, the association of transcription factors with their binding sites would only result in a repressing effect in the absence of DF-1 and this effect may be either, specific for the enhancing function of TEF-1 on an Sp1 containing promoter, or more general. The fact that mutation of the RF-1 site within a previously mutated on the DF-1 site 241 bp enhancer does not restore activity implies that transcription factor(s) interfering with TEF-1 function can be associated with the enhancer region when DF-1 is mutated even when RF-1 is mutated as well.

The gel shift competition pattern generated by the wild type and mutated 62 bp fragments, mentioned above *(621)*, suggests that a potential candidate for the additional sequences contributing to complex formation in the 1-80 bp region, that account for either repressor or derepressor activities, is the region that is located between RF-1 and DF-1. This region (*nucleotides 34-54, termed AF-1*), contains an imperfect direct repeat (GCAA:TTTCTGCTGCAAATTT). This direct repeat overlaps with regulatory regions of other placentally expressed genes such as the mPL-I gene and is very similar to a 11 bp sequence (gTTCTtCCTat, nucleotides -585 to -575) present in the 5' region of the rat

PRL gene *(242)*. Although the AF-1 region has a disrupted ets core, the mPL-I 10mer contains an ets core at a 3' to 5' orientation (CAGGA:G, the reverse complement is underlined in the 10 bp sequence above) and the rPRL 11mer contains an ets related PEA3 element (AGGAAG, nucleotides -577 to -582). Further, the rPRL 11mer is located immediately upstream of a D8-like site (TGTTGCAAAC, nucleotides -572 to -563). D8 is a 10 bp perfect, PSF-A like, palindrome [TGTTGCAACA versus TGTTGGTTGCCAACA (*509.*]) that is present within a 310 bp negative regulatory region 2 Kb upstream of the pituitary hPRL promoter *(538)* and for which preliminary experiments have raised the possibility that it may play a role in the lactotroph-specific expression of the hPRL gene *(622)*.

Preliminary analysis of the possible function of AF-1 by site directed mutagenesis of the hCS-B enhancer region suggests that it may participate to the derepression mechanism *(621)*. This strengthens the possibility that the inactivity of the hCS-A enhancer *in vitro* may be indeed due to the three nucleotide substitutions over the DF-1 and RF-1 regions.

II. Possible mechanisms for repressor and derepressor activities

A number of possibilities exist for the mechanism of the detected repressor activity. Nucleosomes, as well as histone H1 (*623,624*), are considered general repressors of transcription by inhibiting the binding of the basal transcription apparatus to the TATA box *(59-62)*. However, similar considerations should apply for the binding of nucleosomes over other regulatory regions such as the enhancers. In this context,

nucleosomes may prevent the binding of an activating factor on its recognition sequence in an enhancer region. For a gene, such as the hCS-B gene, that transcriptional activation is dependent on enhancer function, blockade of the interaction between the major enhancer element (TEF-1) and its corresponding transcription factor would abolish transcriptional activity. In contrast to its repressing activities however, nucleosomal binding may be a part of a positive regulatory mechanism if it is directed to a specific region which may result in the placement of regulatory elements in accessible positions, such as linker DNA sequences (*625*), or may allow juxtaposition and optimization of the interaction between two distinct regulatory elements normally present at a distance from each other (*568*).

A more recent concept focuses on the relief of chromatin repression by global transcriptional regulators. A characteristic example of this concept is represented by the SWI1, SWI2, and SWI3 genes that are required for the activation of several yeast genes. These proteins appear to act via rather non sequence-specific DNA elements and may act in a multiprotein complex as coactivators (or co-antirepressors) to modulate chromatin structure (*626*). This is supported by the fact that a number of SWI1 mutant suppressors (that is clonal populations that bear mutation(s) that reverse the phenotype that has resulted from mutagenesis of the SWI1 gene) are related with mutations of chromatin related proteins such as an HMG-1 related gene, histone H3, H2A and H2B (*reviewed in 62*).

