CLONING AND REGULATION OF THE RAT INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-1 GENE

BY

CHARITHA SENEVIRATNE

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

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ABSTRACT

Insulin-like growth factors (IGFs) are a family of polypeptides that are structurally and functionally similar to insulin. Like insulin, IGFs stimulate growth, differentiation and metabolism but unlike insulin, does not have a major storage organ.

IGFs exist in the circulation complexed to IGF-binding proteins (IGFBPs) which are thought to subserve the function of storage as well as modulate function of IGF. Six members of the mammalian IGFBPs have been cloned to date.

In this thesis, I describe (i) cloning of a full length rat IGFBP-1 cDNA, (ii) use of this cDNA in studies of IGFBP-1 RNA expression and regulation, (iii) cloning of the rat IGFBP-1 gene and (iv) some characterization of this genomic clone.

A full length rat IGFBP-1 cDNA (CS15) was cloned and sequenced. It consists of approximately 1500 nucleotides (nt) with an open reading frame of 816 nt in length which begins at an in-frame ATG site located 93 nt down stream from the 5' end of the clone. At the 3' end is a poly (A)+ tail of 56 nt immediately preceded by a consensus poly (A)+ signalling sequence. The deduced protein was 272 amino acids (aa) long with a predicted relative molecular mass (M_r) of 29.6 kilodalton (kD); it exhibited 79% homology with the human counterpart with conservative substitutions.

Fasting for 24h increased the rat hepatic IGFBP-1 mRNA 10±2.2-fold compared to control rats. Refeeding for 1h, decreased the message to non-detectable levels by Northern blot analysis.

In growth hormone (GH)-deficient hypophysectomized (hypox) rats, hepatic

IGFBP-1 mRNA abundance increased significantly compared to pituitary intact (sham-operated) control rats (6.5±1.7-fold; n=9; compared to sham-operated control rats, $p \leq 0.05$) while chronic GH treatment reduced the message significantly compared to the hypox levels suggesting inverse GH regulation of IGFBP-1 gene expression.

Cloning of the rat gene for IGFBP-1 from two rat genomic libraries including cloning and sequencing of 1627 nt of 5' flanking region was achieved. This (IGFBP-1) single copy gene spans about a 5kilobases (kb) in the rat genome and is organized into four exons with sizes of 545 nt (exon 1), 186 nt (exon 2), 129 nt (exon 3), and 646 nt (exon 4). The introns were 1.2 kb (intron 1), 0.8 kb (intron 2), 1.4 kb (intron 3) in length, estimated by Southern blot analysis. All splice sites conformed to classical GT/AG rule. The phase and the amino acid interrupted by the intron were conserved between rat and the human genes. The mRNA cap site was located 173 nt 5' to the translation start site. The 3' untranslated region was 514 nt in length.

Cloning and sequencing of 1627 nt of 5' flanking region and subsequent analysis with the aid of a computer, revealed the presence of consensus sequences for CAAT and TATA boxes, three insulin response elements (IREs), two glucocorticoid response elements (GREs), one AP-1 protein binding site, four AP-2 binding sites and two octamer binding sites (OTFs). In collaboration, a 37 bp region of -119/-83 in the IGFBP-1 has been shown by chloramphenicol acetyltransferase CAT) assays, to exhibit insulin responsiveness. Also, in a very preliminary study, scanning the 5' flanking region from -728 to -83 with mobility-shift assays, revealed a 196bp region from -278/-83 showed a slight growth hormone response.

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LIST OF ABBREVIATIONS

REAGENTS

$CaCl_2$	calcium chloride
CsCl	cesium chloride
DNase	deoxyribonuclease
DMEM	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
FBS	fetal bovine serum
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
I	iodine
KCl	potassium chloride
K_2HPO_4	dipotassium hydrogen phosphate
MEM	minimum essential medium
$MgSO_4$	magnesium sulphate
PMSF	phenyl methyl sulphonyl chloride
PNK	polynucleotide kinase

NaCl	sodium chloride
Na_2HPO_4	disodium hydrogen phosphate
NaOH	sodium hydroxide
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
RNase	ribonuclease

Restriction enzymes

Av	Ava 1
Ap	Apa 1
B	Bam HI
E	Eco RI
H	Hind III
Hp	Hpa II
Hae	Hae III
P	Pst 1
Sc	Sac 1
Sl	Sal 1
X	Xba 1
Xh	Xho I
R	Rsa 1

BIOLOGICAL FACTORS AND PREFIXES

ACTH	adrenocorticotrophic hormone
ALP	acid labile protein
b	bovine
CNS	central nervous system
CSF	cerebrospinal fluid
CTF	CAAT box binding transcription factor
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FGF	fibroblast growth factor
GH	growth hormone
GHRF	growth hormone releasing factor
h	human
Hypox	hypophysectomized
IDDM	insulin dependent diabetes mellitus
IGF-I	insulin-like growth factor- I
IGF-II	insulin-like growth factor-II
IGF-IR	insulin-like growth factor-I receptor
IGF-IIR	insulin-like growth factor-II receptor
IGFBP	insulin-like growth factor -binding protein

m	mouse
MSA	multiplication stimulation activity
NF-1	nuclear factor-1
NSILA	non-suppressible insulin-like activity
p	porcine
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PKA	protein kinase A
PKC	protein kinase C
r	rat
SFA	sulphation factor activity
TGF-β	transforming growth factor -β
TF	transcription factor

UNITS

mA	milliamperes
C	degrees centigrade
Ci	curies
mCi	millicuries
μCi	microcuries
dpm	disintegrations per minute

cpm	counts per minute
g	gravity
gm	gram
mg	milligram
μg	micro gram
h	hour
kD	kilodalton
l	litre
ml	millilitre
μl	microlitre
M	molar
mM	millimolar
μM	micromolar
mmol	millimoles
rpm	revolutions per minute

MISCELLANEOUS

Amino acids

A	Ala	alanine
B	Asx	asparagine or aspartic acid
C	Cys	cysteine

D	Asp	aspartic acid
E	Glu	glutamic acid
F	Phe	phenyl alanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine
P	Pro	proline
Q	Gln	glutamine
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valine
W	Trp	tryptophan
Y	Tyr	tyrosine
Z	Glx	glutamine or glutamic acid

ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate

bp	base pairs
CAT	chloramphenicol acetyl transferase
CO_2	carbon dioxide
COOH	carboxy
DHRF	dihydrofolate reductase
GRE	glucocorticoid response element
FL	flanking
^3H	tritium
ip	intraperitonial
IRE	insulin response element
dNTP	deoxynucleotide triphosphate
A	deoxyadenosine
T	deoxythymidine
G	deoxyguanosine
C	deoxycytidine
U	uridine
MCS	multiple cloning site
mol wt	molecular weight
M_r	relative molecular mass
MT	metallothionine
NH_2	amino
nt	nucleotide

OTF	octamer binding transcription factor
PAGE	polyacrylamide gel electrophoresis
PEPCK	phosphoenolpyruvate carboxykinase
RE	restriction enzyme
RNA	ribonucleic acid
hnRNA	heterogeneous nuclear RNA
mRNA	messenger RNA
RT	room temperature
rpm	revolutions per minute
sc	subcutaneous
SnRNP	small nuclear ribonuclear protein particle
UT	untranslated
UV	ultra violet
vol	volume
v/v	volume/volume
%	percentage

INTRODUCTION

Preamble

Insulin-like growth factors (IGFs) are polypeptides similar in structure and function to insulin. Over the years much energy has been expended in trying to understand multiple activities and complex regulation of IGFs.

Current research is focused on understanding the role of IGF-binding proteins (IGFBPs) in modulating the action of IGFs. To this end, rat IGFBP-1 gene was cloned and some of its metabolic and hormonal regulation was elucidated.

In order to place this work in the context of the somatomedin hypothesis, I will begin by describing the structure and the functions of IGFs, insulin and growth hormone (GH) . This will be followed by a section on their (IGFs, insulin, and GH) receptors.

In the second chapter, function, purification and characterization, gene structure as well as regulation of IGFBPs will be reviewed. In the third chapter, interactions between IGFs, and IGFBPs with GH in effecting organismal growth within the context of the somatomedin hypothesis will be briefly presented. In the fourth and final chapter, structure, regulatory elements of eukaryotic genes as well as flow of genetic information from DNA to protein will be reviewed.

LITERATURE REVIEW

1. Biology of insulin-Like Growth Factors

Insulin-like growth factors (IGFs), as the name implies are a group of polypeptides that are structurally and functionally similar to insulin. Like insulin, IGFs have relative molecular masses (M_r) around 7,000 dalton (dal); IGF-I with 70 amino acids and 7,646 relative molecular mass (M_r) is slightly bigger than IGF-II which has a M_r 7,471 dalton and a 67 amino acid polypeptide chain (1-5).

1.1. History

The IGF polypeptides were first characterized as specific functional entities of serum; (a) sulphation factor activity (SFA), (b) non-suppressible insulin-like activity (NSILA) and (c) a multiplication stimulating activity (MSA).

(a) SFA: Now classical experiments of Salmon and Daughaday in 1957 led to the discovery of IGFs. When rat cartilage was immersed in serum from normal rat, sulphates were incorporated to peptido-glycans of the cartilage while in the serum from a hypophysectomized (hypox) rat failed to do so, even when growth hormone (GH) was added to the serum, in vitro. Further, when hypox rats were treated with GH before the withdrawal of the serum, the SF activity observed in normal animals was restored suggesting that some factors that were induced by GH were responsible for this SF

activity. This observation gave rise to the now classical Somatomedin hypothesis of Salmon and Daughaday (1a).

(b) NSILA: Froesch et al in 1963 (65) observed a non-suppressible insulin-like activity (NSILA) in human serum. There was far more insulin-like activity than could be accounted for by immunoreactive insulin, hence the name NSILA was given.

(c) MSA: In 1973, Dulak and Temin (66) searched for serum growth factors that stimulate multiplication of chicken embryo fibroblasts in culture, and isolated such a factor from the BRL-3A cell line (a buffalo rat liver cell line) conditioned media, which was called multiplication stimulation activity (MSA).

It was realized that all these three activities have the properties of a group of similar substances, and investigators in the field in 1972 decided to name these factors as somatomedins, defined as mediators of GH-stimulated somatic growth and insulin-like activity (1).

However, it took until 1978 to purify two members of this family of proteins from human plasma; this was accomplished by the Zurich duo of Rinderknecht and Humble (3-4) as NSILAs. Ten years after the purification of these peptides, it became abundantly clear that only IGF-I fitted the somatomedin definition in that it is regulated primarily by GH, while IGF-II was not. Thus, in 1987 the term "insulin-like growth factors" (IGFs) was coined to best describe this family of peptides (1).

1.2. Structure

Adult human plasma was the source for the IGF-I and -II isolation accomplished by Rinderknecht and Humble in 1978 (2-4). The polypeptide chain contains 70 amino acid (aa) residues in IGF-I while that of IGF-II is 67 aa; relative molecular masses are 7646 and 7471 dalton (dal) respectively for IGF-I and -II. There are intrachain disulfide bonds present in both IGF-I and -II and 62% aa residue homology exists between the two peptides. Whereas with proinsulin, IGF-I has 43% aa residue homology, it is decreased to 41% with IGF-II.

As seen in Fig 1A, domains B, C, and A of the polypeptides, IGF-I, -II and proinsulin, are structurally similar, but IGF-I and -II are extended at their carboxyl (COOH) ends to include a D domain and, in some cases an E domain which is absent in proinsulin. Also, the equivalent of domain C of IGFs that connects B and A, is removed when proinsulin is processed to insulin (1). In other words, proinsulin is closer than insulin to the structure of IGF-I and -II. Variants of IGF-I have been isolated both at protein and gene transcript levels. Recent studies with some of these variants, for example, the variants of IGF-I with the tripeptide truncation at the amino terminal (des-IGF-I), demonstrated higher potency than intact IGF-I in stimulating [^{3}H] thymidine incorporation into fetal rat brain cells (1). These peptides were initially isolated from human brain and bovine colostrum (5, 6). IGFs are becoming increasingly recognized as important in the central nervous system (CNS) as neuropeptides and are also implicated in brain development and differentiation (5). Searching for growth factors in human

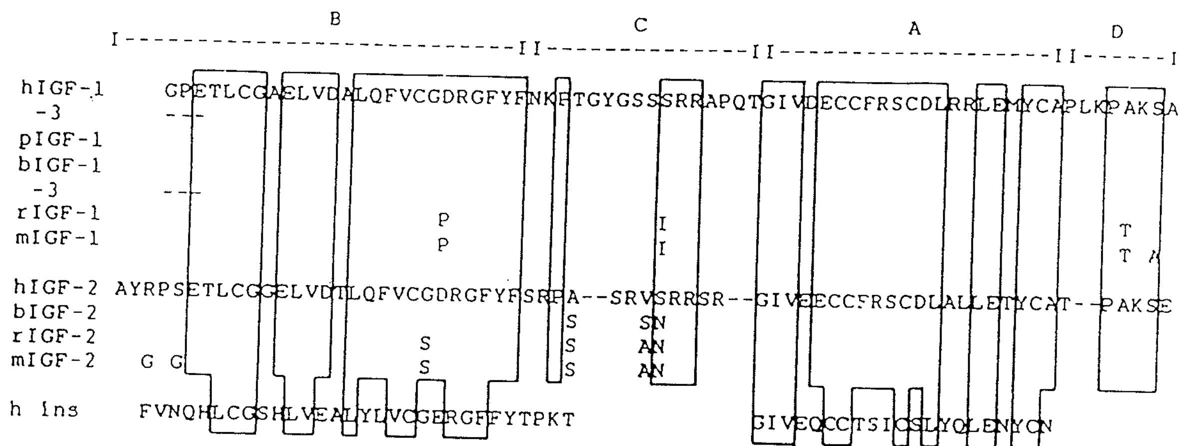


Fig 1A Primary structures of human IGF-I, IGF-II, and insulin. Alignment has been chosen to give maximal homology; a dash indicates truncation or introduced gaps. Comparison to porcine (p), bovine (b), rat (r), and mouse (m) IGF-I and bovine, rat, and mouse IGF-II is shown by giving the residues that differ. Residues conserved between human (h) IGF-I, IGF-II, and insulin are boxed.

Reproduced from Sara et al (1).

colostrum that induced protein synthesis in L6 myoblast cells, resulted in the purification of IGF-I from this source (6). Furthermore, these studies suggest that the truncated IGF-I form (des-IGF-I) isolated from colostrum is a post-translationally modified IGF-I (7). The various rat IGF-I gene, mRNA and protein structures are shown in Fig 1B.

1.3. Function

IGFs can be considered in general terms, to be anabolic hormones. Further, action or the function of IGF can be summarized into two main themes:

A. Mitogenic activity B. Insulin-like activity.

A. Mitogenic activity

The mitogenic activity of IGFs includes cell proliferation and differentiation, which are observed in vitro as well as in vivo. Recombinant IGF-I is available, while only purified preparations of IGF-II can be obtained. In many cell culture systems such as chondrocytes, astrocytes from the CNS, myoblasts, hepatocytes, kidney cells, and embryonic stem cells, IGFs promote cell proliferation and ^{3}H-thymidine incorporation to DNA (1 and references therein).

In many in vitro systems such as primary cell culture, established cell lines, and in organ explant, it has been demonstrated that both IGF-I and -II stimulate DNA synthesis and cell proliferation (8-10). There are two types of growth factors; competence-

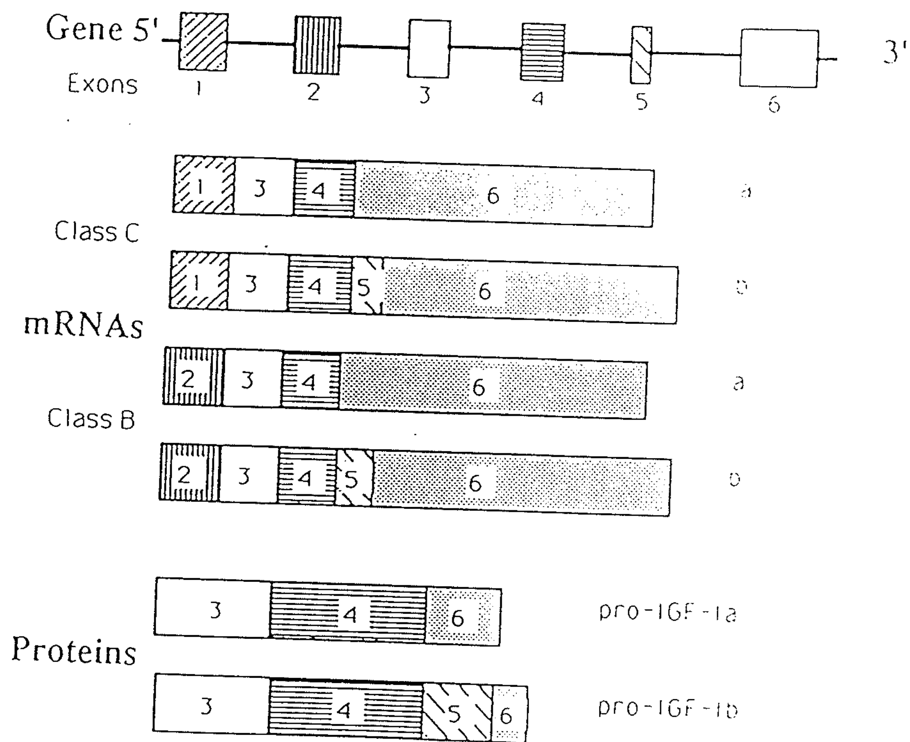


Fig 1B . Structure and expression of the rat IGF-I gene.

Reproduced from Luo (91).

and progression-factors. IGFs belong to the former type where as fibroblast growth factor (FGF) and platelet derived growth factor (PDGF), belong to the latter (11). Once the competence factor initiates the induction of cells to the cell cycle, progression factors are thought to propagate the cells through the DNA synthesis phase of the cell cycle. Thus a combination of these two types of growth factors, such as PDGF and IGF has exhibited potency to the same level as serum (6,11). Meiotic division of oocytes is stimulated by IGFs (11a). A host of other effects are elicited by target cells in response to these peptides and the response depends on the type and the state of the target cells. For example, IGFs induce differentiation in osteoblasts (15), myocytes (14, 16), adipocytes (17) and oligodendrocytes (18). They induce erythropoiesis (19), granulopoiesis (20, 21) as well as chemotaxis in endothelial (22) and melanoma cells (23). The genes c-fos and c-jun encode proteins C-FOS, C-JUN, and JUN-B which are transcription factors. The expression of these transcription factors is increased in response to various extracellular growth signals and precedes cell proliferation (195). In skeletal muscle of rat both IGF-I and GH stimulate the expression of c-fos (24). In addition GH also stimulates c-jun and jun B gene expression. Therefore, GH and IGF-I may regulate cell proliferation through the transcription factors C-FOS, C-JUN, and JUN-B. The heterodimers formed between C-FOS and C-JUN or JUN-B, interact with *cis*-acting DNA element AP-1 (195). IGFs have growth promoting effects on the neural system in that they (IGFs) are shown to induce neurite outgrowths in glial cells (28, 29). Receptors for both IGF-I and -II are present in the nervous system of several mammals including human, and IGF-II has been detected in the cerebro-spinal fluid (CSF). When tadpoles, frogs and humans were

injected with GH, an enhanced ratio of neurons to glials was observed (27), suggesting that IGFs may play a significant role in growth and development of the brain. Growth hormone treatment of children with less than normal head circumferences, induced faster catch-up growth of the head and improved IQ further suggesting the important role of IGFs in brain development (27). An IGF-mediated increase of neurotransmitter (acetylcholine) release also has been observed in brain slices (30) as well as catecholamine release from chromaphin cells (31).