SWI1, SWI2, and SWI3 genes are also required for the activity of the GR expressed after transfection in yeast (*627*). This is particularly interesting since the GR action on the MMTV promoter (*625*) and on an enhancer region of the rat tyrosine

aminotransferase (TAT) gene (*628*) represent classical examples for the displacement of a nucleosome from a regulatory region. Similarly, several global factors, such as the yeast GRF1 and GRF2, GCR1 and CPF1, maintain regulatory sequences free of nucleosomes and accessible to other transcription factors, likely by binding initially to the internucleosomal region which is followed by displacement of adjacent nucleosomes.

A characteristic, for these general regulators, is their minute enhancing activity when tested alone, however, they are strong enhancers of the activity of factors binding on neighbouring regulatory sequences *(62, and references there in)*.

Recently the partial purification of the human SW1/SNF complex was reported *(629,630)*. These factors destabilize nucleosomes and facilitate the binding of transcription factors such as GAL4 and the TATA binding protein on nucleosome associated binding sites. It appears that the facilitated binding of GAL4 on nucleosome assembled templates is dependent on the presence of the activation domain of GAL4. In contrast, the binding and transactivation potency of GAL4 on naked DNA does not require its transactivation domain. This suggests that although SW1/SNF factors may lack sequence specificity for DNA binding, their functions are sequence specific as they function and increase transcription dependent on the presence of sequence specific transcription factors.

It is conceivable that a similar mechanism, involving chromatin and/or nuclear matrix mediated repression and derepression of hCS-B activity, may be orchestrated by the multiple elements identified within this potent regulatory region. However, analysis of this possibility would require a very detailed examination of the comparative behaviour of wild type and mutant hCS-B enhancer regions in assays assessing the accessibility of chromatin reconstituted templates to transcription factors. Similarly, a detailed

examination of nucleosome positioning, distribution and association with proteins of the nuclear matrix of transiently and stably transfected reporter constructs that carry wild type or mutant hCS-B enhancer sequences might be very informative.

This type of analysis may provide insight into the molecular basis for the dominant role of DF-1 in the regulation of the hCS-B enhancer region and the mechanism involved in the neutralization of the repressor effects. This information, combined with the information provided by the analysis of temporal and tissue-specific distribution of DF-1 and RF-1, once they are cloned, will likely allow to determine whether the onset of placental expression of DF-1/ets is the signal for hCS-B expression and whether the repressor sequences function to assure absence of expression in non placental tissues.

III. Identity of DF-1 and RF-1

The similarity of the DF-1 site with the ets-1 binding site, and the presence of the only nucleotide substitution (tAGGATGT) out of the core of the DNA binding site (AGGA) suggests that DF-1 is, either a placenta-specific or a partially-tissue-specific, ets-like protein factor. Direct confirmation of this possibility, will require the use of gel supershift assays using polyclonal and monoclonal antibodies for different members of the ets family, however, ultimate proof will be provided only through the cloning of this factor. Similarly, since the cloning of TEF-2 has not been reported, cloning of RF-1 will be the only way to elucidate the identity of this factor, as well.

IV. Molecular basis for subloci control within the hGH gene family locus

a. The P sequences

Nachtigal et al (*509*) provided data for the pituitary inactivation of the active CS/GH-V promoters by repressor sequences in the 5' flanking regions of all placental genes. This indicated that there may be a level of subloci organization operating as part of a restrictive mechanism in pituitary. Does this restrictive mechanism depend on pituitary specific factor(s) such as GHF-1/Pit-1? If yes, this would be rather enough to explain the absence of this negative control in placenta. If not, how is this negative regulatory system neutralized in placenta? Interestingly, similarities between elements identified by Nachtigal et al (*509*) and sequences within the 3' CS-B enhancer region identified by Walker et al (*507*) were revealed during the studies presented in this thesis. The idea of interaction between 5' and 3' elements is attractive, and this hypothesis is supported by the results of Nachtigal *et al (509, and unpublished)* that suggest a potentiation of the repressing effects of PSF elements in the presence of hCS-B enhancer sequences and a loss of hCS-B enhancer activity in placental cells in the presence of mutated but not wild type PSF-A/B elements.

Further, the idea of sublocus control is consistent with the demonstration of distinct patterns of expression for individual members of the hGH family in the placenta *(123)* and in choriocarcinoma/HM as presented here. The CS-A enhancer, bearing a modified DF-1 was shown by Jacquemin et al *(540)* to lack enhancer activity . However, hCS-A RNA expression was reported to increase relative to hCS-B with the progression

of pregnancy *(123)*.