In vivo growth promoting action of IGFs has been shown under many experimental conditions. Snell dwarf mice are pituitary deficient from birth and therefore remains at an early developmental stage, both in terms of growth and skeletal maturity. When Snell dwarf mice and hypophysectomized (hypox) rats were given IGF-I intravenously, elevated levels of growth were observed (29). The IGF-I and -II growth promoting effect was significant in Snell dwarf mice but in hypox rats, the response was weak. Also, when IGF-II secreting tumors were transplanted to nude mice, no growth promotion was observed (33). In the case of IGF-I, long term subcutaneous infusion of the recombinant peptide stimulated growth in hypox, Snell dwarf, and insulin-dependent diabetes mellitus (IDDM) rats (39). In hypox rats, GH was more potent than IGF-I in promoting growth. It was then thought to be due to the ability of GH to stimulate endocrine, paracrine and autocrine production of IGFs, as well as the down-regulation effect on insulin-like growth factor binding proteins (IGFBPs) that inhibits IGF action. For example, if these binding proteins(IGFBPs) reduce the available "free" IGFs by complex formation, the down-regulation of these proteins (IGFBPs) would result in higher

availability of "free" IGFs; resulting in higher mitogenic activity. This point will be further reinforced later in the discussion. Further in vivo evidence for the role of IGFs in growth promotion, comes from transgenic mice. The predominant adult human liver IGF-I transcript is IGF-Ia. A fusion gene where the corresponding cDNA (complementary to IGF-Ia mRNA) driven by mouse metallothionein I promoter was constructed. Transgenic mice carrying this human IGF-Ia fusion gene exhibited increased body weights compared to their normal siblings, attributable to organomegaly and hyperplasia. But the bone mass was unchanged and the circulating IGF-I levels were only slightly elevated. The endogenous gene expression of IGF-I and GH was inhibited (36).

There is some controversy regarding the growth promoting activity of IGF-II. But the transgenic mouse model for IGF-II supports the growth promoting role of the peptide. Trangenic mice were made with IGF-II gene disrupted by homologous recombination; homozygosity was lethal while the heterozygotes were significantly smaller than their normal siblings (37).

B. Insulin-like metabolic activity

Zapf et al (38) observed that when a bolus of human recombinant IGF-I was given to normal rats intravenously, the treatment induced hypoglycemia, increased glucose uptake from the blood plasma and incorporation into glycogen. However, only minor stimulation of lipogenesis occurred. This was also the case with IGF-II but the potency was greater for IGF-I. Skottner et al (39) also showed similar results in hypox rats, in

Gottinger miniature pigs as well as in healthy adults. But in all these insulin-like effects, insulin itself was far more potent than IGFs (40-41).

Other insulin-like anabolic action, for example, the stimulation of the uptake of amino acid and protein synthesis in rat cartilage and human skin fibroblasts were observed in response to IGFs. Also, in insulin-target tissue like adipocytes and muscle, IGFs potently induced glucose transport and metabolism. Insulin-like growth factors have acute insulin-like action, while chronic action is mainly mitogenic (1). Inhibition of lipolysis was observed in response to IGF-I as is the case with insulin in healthy human subjects, but again insulin was much more potent than IGF-I (41). In the same study, when the hypoglycemic effects of the two agents were measured (IGF-I vs insulin), IGF-I was as only 6% potent as insulin on a molar basis.

2. Growth Hormone

The classical somatomedin hypothesis of Salmon and Daughaday is depicted in Fig. 2 (27). Growth hormone can act directly on target tissues without its proxies, the IGFs, and in certain cases has stronger effects than the IGFs.

The whole concept of or the GH-IGF-Growth axis in toto has relevance only in post-natal life, because fetal growth and development is pituitary-independent, hence GH-independent. Therefore, it is thought that IGFs regulate growth during this period (39). GH-mediated biological activity has been demonstrated in many in vitro systems. For example, erythropoiesis and lymphopoiesis but not granulopoiesis are under pituitary GH

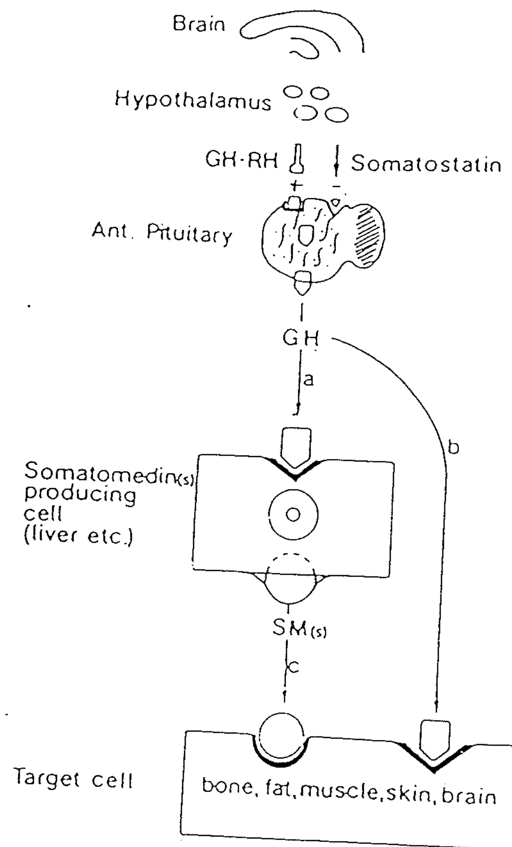


Fig 2. Somatomedin hypothesis

a: depicts the direct GH action on liver while b depicts the direct action of GH on taget cells of somatomedins (SMs = IGFs). GH-RH; growth hormone releasing hormone, c; IGF-1.

control. GH stimulates erythropoietic and lymphopoietic cell colony formation in vitro . This is a direct GH effect rather through IGFs (19,20,42). Smooth muscle is another tissue that responds directly to GH in vitro with simulated growth (43-45); rat pancreatic β cells also respond in serum-free medium to GH by increased [^{3}H]thymidine incorporation and DNA content (71). Adipose conversion is carried out by GH and other yet to be identified plasma factors. This conversion was shown to occur in cell cultures of preadipocytes derived from 3T3 cells when GH is added alone with 1% serum. Adipogenesis acts at distal sites from the GH receptor, because not all cells possessing GH receptors, respond to the hormone (GH) with adipose conversion (42,146,147).

When GH is enzymatically fragmented, the different fragments elicit different profiles of biological activity (42). Metabolic action of GH in the whole organism (in vivo) can be summarized into two main topics: I. insulin-like activity, and II. anti-insulin (antagonistic) activity. The anti-insulin activity of GH is diabetogenic and anti-lipogenic (lipolytic) in nature (150), and at first glance appears paradoxical with the insulin-like activity of GH.

Endogenous GH prevents the expression of insulin-like effect of ectopic GH in the form of a transgene (235) or exogenous preparations, but this effect (insulin-like) is transient, and is followed by a refractory period (146,230). The paradigm emerging from experiments above is that GH, like IGFs, has insulin-like effects.

In the male rat, GH secretion is episodic and such episodes occurs every 3-4 h (42) while in human male, this occurs every 90 minutes (95). Therefore, under experimental conditions, a daily dose of GH administration may not fully mimic the

in vivo situation.

GH and IGF-I can have distinct growth patterns. For example in hypox rats, kidney, spleen and thymus were more sensitive to IGF-I, while in the skeletal muscle GH was more effective (141). Furthermore, in somatomedin-ablated mice (genetically) when compared to normal GH-expressing and IGF-I transgene-carrying siblings, growth hormone expression was required to reach normal liver size. However for the brain, expression of IGF-I was of greatest effect (236).

3. Receptors for IGFs, insulin and GH

Key players in the somatomedin hypothesis are IGF-I and -II, insulin and GH. Thus, it will be pertinent to review some of the literature of their (IGF-I and -II, insulin and GH) receptors.

The nomenclature (type I and type II IGF receptors) is due to the fact that both peptides, i.e. IGF-I and -II, bind to each others receptors, 10-100%; i. e. IGF-I binds type I receptor 100% and type II receptor at 10% affinity of IGF-II affinity to type II receptor, and vice-versa. Because of this extensive cross-reactivity, they are called IGF type I and type II receptors (63). IGF type I and insulin receptors are both heterodimers of $\alpha_2\beta_2$ configuration, whereas the IGF-II receptor is a single chain polypeptide (63).

Insulin receptor

One copy of this receptor gene is present per haploid genome of mammals. The receptor is of $\alpha_2\beta_2$ configuration with each β chain about 90kD while α chain about 125 kD; under non reduced conditions, M_r of native receptor is 350-400 kD. There are a number of tyrosine residues which become autophosphorylated in both α- and β- sub-units. The catalytic sites of the receptor are located in the β-subunits. The α- and β-subunits are held together by disulfide bonding and both subunits i.e. α and β are glycoproteins (63,153).

Many growth factor receptors like those for the IGFs and insulin are tyrosine kinases, and other examples are epidermal growth factor receptor (EGFR), and platelet derived growth factor receptor (PDGFR). Usually it is the serine/threonine phosphorylation that is more abundant in mammalian cells as opposed to tyrosine phosphorylation (153).

Phosphorylation activates the receptor as a kinase, while dephosphorylation does the opposite. It has been shown that β-adrenergic agonists, acting through cAMP and protein kinase A (PKA), decreases receptor kinase activity resulting in anti-insulin (diabetogenic) action (63).

Therefore, tyrosine and serine/threonine phosphorylation of the insulin receptor modulated the ligand binding and kinase activity (153).

IGF type I and II receptors

The IGF type II receptor is a single chain polypeptide receptor traversing the plasma membrane with intra-chain disulfide bonds, and different in configuration to type I IGF and insulin receptors (63, 153). Under non-reducing conditions affinity labelling of IGF-I receptor with (^{125}I) IGF-I gave a M_r of >300,000 dalton (^{125}I IGF-I plus receptor complex) when treated with monoclonal antibodies for IGF-I, and when disulfide bonds were disrupted, this (M_r) decreased to 130,000 dalton.

Ligand affinities are as follows: IGF-I bind at the highest affinity to type I receptors, but IGF-II as well as insulin bind this receptor with lower affinities; type II receptor binds to IGF-II with the highest affinity, IGF-I with lower affinity whereas insulin does not bind the type II receptor (63).

Immunoprecipitation with monoclonal antibodies of IGF type I receptor after biosynthetic labeling, demonstrated not only an α subunit but also a 92,000-98,000 dalton species. The total M_r of IGF type I receptor has been calculated to be 402,000 dalton, based on many biochemical parameters (63). Therefore, type I receptor is a heteromeric structure made up of α(M_r= 130,000) and β (M_r= 92,000-98,000) subunits connected by disulfide bonds, with $\alpha_2\beta_2$ stoichiometry (63).

The type II receptor on the other hand is a monomeric structure with a M_r of 250,000 dalton. The purified 250,000 dalton receptor is sufficient to bind IGF-II. Detergent-solubilized receptor, exhibited a Mr of 210,000 dalton on SDS-PAGE under non-reduced conditions whereas it increased to a 250,000 dalton under reduced conditions

(63,156).

The β subunit of the type I receptor is hyper-phosphorylated in response to IGF-I binding. This occurs even at 2.5ng/ml IGF-I concentration in 1M-9 cells, a human lymphocyte cell line (64). The insulin receptors did so at much higher concentration. Once the type I receptor is phosphorylated, it in turn phosphorylates tyrosine residues of cellular substrates. In bioassays, the IGF-IR is able to phosphorylate synthetic substrates like poly (glu, tyr) 4:1 (154). Phorbol esters also cause phosphorylation of IGF type I receptor (IGF-IR) probably acting via protein kinase C (PKC). The phorbol esters phosphorylate the insulin receptor at a site distinct of insulin itself, but whether this is also the case with IGF-IR is not yet known (155).

The type II receptors also autophosphorylate in intact cells, such as rat embryo fibroblast, H-35 hepatoma cells and BRL-3A2 rat liver cells, in response to IGF-II binding. Also, type II receptor binds Mannose-6-phosphate at a distal domain to that of IGF-II. Thus it is often referred to as the IGF-II/mannose-6- phosphate receptor (157).

GH Receptor

In 1973, a radioreceptor assay for GH was developed (160) in cultured human lymphocytes (60). Receptor work has been slow according to Hughes and Friesen (60) due to the difficulty in defining an <u>in vitro</u> biological response to GH. Also "GH receptor" is technically incorrect because in most tissue, GH action is not mediated though GH binding sites (61); many forms of GH (ligand) thus many forms of receptors can be

anticipated. Further, there are overlapping specificities with receptors such as prolactin (62).

GH receptors are widely distributed among mammalian tissues and organs including liver, adipose tissue, intestine, heart, kidney, lung, pancreas, brain, cartilage, skeletal muscle, corpus luteum, and testis (260).

Purified GH receptor from rabbit liver showed a relative molecular mass of 130 kD when analysed by SDS-PAGE (261). Several studies have suggested the presence of multiple forms of the GH receptor including GH binding studies with rabbit liver membranes (61), epitope mapping studies (262), and cross-linking studies in mouse liver (263), in human liver (264), and in RIN cells, a rat islet tumor cell line (265).

Mammalian GH receptor cDNA including rabbit (266), and human (261) have been cloned. The deduced protein is 620 amino acids in the rabbit; consists of a 246 amino acid extracellular hormone-binding domain, a single transmembrane region, and a cytoplasmic domain of 350 amino acids. The extracellular domain contains seven cystine residues and five potential N-linked glycosylation sites (266). Site-directed mutagenesis analysis of the extracellular domain of the GH receptor suggested the involvement of acidic residues E42 and E44 in the first cysteine loop, R 70 and R 71, W 76, residues T 101, S 102, I 103 and W 104, P 106, residues V 125, D 126, E 127, I 128 and D 132 in the receptor binding site (267).

The GH receptor primary amino acid sequence did not provide any clues into the mechanism of signal transduction; no consensus sequence in the GH receptor is found homologous to known tyrosine kinases (260). New insight into GH signalling mechanism

was provided by the demonstration that a tyrosine kinase is present in a complex with GH receptor prepared from GH-treated 3T3-F442A fibroblast cells (268). Additional studies in this same cells showed a rapid GH-dependent tyrosine phosphorylation of multiple proteins including stimulation of microtubular-associated protein kinase activity as well as inhibition of these actions by inhibitors of GH receptor-associated tyrosine kinases (269) suggesting a pivotal role for the GH receptor-associated tyrosine kinases in GH signal transduction. Two such kinases have been identified to date. First tyrosine kinase is 121 kD and the second is JAK 2, which is a 130 kD tyrosine kinase (271). GH also stimulates the phospholipase C activity in vitro with the production of inositol triphosphate and diacylglycerol in basolateral membrane of canine kidney (272). In OB 1771 mouse preadipocyte cells, GH has been shown to stimulate the production of diacylglycerol by means of phophotidylcholine breakdown, involving a phospholipase C coupled to the GH receptor (273). In isolated hepatocytes, GH receptor is also able to stimulate rapidly the production of diacylglycerol without changes in the inositol phosphate concentration (274). Diacylglycerol is a known protein kinase C activator; therefore, the data suggest that protein kinase could mediate at least some of the actions of GH.

4. Insulin-Like Growth Factor Binding Proteins

4.1. History and Discovery

With the isolation and purification of IGFs from human plasma in 1974, it was observed that the native form of these peptides was much larger. Affinity labeling of plasma proteins with (^{125}I) IGF-I as a probe resulted in two sets of bands on SDS-PAGE. The first set migrated around 150kD while the second set was smaller at 20-40kD. When IGFs were secreted to media from cell lines, they were in their predicted M_r range of 7kD (1, 158, 159) based on cDNA sequence.

Pioneers in the field like Hintz and Liu from Stanford (161), Froesch's group in Zurich (180) and others (159) affinity labelled human serum protein with (^{125}I) IGF-I followed by size exclusion chromatography, dermonstrated the presence of high affinity binding sites for IGF-I in the 70,000-150,000 dalton molecular mass range. Only unlabelled IGF-I could displace the binding of the radiolabeled counterpart (IGF-I) from these sites, whereas insulin, ACTH and human growth hormone failed to do so (169). Also it was noted that upon acetic acid/NaCl treatment of these complexes of 70,000-150,000 dalton, biologically active IGF eluted in the void volume of Sephadex G-75 size exclusion chromatographic columns. These observations suggested the presence of high-affinity binding proteins for IGFs in the plasma.

4.2. Function

The biological or physiological reason suggested for the existence of IGFBPs are as follows:

Unlike insulin, which has a major storage and secretion organ in the pancreas and β-cells of pancreas respectively, IGFs are produced by many cell types situated in different tissues and organs in the body. Furthermore, insulin acts through the classical endocrine mechanism, where various neurological, nutritional and hormonal conditions regulate its secretion, and then target organs respond to its metabolic (acute), and mitogenic (chronic) actions (163) when present in circulation. On the other hand, 80% of IGFs are produced by the liver, and the reservoir of these peptides is thought to be the binding proteins (IGFBPs) that exist in the circulation (71). Glimpses of IGFBPs function come from the observations of half-life prolongation from a few minutes (10 min) for "free' IGFs to a few hours (10-11h) when bound to their carrier-proteins (1, 71, 102).

Then the question arises, what else do these binding proteins do?

Since the mid 70's investigators in the field, have sorted-out answers to this central question. The interest in the area can be judged by the tremendous amount of literature available, because IGFBPs seems to be the latest addition to somatomedin hypothesis regarding growth and differentiation function. This somatomedin system consists of growth hormone secreted by the pituitary, IGFs mainly secreted by the liver and under GH control (synthesis, and secretion in other cell types in peripheral organs and tissues), and finally the binding proteins (IGFBPs) that modulate their (IGFs) action, both

metabolic and mitogenic (1,71).

4.3. Purification and Characterization

4.3.1. IGFBP-1

The very first IGF binding protein to be characterized was human IGFBP-1 and this thesis is about the cloning and regulation of the rat IGFBP-1 gene .

In the late 1970's (1979), Drop et al working in Barry Posner's group of Montreal, purified the human protein (hIGFBP-1) from amniotic fluid; it was named IBP-1 (inhibitory IGF-binding protein-1) or BP-28, since the M_r was 28,000 dalton under non-reduced condition (165). Later, another BP was isolated from human placenta, and called placental protein 12 (PP12), which was found subsequently to be identical to IGFBP-1 (71).

4.3.2. IGFBP-3

This protein is the predominant form in the adult, in both rat and human. It exists as an 150-200 kD complex which consists of a β subunit (40 kD IGF binding protein-3), an α subunit which is the acid labile protein (ALP) and is a 85 kD glycoprotein, and finally a γ subunit which is IGF-I or IGF-II (179). In collaboration with Baxter's group in Australia, Leong et al (179) have cloned the α-subunit (ALP) encoding gene and

demonstrated that ALP upon binding to the binary complex of IGFBP-3/IGF-I (IGF-II), will give rise to the final ternary complex. This ternary complex has the longest half-life compared to IGFs or the IGF/IGFBP-3 binary complex. Our studies have shown that 60% of the available IGF-I sites in the adult rat serum reside in IGFBP-3 (102).

4.3.3. IGFBP-2

This protein was first isolated from a rat liver cell line (BRL-3A) conditioned media by Rechler's group at NIH (166). Plasma form of this protein exists in human and rat sera with a M_r of 30-36 kD depending on the experimental conditions of the SDS-PAGE (166-168).

4.4. Gene Structure

4.4.1. IGFBP-1

The human gene for IGFBP-1 consist of 4 exons and three introns spread over a 5 kb of genomic DNA in chromosome 7 (170). This single copy chromosomal gene encodes a 1.55kb transcript in the liver which translates into a 25.275 kD mature protein.

The 5'flanking region of the gene contains a TATA beginning at -28 bp and a CCAAT promoter element beginning at -72 bp from the transcription initiation site. Further, Suwanichkul et al (142) delineated the basic promoter of the gene to a 22 bp

region spanning from -82 to -61.

4.4.2. IGFBP-2

The gene encoding IGFBP-2 consists of 4 exons in a 8 kb region of the human chromosome 2; a fragment of 1260 nt in the 5' flanking region of the gene demonstrated promoter activity. This promotor did not contain either TATA or CAAT boxes which are typically found in house keeping genes and inducible tissue specific genes such as IGFBP-2 (126, 164). Growth factor receptor genes such as epidermal growth factor receptor (173), nerve growth factor receptor (174) and insulin receptor are also TATA-less (175, 176). IGFBP-2 is GC-rich in the first exon and first 120 nt of the 5' flanking region (164). These GC-rich areas may be recognized by transcription factors like Sp1 or ETF. There is a potential Sp1 site in -138/-130 5' flanking region of this (IGFBP-2) gene (164). ETF-binding elements are more active in TATA-less promoters compared to ones with TATA and those (promoters) containing four Cs (C4) are more active than that of four Gs (G4); fifteen G4 and C4 sequences in IGFBP-2 promoter and a variant Sp1 site exists at -158/-150 (164). In the low density lipoprotein receptor (178), a variant Sp1 sequence in tandem with a consensus Sp1 site appears critical to the regulation of transcription by sterol. Also the human IGFBP-2 gene has been cloned (126). The human cDNA of IGFBP-2 has been cloned from libraries of fetal liver (167) and human hepatoma cell line Hep G2. Extensive homology between human and rat was observed in the predicted amino acid sequence (>85%) and both human adult and fetal liver

contained 1.6-1.8 kb IGFBP-2 transcript (85).