Finally, when considering the repetitive presence of DF-1/ets, RF-1/TEF-2, Sp1 and PSF-A/B elements there is an overall similarity of the hGH/CS locus with the well characterized ß-globin locus *(631-633)*.

A locus control region (LCR), identified on the basis of its DNAase I hypersensitivity, is responsible for activating, one at a time, the genes of the ß globin locus (ϵ,τ and ß) depending on the developmental stage of erythroid cells. The LCR consists of four 300 bp DNAase I hypersensitive regions (LCR cores) that are spread over more than 10 Kb of genomic sequences. The LCR establishes an open locus configuration and functions through exclusive interaction with a single globin promoter at a time *(633)*. The specific globin promoter-LCR interaction may be based on competition of the different promoters for LCR. Competition, however, may depend on LCR or promoter binding by activators or repressors, such as the YY1 bifunctional repressor/activator that has been implicated in the silencing of the ϵ and τ gene promoters during development *(633)*.

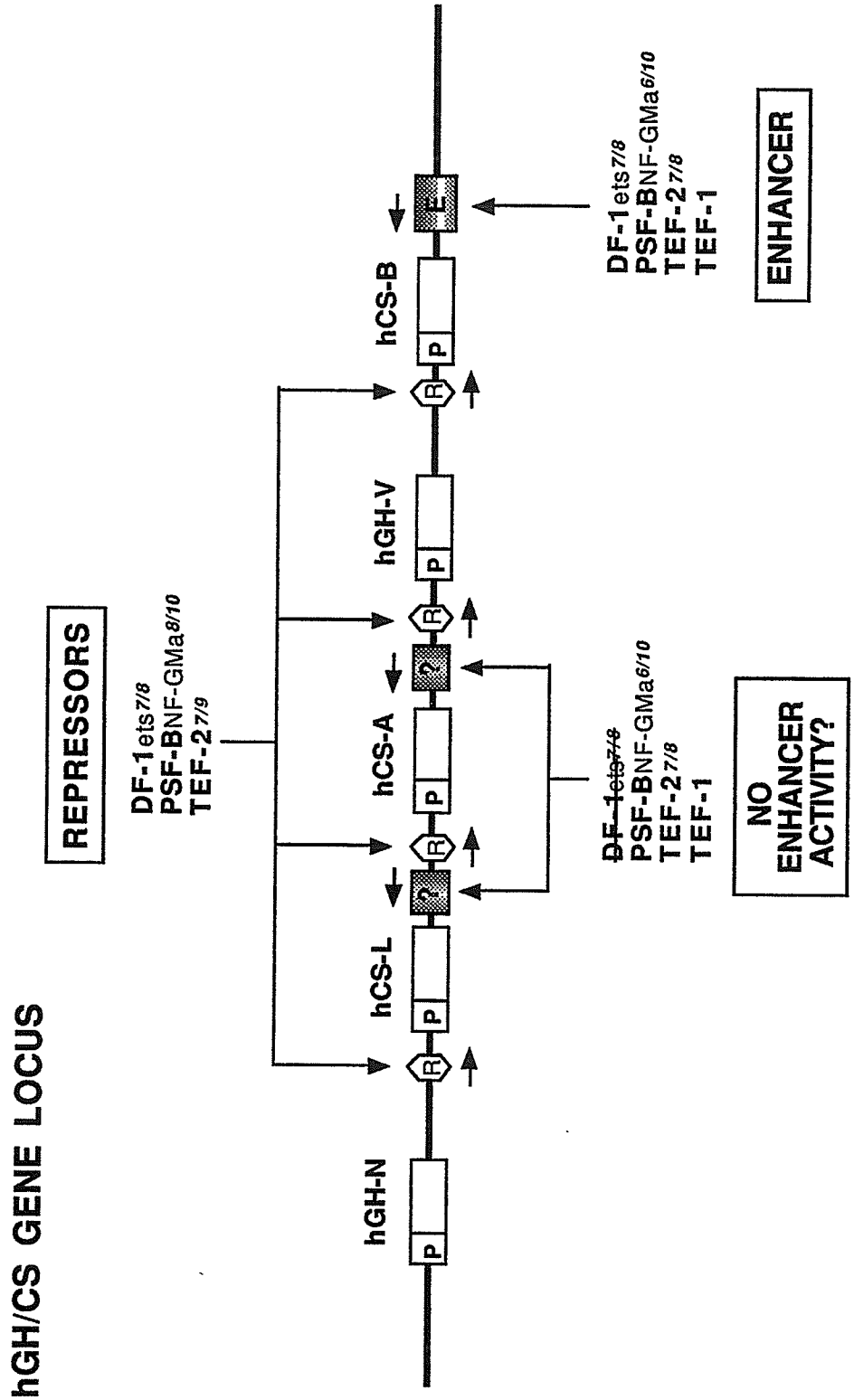
Three types of binding sites appear important for globin gene activity: GATA-1, AP-1 and GGTG/CACC sites corresponding to the binding sites of various factors including Sp1 and TEF-2 *(633,634)*. GATA-1 and CACC/GGTG sites are spread throughout the LCR cores and promoter regions and a GATA-1 site in the TATA-box of the chicken ß globin gene promoter is required for activation by the 3' enhancer of this gene *(633)*. The similarity of this system with the ets and TEF-2 sites distribution patterns is striking. TEF-2 sites belong to the GGTG/CACC group of sites, DF-1/ets and RF-1/TEF-2 have been implicated by the present studies in the activity of the 3' enhancer