The IGFBP-2 transcripts of 1.6 or 2.0 kb are expressed in BRL-3A cells, and in multiple fetal rat and tissues including liver, kidney, stomach, intestine, lung and brain (167). The IGFBP-2 mRNA is highly expressed in fetal and neonatal rat liver; in the term gestation rats, this binding protein is most abundant in the liver than in other tissues (167). IGFBP-2 mRNA abundance is persistently high in adult rat brain tissue. This may be the reason why IGFBP-2 is present in the CSF at high levels (166, 167).

4.4.3. IGFBP-3

According to Cubbage et al (107) the human IGFBP-3 spans 8.9 kb and exists as a single copy in the chromosome 7. The protein encoding region of the gene is divided into 4 exons and the fifth exon encodes all but 15nt of the 3'-untranslated region.

The mRNA cap site (transcription start site) is located 132 bp 5' to the ATG translation initiation codon. A consensus TATA box and a GC rich region were located 30 bp and 97 bp respectively 5' to the mRNA cap site, an organization common to many eukaryotic genomic promoters.

4.5. Regulation

4.5.1. IGFBP-3

In GH hypersecretory conditions such as acromegaly, the expression of IGFBP-3 is increased, while the reverse is true in the hypopituitary conditions (114), suggesting positive GH and/or IGF regulation of IGFBP-3. Further, in vivo evidence comes from chronic and acute studies correlating GH levels of sera with growth parameters such as height and weight gains. These studies also exhibited positive correlation between growth and GH as well as IGFBP-3 levels in the serum (185, 187).

IGF-I has been implicated as the mediator of IGFBP-3 regulation of GH, because even human patients with Laron's dwarfism which is due to a defective GH receptor, show the same levels of IGFBP-3 in the serum compared to that of normal healthy adults (114).

In vivo, when hypox rats were treated with GH, increased levels of IGFBP-3 complex (150-200 kD) in the serum was observed (189, 190); in vitro, no such effect was observed in response to GH treatment of human fibroblasts (185). Fasting and protein restricted diets did lower the 150-200 kD complex in serum; recovery was observed when nutritional restrictions were removed (189).

In type I diabetics, both rats and human, IGFBP-3 levels were decreased; treatment of rats with insulin at high doses (10 u/ 100 gm body weight per day) resulted in an increase in these levels (119-121). Estradiol also increases IGFBP-3 in vivo in menstrual

cycles (191); treating osteoblastic-like chondrocytes in vitro with estradiol resulted in the stimulation IGFBP-3 production (16a).

In pathologic states arising from glucocorticoid deficiency such as Cushing's disease, increased levels of IGFBP-3 containing complex (150-200kD) were demonstrated; exogenous dexamethasone (synthetic glucocorticoid) administration resulted in reversal of the trend (i.e. decreased 150-200 kD complex levels). In the plasma of normal rats, IGFBP-3 levels as well as hepatic mRNA of IGFBP-3 were decreased when treated with dexamethasone, suggesting inverse glucocorticoid regulation of IGFBP-3 (91). Cortisol reduced the IGFBP-3 production from a rat osteoblastic cell line (237), whereas vasopressin, PDGF, EGF as well as TGF-β increased the production of this binding protein in vitro, in cells (238,240). Also in human pregnancies, immunoreactive IGFBP-3 levels increased compared to non-pregnant levels (187).

4.5.2. IGFBP-1

Although present in low concentration in the normal adult, this protein increases significantly in certain physiological and pathological conditions (71,102). In pregnancy, elevated protein content has been reported in the decidualized endometrium of humans. The protein was purified from pregnancy related tissues such as the amniotic fluid and the placenta as mentioned before. In the rat, age related decrease of the expression also has been reported where the hepatic mRNA was high in the fetus and decreased in the adult (98). In many pathological conditions, IGFBP-1 seems to be regulated in a opposite

manner to that of the IGFBP-3. In hyperpituitary conditions such as in acromegaly, increased protein levels of IGFBP-3 in the serum are found, whereas the corresponding IGFBP-1 levels decreased. In patients with familial or genetic hypopituitary conditions, the opposite was true with increased serum IGFBP-1 levels and decreased of levels IGFBP-3. Upon GH treatment, the IGFBP-3 levels increased and that of the IGFBP-1 decreased compared to the hypopituitary situation suggesting positive GH regulation of IGFBP-3 and the negative regulation of IGFBP-1. In patients with Laron's dwarfism which is due to the defective GH receptor, as well as in genetic or idiopathic GH deficiency, the IGFBP-1 levels in the serum were decreased compared to normal levels, and under GH treatment the levels (IGFBP-1) returned to normal suggesting inverse GH regulation of IGFBP-1.

In type I diabetes which is characterized by low circulating insulin levels, increases in IGFBP-1 levels were observed in humans while treatment with insulin reversed the trend suggesting inverse insulin regulation of IGFBP-1 (119,120). Also in the insulin-deficient streptozotocin-induced diabetic rats, increased hepatic message for IGFBP-1 was observed whereas insulin treatment decreased the message levels, again suggesting inverse insulin regulation of IGFBP-1 expression (121).

4.5.3. IGFBP-2

The mRNA abundance of IGFBP-2 exhibits age dependence. This is the predominant IGFBP in fetal tissue including liver, kidney, lung, heart, and muscle with

liver showing the highest abundance (171). In the adult, expression is limited to the CNS (171-172). Also IGFBP-2 exhibits a 10-fold preference in the binding of IGF-II compared to that of IGF-I, and ontogeny and tissue distribution are very similar to IGF-II (166-168).

4.6. IGFBPs 4, 5, and 6

IGFBP-4

Mohan et al were the first to purify this protein from a human osteosarcoma cell line conditioned media (126a). Shimasaki et al (129) cloned the cDNA. They first isolated protein from both the human serum and a human osteosarcoma cell line and then oligonucleotides were synthesized which were used as probes to screen cDNA libraries. Also, they demonstrated its presence in the adult rat serum. The transcript from the IGFBP-4 gene is 2.6 kb and has an ubiquitous tissue distribution in the adult human; the highest expression is in the liver. The calculated M_r is 26 kD (25,980 dalton); interspecies homology between rat and human is about 92% at the amino acid level. The gene is located on chromosome 17 in humans (129). The chromosomal gene is yet to be cloned.

IGFBP-5

The cDNA has been cloned from rat ovary, human placenta and osteosarcoma cDNA libraries. Under non-reducing condition, IGFBP-5 migrates as a 31-32 kD band in

SDS-PAGE. It displays the highest affinity for both IGF-I and -II compared to other cloned IGFBPs; it also displays fifty-fold excess affinity compared to IGF-I and II receptors at pH 7.4. The message of 6 kb is detected in multiple adult rat organs with highest expression seen in the kidney. But transcripts of 1.7-1.8 kb were also detected in rat ovarian tissue and human osteosarcoma cells (128,29). The gene is located on chromosome 5 in humans.

IGFBP-6

The IGFBP-6 protein has been isolated from several sources including serum (129), cerebrospinal fluid (194), and conditioned media of fibroblasts (137). IGF-I binds to IGFBP-6 at 10 to 100-fold higher affinity than IGF-II and overall affinity is similar to IGFBP-3. The gene is situated on chromosome 12; the mRNA is 1.3 kb in length and the deduced relative molecular masses are 21,416 dalton for rat and 22,847 dalton for human, respectively (129). This protein was first reported to be purified by Roghini et al (194), then Martin et al isolated it from SV40 transformed human fetal fibroblast cells (137).

4.7. Structure and Function of the IGFBPs

The structure of IGFBPs should contain IGF (ligand) binding sites, a site that interacts with the IGF receptors, and possibly other cell surface receptors such as integrin. Like many matrix proteins including elastin, fibronectin, and relaxin, all the six IGFBPs

cloned so far contain Asp-Gly-Gln (RGD) tripeptide that is important in interaction with the integrin family of cell surface receptors. The deduced amino acid sequences of IGFBPs consist of 200-300 amino acids with a signal sequence of 40 amino acids. Eighteen cysteine residues are scattered in both NH_2 and COOH ends of the molecules and are conserved across the six IGFBPs cloned to date; also, the alignment of these cysteines suggests the participation in various active sites/functional domains, probably via the formation of disulphide bonds. In addition, there are two more cysteine residues in the rat and human IGFBP-4 (71). The COOH and NH_2 terminal and portions of the molecules are most conserved regions, while the middle 2/3's of the peptides and the deduced signal peptide are the most diverse, suggesting functional importance of the conserved regions (98,126,129).

The IGF-binding proteins (BPs) undergo post-translational modifications; in the case of BPs 4 to 6, they are glycosylated to varying degrees, as is the human BP-1 (71).

5. GROWTH HORMONE

Pituitary growth hormone, according to the classical somatomedin hypothesis (Fig 2), targets the liver and stimulates it to secrete IGFs which in turn stimulate growth, differentiation and metabolic activity in peripheral target cells (27). But, GH also can act directly rather than through IGFs. For example, when hypox rats were treated with GH or IGF-I, and the response measured in terms of body weight gain, increased tibial epiphyseal width and ^{3}H-thymidine incorporation in to costal cartilage, the magnitude of

the effects were smaller with the IGF-I than the GH (35).

Also, some organs responded to GH better than IGF-I; studies in hypox rats treated with GH or IGF-I revealed that for the muscle and liver, GH was more potent in promoting growth than IGF-I (35). Also effects such as increased epiphyseal width, body weight gain, lipolysis as well as skeletal muscle growth were greater with recombinant human GH (rhGH) than with rhIGF-I (72).

Further evidence that GH is superior in promoting growth comes from transgenic mice models. When transgenic mice for IGF-I (236) were compared with mice that were transgenic for GH or GH releasing factor (GHR) (59), the latter were significantly bigger. Phenotypically body weight gain and skeletal growth were higher in GH transgenics compared to that of IGF-I transgenics. When internal organs were analyzed, liver, skeletal muscle, and bone were more responsive to GH than IGF-I, while brain responded more to IGF-I compared to GH. Thus, GH has a distinct pattern of growth promotion than that of IGF-I (59, 70). Current thinking is that GH is a better mitogen

because it has not only endocrine action but also paracrine and autocrine action via IGFs; it also acts by modulating IGFBPs, resulting in a distinct pattern and higher potency for GH-mediated growth compared to IGF-1 (1, 71).