of the hCS-B gene, and multiple ets sites were observed throughout promoter and upstream regulatory regions as well as in proximity to the TATA box (nucleotides -50 to +10) of placentally and pituitary expressed analyzed above *(Tables 13-15)*. Further, the activity of an erythroid-specific enhancer present at the 3' flanking sequences of the cytosolic glutathione peroxidase gene was shown, by side directed mutagenesis, to be dependent on the of a cluster of GGTG/CACC binding sites as well as binding sites for members of the ets and GATA families *(507)*. These similarities raise the possibility that ets, TEF-2 and Sp1 sites spread in the hGH/CS locus *(Figure 40)* may be important for all levels of regulation of hGH/CS gene expression having a role also in the chromatin configuration of the locus.

b. The Promoter

The promoter regions of the hGH and hCS-A and hCS-B genes share extensive similarities. A number of transcription factors can bind on the hGH promoter region, apart from Sp1, such as glucocorticoid and thyroid receptors, USF, NF-1 and AP-2 *(553)*. Interestingly, in spite of their similarities, the hCS-B enhancer was reported to activate the hCS-B promoter region 3-5 times more efficiently than the hGH promoter *(634?)*. In an analysis of the sequence requirements for the hCS-B promoter to allow efficient transcriptional activation in the presence of the hCS-B enhancer region, deletion of nucleotides -142 to -129 resulted in an 8-fold decrease in CAT activity *(Table 16)*. This region contains a sequence similar to a binding site for Sp1 and an Sp1 oligonucleotide could compete for complexes formed on the hCS-B promoter.

FIGURE 40: Schematic representation of the hGH/CS locus. P, promoter regions, include Pit-1 and Sp1 elements; R, repressor p sequences; E, enhancer sequences. Putative ets, TEF-2 and NF-GMa elements in the repressor and enhancer regions and their orientations are indicated. The DF-1/ets site in the hCS-A and hCS-L regions bears a core nucleotide substitution that would likely impair putative ets functions.



None of the 7-8 nucleotide differences between the hCS (hCS-A and hCS-B) and hGH promoters is present within the -142/-129 region. Three nucleotide differences are present between -62 and -48 where two half sites for thyroid hormone receptor binding are present, an additional nucleotide difference is present at the edge of the distal Pit-1/GHF-1 site at nucleotide -110. This difference has resulted in the impairment of Pit-1 binding on the distal site of the hCS promoter *(216)* and thus facilitates the binding of Sp1 considering a mutually exclusive mode of binding for these two factors. Finally two nucleotide differences are present at the initiation site one of which in a sequence CAAGGATCC in the hGH and CTAGGATCC in the hCS promoter that differs in two nucleotides from DF-1 (CTAGGATgt) and represents a ets$^{6/8}$ site as well (tAGGATcC).