6. EUCARYOTIC GENES

6.1. Structure

Introns or intervening sequences set eucaryotic genes apart from those of the prokaryotes. Exons are that part of the genetic code (DNA) that is represented in the mRNA; the last and the first exon contain untranslated regions, 5' untranslated (5' UT) and 3' untranslated regions respectively. Translation usually starts at an inframe ATG (coding a methionine residue), and continues to a translation termination codon. It is also known that in some eucaryotic genes translation initiates at a leucine codon instead of a methionine codon. There are three translation termination consensus codons for eukaryotes, i.e. UAG, UAA, UGA in the mRNA (222); These are also known as "non-sense" codons.

Exons encode structural/functional motifs of proteins, and introns therefore make it possible to mix and match various motifs (exons), resulting in different types of protein. This may be one of the ways that the organism can adapt to long-term environmental challenges during evolution.

The functional significance of introns are signalled by their remarkable conservation through evolution from Zea maize to human (221). Furthermore, the presence of introns in the genome allows changes in the transcribable complement, with relative ease. For example, the IGF-II gene consists of 8 exons (exons 1, 2, 3, 4, 4B, 5, 6, and 7) and encodes multi-transcripts. In the 5.3 kb transcript (IGF-II), 1,2,3,5,6, and

7 exons are spliced together whereas the 6 kb message includes exons 4,5,6, and 7. Thus exons 1,2 and 3 are part of the 5.3 kb transcript, but part of the 5' flanking sequence in relation to the 6 kb transcript. Also, exon 4 in the 6 kb mRNA is part of the intronic sequence in relation to the 5.3 kb mRNA (1).

Introns also allow splicing together of different exons which is another mechanism of gene regulation. One combination of exons will result in a protein that is different from another combination. The human gene encoding IGF-1 is a good example; it consists of at least 5 exons (Fig 1B) and produces multiple transcripts. Splicing of exons 1, 3, 4 with 6 or 5 and 6, gives rise to IGF-1 class C transcripts while splicing of exons 2, 3, 4 with 5 or 5 and 6 results in class B transcripts of IGF-1 (1). The biological significance of two IGF-1 protein species is still not known.

Multiple transcription initiation sites and alternative splicing are also exhibited by the human IGF-II gene (1, 182).

6.2. Transcriptional Regulation

The venue of transcription in the eukaryotic cell is the nucleus, where the transcriptional machinery makes a heteronuclear RNA (hnRNA) using a part of the genome as a template. This (transcription) is a major point at which gene expression is regulated.

As can be appreciated in the RESULTS section of this thesis, there is evidence that rat IGFBP-1 gene expression is transcriptionally regulated by GH. Therefore it is

pertinent to review some of the transcriptional controls of eukaryotic genes.

6.2.1. Regulatory elements of DNA

Promotor: According to Pamella Mitchell, Robert Tjian and Tom Maniatis et al (198, 199), the DNA elements residing in the close vicinity of the transcription start site (100-300 bp), are defined as the basal promoter; activity of these promoter elements are orientation dependent. In contrast, enhancer elements regulate gene expression from a distance and are orientation independent; distances from the transcription start site can vary from a hundred base pairs to 30 kb (199).

The TATA box ensures the efficient and accurate initiation of transcription by the RNA polymerase II (pol II) containing basic transcriptional machinery. Pol II binds the TATA box via transcription factor (TF) IID [TF IID] along with 8 other transcription factors including TFIIA, TFIIB, TFIIE/F which interact in a coordinated manner with the core promoter on TATA box sequence to form the "preinitiation complex". Usually this (TATA) sequence is located about 30 bp upstream in the promoter relative to transcription start site (200). Mammals also have TATA box sequence associated factors (TAFs) which help to stabilize this ("pre-initiation") complex. So far about ten such TAFs have been identified in mammals and these nuclear proteins are of 10-200 kD in molecular mass (199). The above complex ("pre initiation") assembly is promoter and RNA polymerase specific. In other genes that do not possess a TATA box, a sequence called "initiator", takes over the function of the TATA box (101). It is postulated that TATA-less genes

without CpG islands are predominantly found in the house-keeping genes, whereas when CpG islands are present, it's a promoter of a inducible gene (101). Brown and Rechler reported such a TATA-less gene promoter in the rat IGFBP-2 gene (164).

The "CCAAT box" with a consensus sequence of GGPYCAATCT is the most highly conserved in the basal promoter. Deletion analysis revealed its importance in basal promoter activity (199). Several CCAAT transcription factors (CTF) binding the DNA motif have been isolated and cloned. These CTFs arise by alternative splicing of a single gene (201-203). The four CTFs known so far are : 1. Nuclear Factor-1 (NF-1) reported by Jones et al (227,228), 2. CAAT binding protein (CBP) reported by Graves et al (226), 3. NF-Y and 4. NF-Y^* binding proteins. The last two CTFs were demonstrated by Dorn et al in 1987 (205). The "CAAT box" (cis-element) is equally active in both orientations. The Y box is a 14 bp sequence that is highly conserved in the 5' flanking region of class II major histocompatibility complex (MHC) genes. It is also a "CAAT" box in reverse orientation (ATTGG) and binds nuclear factors-Y and -Y^* isolated from B lymphoma cells.

The Sp1 transcription factor binds GGGCGG (G/C box) in the basal promoter of many genes and the consensus decanucleotide for Sp1 is 5' G/T GGGCGG G/A G/A C/T 3' (206). This transcription factor was purified from HeLa cells; binds only some GGGCGG hexanucleotides and only in one orientation; Sp1 sites are found in promoters either alone [example, human Metallothionein -II_A (human MT-II_A)] or in many copies [example, SV40 early promoter (SV40 P_E) contains a tandem repeat of six], or as scattered repeats [example, in herpes simplex immediate-early (HSV 1E-3) promoter, or

dihydrofolate reductase (DHFR) gene] (206,207).

Enhancers: As the name implies, these DNA elements enhance or facilitate gene transcription from a promoter. The location can vary from a few hundred to few kilobases from the transcription start site; in contrast to the promoter elements, activity of enhancers is independent of orientation. Then again, some *cis*-elements in the promoter also are orientation-independent, hence the distinction between enhancer and promoter elements is somewhat arbitrary (199, 200). Very simply, one can divide enhancers into two categories; temporal/spatial and inducible enhancers (207-209). The former include cell-specific (207,208) enhancers, while examples for the latter include the cAMP response element (209) and steroid hormone receptor binding elements (210). In the recent past this area of research has expanded dramatically resulting in the characterization of *cis*-acting enhancers and corresponding *trans*-acting factors for a large number of genes (196). As mentioned above, enhancers can be located almost anywhere relative to the transcription start site; the cell-specific element of the T-cell receptor alpha-locus exerts its influence over 69 kb (212). Also enhancers can be present in the 3' flanking region of a gene (198,200). Regulatory elements to date have been best characterized in the 5' flanking region of genes, thus making it the most logical place to start looking for such elements. But there is an increasing body of evidence suggesting the presence of such regulators elsewhere in the genes (216,217). For example,in the human IGF-11 gene, when promoter 1 (P1) operates, exons 1 to 4 are found in the mRNA, but when the promoter 2 (P2) is used, the first three exons become 5' flanking DNA, and when the functional promoter

is P3, all first four exons become 5' flanking DNA (218). There is some recent evidence to suggest that repetitive sequences such as Alu can play a part as gene expression region regulators (219). Thus, intronic sequences are not just 'junk' DNA but may contain important regulatory functions.

Another concept in gene expression regulation is the presence of "silencer" elements; promoters and enhancers generally confer a positive effect on transcription. But as in any other regulation, negative controls of transcription are also important. Insulin target genes are good examples to elaborate such controls. Insulin has an antagonistic effect on both human GH (220) and phosphoenolpyruvate carboxykinase (PEPCK) gene transcription (229); insulin response silencer (IRS) elements have been located in both 5' flanking regions of these genes.

6.3. Processing of hn RNA→mRNA

The maturation of the primary transcript (hnRNA) to the messenger RNA (mRNA) occurs in the nucleus. The hnRNA runs from the transcription initiation (start) site which is usually an adenine (A) nucleotide, to a transcription termination site. Immediately preceding the transcription start site, is an added G, methylated on the 7-position of the base and linked to the intron nucleotide by an unusual 5'-5' triphosphate linkage, ("cap" site). The 5' untranslated region (5'UT) spans from the transcription initiation site to the translation start site. Although the exact function of the "cap" is unknown, there is evidence to suggest its importance in ribosomal binding during translation, in the

inhibition of mRNA degradation and translocation of mRNA into the cytoplasm. At the other end, the 3' UT region-from translation to transcription end- contains polyadenosine (40-100 residues long) tail, added to mRNA after splicing but before translocation of the message to the cytoplasm. It is thought that the poly $(A)^+$ tail increases stability or functions as an anchor in attaching mRNA to membranes. But not all mRNA contains poly $(A)^+$ tails as in the case of histone message. Addition of this tail by a special polymerase where action is triggered by the AAUAAA consensus poly $(A)^+$ addition signal present in the 3' UT of the mRNA (221).

In 1977 the discovery that eukaryotic genes are interrupted by introns shattered then held views of these genes. The hnRNA (primary transcript) contains information from transcription initiation to transcription end with intronic sequences between exonic information. The splicing of the exons by cutting off the intronic sequences (RNA splicing) takes place in the nucleus via an elaborate series of biochemical reactions(221). For these reactions molecules are involved termed small nuclear ribonucleoprotein particles (SnRNP). These include UI and U2 RNA molecules which are small molecular weight RNA species complexed with proteins. These SnRNPs form kinetic intermediates by interacting with hnRNA 3'-and 5'- splice sites and other proteins (the kinetic intermediate complex) which are called the splicesomes (221).

The analysis of 400 vertebrate genes for exon/intron boundaries has led to the identification of consensus splice site sequences as shown in Table 1A. The consensus region evident in the matrix extends from two last nucleotides in the exon through the first six nucleotides in the intron at the 5' splice site AG:GU(A)AGU (colon denotes the

site of cleavage and ligation). The consensus at the 3' splice site extends from at least the last 15 nucleotides of the intron through the first nucleotide of the exon to give a consensus of $(U/C)_{11}$NCAG:G (221).

Differential splicing as exemplified by a host of genes including IGFs, is thought to be an important mechanism of gene expression regulation.