c. The Enhancer

Interestingly, the Sp1 site in both the hCS and hGH promoter regions is overlapping with an ets$^{7/8}$ sequence (GAGGA-GC) that contains a palindrome (GAGGAG) observed in a number of ets related sequences in the hGH/CS locus *(see Table 13 p. 200)*. An ets-1/Sp1 cooperative interaction has been proposed for two sequential binding sites for these two factors present in the HTLV1 long terminal repeat *(585)*. The ability of Sp1 and ets to interact emphasizes, also, on the importance of the DF-1/ets sequence identified by these studies in the hCS-B enhancer region supporting a loop of interactions between TEF-1, Sp1 and ets. In this context ets may function as a cofactor for TEF-1, mediating its interaction with Sp1, in the same manner as TH-ets appears to be a cofactor

Table 16. Deletional analysis of the transcriptional activity of the hCS-B promoter in the presence of the hCS-B enhancer region.

HCS PROMOTER REGIONS	PERCENTAGE OF PROMOTER ACTIVITY (RELATIVE TO -1200/+2)
-1200/+2	100%
-390/+2	121%
-390/+2 δ:-152/-129	5%
-232/+2	109%
-232/+2 δ:-129/-77	119%
-232/+2 δ:-142/-77	12%
-142/+2	69%
-129/+2	8%
-86/+2	16%
-77/+2	33%

for the CREB binding protein (CBP) *(608)*. Experimental verification of these putative interactions could be provided by DF-1 gel supershift assays using Sp1, TEF-1 and ets antibodies.

d. The rPRL Promoter: a "lead" on the molecular basis of hGH subloci control

The overlap between Sp1 and the ets sequence is further complicated by the fact

that Sp1 overlaps, partially, with the distal Pit-1 site of the hGH promoter, as well. No function has been described for this ets sequence in the hGH promoter, however, the study of the rPRL promoter offers some insight on the possible importance of ets sequences for the expression of this Pit-1 dependent gene. Three ets sequences are present within the rPRL promoter region. Two of these sequences are 8/8 and 7/8 consensus sequences and are found in proximity to (-71/-64: GAGGATGC) or overlapping with (-157/-164: CAGGAAGa) Pit-1 sites (-51/-42 and -156/-149). A third ets$^{6/8}$ sequence (-178/-185: aAGGAAGg) contains a PEA-3 motif (AGGAAG). The region containing these ets sequences has been mapped as the region that mediates the effects of the Ras signaling pathway, in pituitary cells, representing a Ras response element-RRE *(635,636)*. Further a functional interaction between ets and Pit-1, resulting in a synergistic enhancement of Ras induced promoter activity, was shown by co-transfection experiments in Hela cells and the binding of both ets and Pit-1 on the RRE was shown by gel shift assays *(636)*.

The demonstrated interaction between Pit-1 and ets in the pituitary tissue, in this study, and our data suggesting interactions of Pit-1 with PSF-A and PSF-B *(509,637)*, and RF-1 with PSF-A, PSF-B and DF-1/ets in pituitary nuclear extracts, point at the direction of a very complex mechanism regulating hGH family subloci control. The competition of RF-1/GC protein complexes, in mobility gel shift assays, by DF-1 as well as PSF-A and -B oligonucleotide competitors, raises the possibility that these elements may have a synergistic effect on the repressor activity exercised by PSF-A and PSF-B on the CS promoter in the presence of the CS-B enhancer *(509)*. Similarly it is possible that interactions between these elements may be, also, of importance for the activation of the placental genes in trophoblasts. If these interactions prove to be of functional

significance, this would provide important information on the synergism between 5' and 3' elements to secure subloci specific expression within the larger hGH gene locus.

e. The level of organization of a putative subloci control mechanism.

An important question is whether a subloci control may be exercised at a gross chromatin configuration level, by DNA folding to 30 nm solenoid structures, or whether two distinct levels of control exist: one that determines the "open" or "closed" state of the whole hGH locus and another that, selectively, blocks expression in spite of an active locus configuration. DNAase I sensitivity experiments *(638)* have detected hypersensitive sites throughout the hGH locus (including the hGH gene) in BeWo and JEG-3 cells implying that the complete locus is open in both cell lines. Detailed hypersensitivity analysis may reveal differences between individual genes that would imply the existence of a second level of subloci control, exercised on an "open" configuration of chromatin. Probes corresponding to the P sequences as well as the CS enhancer regions may help to elucidate this matter, however, an intrinsic difficulty lies again on the extreme similarities between the genomic DNA sequences of the hGH/CS genes. *In vivo* footprinting coupled with PCR could be applied provided that specific primers that can amplify the regulatory region of one gene (e.g. hCS-B enhancer region), and not that of any other (e.g. hCS-A and hCS-L enhancer regions) gene, can be synthesized.