6.4. mRNA→Protein [Translation]

The above flow of genetic information (translation) takes place in the cytoplasm. Ribosomes read the triplet codons of mRNA starting mostly at an inframe AUG which encodes a methionine residue, or UUA and UUG both of which signal a leucine residue and ends (translation) at a "non-sense" codon. The analysis of 699 vertebrate mRNA sequences has revealed a consensus 13 nt translation start site of (GCC)GCCA/GCC<u>ATG</u>G; the ATG start is underlined. Except for the bracketed nucleotides, importance of the others in translation initiation has been demonstrated by site-directed mutagenesis studies whereas the bracketed nucleotides occur at statistically high probabilities (256). Most genes have one translation initiating ATG site except in many proto-oncogenes where more than one are present. In such situations (multiple initiator codons) the most proximal one is favoured. Furthermore according to Kozak (256), not only the position but also the context of the ATG initiator site is important in deciding the functional translation initiating ATG site. Consensus "non-sense" codons UAG, UAA, and UGA are necessary and sufficient (one of them) to terminate protein

Table 1A Consensus sequences of vertebrates[a]

5' splice site

	exon										intron	
%G	23	14	13	77	100	0	32	12	84	18	30	22
%A	34	35	62	8	0	0	60	74	9	15	33	22
%U	15	12	13	8	0	100	5	7	3	50	17	25
%C	29	38	12	8	0	0	3	7	4	17	21	31
	—	—	A	G:	G	U	A	A	G	U	—	—

3' splice site

	intron																exon	
%G	18	13	15	10	10	7	7	10	9	5	5	5	24	0	0	100	55	27	24
%A	17	11	8	9	7	4	9	8	10	8	4	9	26	2	100	0	20	21	19
%U	37	44	46	46	56	59	43	49	41	46	42	46	23	19	0	0	8	32	28
%C	28	33	32	35	27	30	42	33	40	40	49	41	27	78	0	0	17	20	28
	—	—	Py	Py	Py	Py	Py	Py	Py	Py	Py	Py	N	C	A	G:	G	—	—

[a]A tabulation of the sequences at assigned 5' and 3' splice sites in approximately 400 vertebrate genes in the Gen Bank Data Base. All examples were included where intervening sequences began with a GU dinucleotide and terminated with an AG dinucleotide.

Reproduced from Padgett et al (221).

synthesis (translation). This (translation) is also one of the points at which gene expression can be regulated, for example in Xenopus oocytes, sequestering of mRNA is known (222).

Secretory proteins such as polypeptide growth factors (eg. IGF-I, II, insulin etc) contain a signal peptide which is hydrophobic in nature; this signal peptide is recognized by the cellular membrane transport system through which secretion to the extracellular environment is achieved. Signal peptide containing preprohormone polypeptides once secreted from the cell, loses its signal peptide becoming the prohormone form; eg. clipping-off signal peptide of preproinsulin (223).

Rationale and Objectives of the Investigation

Hypothesis of this thesis is that IGFBP-1 gene expression is regulated in response to conditions which influence growth. The molecular mechanisms that confer the regulation will be found in the genetic elements within the IGFBP-1 gene.

At the inception of this investigation, very little was known about the regulation of the function of IGFBP-1. IGFBP-3 was shown to be GH dependent and it was postulated that IGFBP-1 was GH-independent (1, 71).

Although the major IGFBP in adult circulation is IGFBP-3, increased levels of IGFBP-1 were associated with pregnancy (example, presence of the IGFBP-1 at high levels in amniotic fluid and placenta), suggesting its importance in fetal growth and development.

In order to better understand the function and the regulation of IGFBP-1, the

following objectives were set: (1) Cloning a full length cDNA for the rat IGFBP-1, (2) Using the above cDNA, to elucidate the nutritional, GH and insulin regulation of IGFBP-1 mRNA abundance. (3) As a first step in delineating the molecular mechanisms governing IGFBP-1 gene expression, cloning of the rat IGFBP-1 gene and elucidate its structure. (4) Regulatory sequences in majority of the genes cloned to date, exist in the 5' flanking region. Thus, cloning and sequencing of 1-2kb of the 5' flanking of IGFBP-1 was also envisaged.

MATERIALS AND METHODS

Isolation and Sequencing of the cDNA

A λgt11 rat liver cDNA library in which the cDNAs were subcloned to the EcoR1 site of the vector was kindly provided by Dr. Dakshinamurti's group at the Department of Biochemistry and Molecular Biology at the University of Manitoba. Phage recombinants containing rat cDNA for IGFBP-1 were selected by filter hybridization using a ^{32}P-labeled 1.1kb partial cDNA cloned by Murphy et al (98) for rat IGFBP-1. Hybridization was performed under stringent conditions at 42^0 C a hybridization solution containing 50% formamide, 20mM NaH_2PO_4(pH 7), 4xSSC, 2mM EDTA, 5xDenhardt's solution (1x=0.02% BSA, Ficoll, and polyvinylpyrrolidone), 0.1% sodium dodecyl sulphate (SDS), and 100 ug/ml sonicated denatured salmon sperm DNA, and was washed first with 2xSSC (1xSSC=0.15M NaCl and 0.115M sodium citrate) +0.1% SDS at room temperature, then with 0.1xSSC +0.1% SDS for 30 min at 65^0 C. The rest of the filter hybridization of the phage library was essentially as described in Maniatis et al (249). The EcoR1 inserts of the positive recombinant phages were subcloned into pGEM 3Z.

DNA Sequencing

All nucleotide sequencing were performed by Sanger's dideoxy chain termination

method (241) in the presence of [α^{32}P]dATP according to United states Biochemical's (Cleveland, Ohio) technical manual. The products of the sequencing reactions were analyzed by denaturing 6% polyacrylamide-8M urea gel electrophoresis at 40-50 watts. Sequencing gels were fixed for 20min in 10 methanol-10% glacial acetic acid and dried using a Biorad model 1125B slab gel dryer. Autoradiography was performed at room temperature for 12-24h.

Animals

Male Sprague-Dawley rats (SD), 150-200 gm body weight (BW), were obtained from the University of Manitoba Breeding facility (Winnipeg MB Canada). In the refeeding experiments rats were deprived of food for 24h period and refed by allowing free access to rat chow. In the insulin effects study, rats that were food deprived for 24h were given an ip injection of insulin (4U; Connaught, Toronto, Ontario, Canada) and sacrificed by decapitation at indicated times after the injection. In the GH regulation/hypox study, male rats at either 80-100gm BW and 3 weeks of age (young hypox) or at 200-250gm BW and 9 weeks of age (old hypox rats) were obtained from Charles River Canada (St. Constance, Quebec) and acclimatized by holding in cages for 2-4 weeks. Age and sex matched, sham-operated rats were obtained from the same source. A group, each from young and old sham operated rats, were chronically treated with a daily subcutaneous (sc) injection of human pituitary GH (3 IU/mg; kindly provided by Dr. Ian Worsely, Department of Physiology, University of Manitoba) at a concentration of 100ug/100gm

BW for 8 days. The liver was carefully dissected from each rat and immediately frozen on dry ice, then stored at -70^0 C.

Determination of Serum Glucose

Serum glucose concentration was measured using a glucose oxidase system (Sigma Chemical Co., St. Louis MO) and according to the protocol supplied by the manufacturer. Essentially, glucose in the serum is oxidized by glucose oxidase to gluconic acid and H_2O_2. This H_2O_2 generated, oxidizes a colorless substrate (o-Dianisidine) catalyzed by peroxidase to produce the oxidized o-Dianisidine (brown). Enzyme mix (glucose oxidase and peroxidase) and color reagent (colorless substrate, o-Dianisidine) was supplied by the manufacturer. The standard glucose solutions and serum samples were incubated with combined enzyme-color reagent solutions as specified by the supplier. The color developed, both in the standards and serum samples was measured by absorbance at 445-475 nm using a spectrophotometer. The serum glucose concentration was determined from the standard curve.

Isolation of RNA

During the course of these studies two methods of RNA extractions were employed. The first, which was more extensively used, was the CsCl cushion method of Chirgwin et al (250); the extraction was executed as described in this publication. This was the method

of choice when the tissue source was over 1 gm. The second method, the modified version of Chromczynski and Sacchi (251) was employed when the starting tissue weighed less than 1gm. In this method (second) the frozen tissue was homogenized using a polytron in a solution of 4M guanidinium isothiocyanate, 0.005% Sarkosyl, 0.025M sodium citrate and 0.007% v/v β-mercaptoethanol at a ratio of 0.5-1.0gm tissue/ml. After a brief homogenization (2 x 45 sec), 0.5ml of 2M Na acetate pH 4.7 +5ml equilibrated phenol + 1.0ml chloroform/isoamyl alcohol was added and the solution mixed vigorously. To separate phases, the mixture was centrifuged at 4000 x g (4 C) for 30 min. The nucleic acids in the aqueous phase were precipitated with an equivalent volume of isopropanol at -20 C for about 45 min. The precipitate was centrifuged at 10,000 x g at 10^0 C. The resultant pellet was resuspended in 3ml TE pH 7.5 and extracted with 2ml of equilibrated phenol + 0.3ml 2M CH_3COONa pH 4.6 + 0.5ml chloroform/isoamyl alcohol, by centrifuging for 15min at 10,000 x g (10 C). The aqueous phase was then extracted with an equivalent volume of chloroform/isoamyl alcohol and centrifuged as above. The RNA was precipitated from the aqueous phase with an equivalent volume of isopropanol at -20^0 C for 30 min. The RNA was pelleted by centrifugation at 10,000 x g for 15 min (10^0 C), then washed with 70 % EtOH, desiccated and dissolved in sterile TE pH 7.5. The yield of RNA was determined by A_{260}.

Poly $(A)^+$-enriched RNA was isolated from total RNA by one cycle of oligo(dT) cellulose chromatography (252). In this technique, 0.5-1 gm of oligo(dT) cellulose was equilibrated for 16h in 1xTEK (0.01M Tris-HCl pH 7.5, 0.001M EDTA, 0.5M KCl) and a 0.5mm column was packed with it. With the aid of an peristaltic pump, the column was

washed with 25ml of 0.1M NaOH, 25ml of sterile double distilled H_2O (ddH2O), and 25ml of TEK. Total RNA (about 1mg or more) was boiled and 2.5M KCl was added to a final concentration of 0.5M KCl and applied to the column; flow through was collected and again reapplied. Column was washed with TEK until all the non-poly $(A)^+$ RNA was removed. Then Poly $(A)^+$ RNA was eluted with about 4ml of TE (65 C) and precipitated with 2.5 volumes of EtOH and 0.04 vol of 5M NaCl and recovered as for total RNA. Poly $(A)^+$ RNA was stored at -70^0 C in TE pH7.5.