The determination of the genomic sequences corresponding to possible matrix attachment regions (MAR) *(639-642)* present in the hGH locus and their possible interactions with the P sequences and enhancer regions, as well as questions on whether transcription factors such as Pit-1/GHF-1, Sp1, TEF-1, PSF-A and -B, DF-1 and RF-1

are involved in cooperative interactions that establish an open-locus configuration, will be the focus of a new series of studies aiming at elucidating further the complex mechanism that controls the tissue specific expression of the hGH genes. Interestingly, it was recently shown that Sp1 can bind nucleosome assembled binding sites, although with 10 to 20-fold lower affinity compared to naked binding sites *(643)*. The binding of nucleosomal DNA by Sp1, which may be associated with its ability to modify chromatin structure, as suggested by a Sp1 binding and Sp1-mediated formation of nucleosome free regions on SV40 minichromosomes *(644,645)*. Therefore, it may also provide the basis for the involvement of additional factors, such as TEF-1 and its cofactors, that through DNA-protein and protein-protein interactions, may modify the specificity or the potency of the Sp1 effects on chromatin.

V. Possible applications of the knowledge generated by the study of the tissue specific expression and regulation of the hGH gene family

Studying the hGH/CS family aims at two distinct targets: (a) the elucidation of the function and roles of the members of this family and (b) the unravelling of the mechanisms that determine their tissue specific gene expression and are likely associated with the growth, development and terminal differentiation of the corresponding tissue.

However, knowledge of the mechanisms that control subloci specific expression will be of virtual importance for the detailed study of the functions of hGH proteins. This knowledge, can be applied for the development of transgenic models targeting primarily the elucidation of the functions of hGH proteins in pregnancy, embryonic development, immune regulation, as well as, in a number of systems, where possible hGH/CS effects

may be masked by the redundancy of functions between different proteins.

The report of pregnancies *(409-413)* that progressed in the absence of hCS brought about some doubt for the functional significance of hCS for the progression of a normal pregnancy. However, subsequent reports failed to establish complete absence of hGH/CS/PRL proteins during pregnancy *(for a review see reference 77)* and in some cases interesting DNA recombination "conspiracies" suggested the possibility of an essential replacement of hCS-A and -B by hGH or hCS-L, a gene that for a long was been considered silent *(77)*.

Elucidating this apparent redundancy and determining the specific effects among members of the GH/CS-PRL family may be facilitated by transgenic models that can be developed using a combination of knock out experiments of mouse placental PRL-like genes and targeting of hGH/CS genes in mouse placenta. However the ability to develop such systems depends on the existence of a certain degree of redundancy between the mouse PRL-like proteins and hGH/CS proteins, in a transgenic organism.

The transgenic approach, requires a thorough experimental design that would allow precise manipulation of the genetic background of transgenic animals. Information on the DNA requirements for pituitary, placenta and lymphocyte specific expression of hGH genes would be used for determining the minimal DNA information necessary for the designing and synthesis of constructs able to confer control over the tissue, time and levels of subloci activation. Ideally a single construct containing all five hGH genes, should also contain the necessary information for the selective activation of any individual gene.

The development and testing of such constructs will in turn increase our knowledge and ability to manipulate eukaryotic genomes. The long term goal would be

the designing of tissue and time specific "gene carrier" vectors for use in gene therapy. Transgenic models can be used for assessing the efficiently of cis-DNA elements and for the construction of DNA "cassettes" able to direct tissue specific expression of exogenously provided genes. Combination of these DNA "cassettes" in vectors for use in gene therapy, will increase our technical ability to intervene and repair defective molecular pathways by replacing defective genes and reconstructing, in a tissue and time specific way, their, constitutive and hormonally regulated, expression profiles.

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