Northern Blot Hybridization

Total or poly$(A)^+$ RNA was fractionated by electrophoresis on 1.3-1.5% agarose-2.2M formaldehyde horizontal slab gels containing 0.5ug/ml EtBr as described (253). These gels were transferred to nitrocellulose filters. Filters were then prehybridized for 16h at 42^0 C in the hybridization solution (same as before). Hybridization was performed in the same solution at 42^0 C. Probe was the gel-purified insert of 1.5kb full length cDNA for the rat IGFBP-1 (CS 15), nick-translated to a specific activity of 10^8 dpm/ug DNA. Filters were also subsequently hybridized with NB29 which is a cDNA encoding a constitutively expressed rat heat shock-like protein (254).

Culturing of COS-1 Cells

COS-1 cells, an African green monkey kidney cell line transformed with SV 40,

were counted with a Coulter counter and grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). To each 10mm culture plate, 5 x 10^5 cells were seeded. These cell cultures were incubated at 37^0 C in an incubator with 5% CO_2. Cells were grown for a 24 h period which resulted in the doubling of the cell numbers to 1 x 10^6 and were subconfluent. At this point, transient transfections were carried out as below.

Transfection and Expression of rIGFBP-1

The mammalian expression vector pSVL from Pharmacia (Bale d'Urfe, Quebec, Canada) was modified as in Murphy, Seneviratne et al (99) to construct the expression vector pSVLJ which contained a unique EcoR1 site down stream of the SV40 promoter. The EcoR1 insert of the pCS15 was ligated to the pSVLJ to produce the pSVCS15(F) containing the rIGFBP-1 in the forward orientation and pSVCS15(R) containing rIGFBP-1 cDNA in the reverse orientation. The pSVCS15 vectors and pSVLJ vector without any insert, were transfected into COS-1 cells using the calcium phosphate precipitation method (249). After transfecting for a 24h period, the medium was removed and serum-free medium (DMEM from SIGMA Inc., St. Louis MO) was added and conditioned for a 48h period. Conditioned medium was dialysed against PBS (2.68mM KCl, 1.5mM KH_2PO_4, 0.14M NaCl, 8.1mM Na_2HPO_4) and concentrated to 1/10th of the original volume using a freeze dryer. The conditioned media was analyzed by polyacrylamide gel electrophoresis by Paul Moreira essentially according to Hardouin et al (114); IGFBP bands were

visualized by ligand blotting with (^{125}I) IGF-1 as the probe.

Isolation of Genomic Clones

Kaspers rat genomic library was prepared by partially digesting Wistar-Fur rat liver genomic DNA with Sau 3A and cloning the resulting fragments into the Bam H1 site of charon 35 vector. This library was kindly provided by Dr. Mary Lynn Duckworth of the Department of Physiology University of Manitoba.

The EcoR1 rat genomic library contains, adult female SD rat liver genomic DNA digested with EcoR1 and cloned into same site in the charon 4a vector; average insert size given is 9.7kb and the number of independent clones are 2.2×10^6.

The Hae III library is also a rat genomic library where adult female rat liver genomic DNA had been partially digested with HaeIII and cloned into the EcoR1 site of the charon 4a vector.The average size of the inserts given is 8.2kb and the number of independent clones are 1.8×10^6.

Both EcoR1 and HaeIII libraries were obtained from Clonetech, Palo Alto, CA.

The Dash-II library is a rat genomic library purchased from Strategene La Jolla, CA, USA, in which male SD rat testis genomic DNA, partially digested with Sau3A1, was inserted into the BamH1 site of the Dash-II vector (modified lambda vector); titre is 2×10^6 primary plaque forming units (pfu) with a average insert size of 22kb.

Filter Hybridization

Filter hybridization was done essentially according to Maniatis et al (249) in which the host E.coli strains-K803 or SRB were inoculated with the phages in a $MgSO_4$-containing LB medium (100mM $MgSO_4$, 0.2% Maltose in LB medium = 1% trypton, 0.5% yeast extract, 0.5% NaCl, all w/v) for 20 min at 37^0 C. Then top agar (LB + 0.7% agar) was added to the above mixture, gently mixed and layered onto LB-glucose (0.2% glucose) plates. Plates were incubated overnight at 37^0 C. The plaques that appeared on the bacterial lawn of the plates were then blotted onto nitrocellulose filters (10mm) and lysed with 0.5M NaOH/1.5M NaCl solution, then neutralized in 0.5M Tris base/3M NaCl pH 7.4 buffer, and finally fixed in 6xSSC (1xSSC=0.15m NaCl-0.115 sodium citrate). Filters were hybridized with nick-translated probes, hybridized in the hybridizing solution under stringent conditions at 42^0 C. Filters were washed first with 2xSSC, at room temperature and 30 min at 65^0 C with 0.1xSSC; autoradiographed on Kodak XAR-5 photographic films at -70^0 C with a DuPont intensifying screen. Positive plaques were purified from three such screenings in duplicate.

Phage DNA purification

Phage DNA purification was accomplished by a modified version of Maniatis et al (249) in which rapid small scale isolation of bacteriophage lambda DNA was employed. An individual positive plaque from the third screen was picked with the sharp end of a

pasture pipette, resuspended in 100ul of SM medium (SM= 100mM NaCl, 8.11mM $MgSO_4$, 50mM Tris.Cl pH7.5, 0.01% gelatine) to which 2.5ul of $CHCl_3$ was added, in order to prevent any phage infection-resistant bacteria from growing. A 25μl aliquot of the above phage stock was preincubated for 20 min at 37^0 C with 100ul of K803 host (all cDNA and genomic libraries except Dash-II) grown in LB broth supplemented with 10 mM $MgSO_4$ and 0.2% maltose (LBMM media) or when the host was SRB (for Dash-II phage library), the incubation was at 39^0 C. This bacterial-phage mix was then layered onto LB-glucose plates (refer Fitter hybridization) and incubated overnight at 37 C (K803 host) or 39 C (SRB host). The confluent plates were flooded with 5ml of SM, then gently agitated for 2-4h. The phage containing SM medium was centrifuged at 500 x g (RT) to remove any resistant bacteria (phage stock). Bacterial hosts were first grown in 100ml of LBMM medium at 37 C, until an OD_{600} of 0.5 was reached, then inoculated with 2ml of phage stock (2 x 10^9 pfu) and grown for another 16 h. Then 1ml of chloroform was added and incubated for another 30 min in a Brunswick shaker to effect the lysis of the bacterial cells. The bacterial debris were precipitated by centrifuging at 3,500 rpm in a PR-7000 IEC centrifuge for 15-30 min at RT. The phage particle containing supernatant was recovered and the bacterial DNA and RNA were digested with DNase and RNase respectively (1ug/ul final concentration) and by incubating at 37^0 C for 30 min. The 100ml initial volume was distributed among four 50 ml polypropylene centrifuge tubes to which 25ml (each tube) of 2M NaCl/20% Polyethylene glycol-8000 (w/v) maintained at 4^0 C, was added and incubated at 0^0 C (ice water) to precipitate the phage particles. The phage particles were then recovered by centrifuging for 20 min at 3,5000 rpm in a IEC-

PR7000 at 4 C. Resuspended phage particles in 2-4 ml of SM medium, were lysed by incubating with 2ml of 10% SDS and 2ul of 10mg/ml proteinase K for 30 min at 37^0 C. Proteinase K was inactivated by the addition of 0.5M EDTA and incubation at 65^0 C for 15 min. The phage DNA was then recovered by phenol/chloroform extraction followed by chloroform extraction as stated in Maniatis et al (249), to get rid of the phenol which can inhibit subsequent enzymatic reactions. The DNA was precipitated by the addition of equal volume of isopropanol at RT for 20 min and centrifuging at 1000 x g for 15 min. The resulting pellet was washed in 70% EtOH and resuspended in approximate volume (200ul) of TE pH 8. The DNA was stored at -20^0 C.

Restriction Endonuclease and Southern blot Analysis

In each reaction, 0.5-1ug DNA was digested in 20ul of reaction mixture. The appropriate reaction buffer from Boehringer Mannheim was used in each case. All restriction enzyme digestions were done for 2h-16h at 37^0 C except for Sma1 where it was at RT.

Products were analyzed electrophoretically in 0.6-1.2% horizontal agarose gels run at 20 V/cm in 1xTBE buffer (1xTBE= 0.9m Tris base, 0.9M boric acid, 18.8 mM EDTA) as described in Maniatis et al (249). The choice of the agarose gels was dependent upon the molecular weight range of the DNA fragments of interest. Visualization of the banding pattern was attained by the addition of EtBr to the melted agarose gel solution which resulted in the visualization of DNA bands under the long wave length of the ultra violet

(UV) light. Fragment length was determined from the distance travelled using a semi-log plot of mobility vs. fragment size derived from known DNA standards. The DNA markers used included lamda DNA digested with Eco R1 and Hind III, lambda DNA digested with Hind III and ϕX174 DNA digested with HaeIII. From the above standard curve, the length of the fragment of interested was determined.

Southern Transfer and Hybridization

DNA from gels was transferred (249) onto nitrocellulose filters and prehybridized as before at 42^0 C for 4-16h and hybridized in the same hybridizing solution with the appropriate nick-translated or random prime-labelled probes prepared as outlined below.

DNA Labelling

The DNA probes were [^{32}P] labelled by one of two techniques. In a nick-translation (249) reaction, 125ng of the DNA was labelled under standard conditions of the supplier (Amersham Canada Ltd., Oakville, Ontario) using 70-100uCi of 3000Ci/mmol [α-^{32}P]dCTP. The unincorporated label was removed by size exclusion chromatography columns prepared with G-75 Sephadex equilibrated in TE pH 7.5. The specific activity of the resultant probe ranged from 1-5 x 10^8 cpm/ug DNA.

The second method was using the random prime labelling kit (293). The advantage was that this method required less DNA. Usually 25ng of DNA was used and only 50μCi

of 3,000Ci/mmol [α^{32}P]dCTP was required when employing this method. The biochemistry behind this method is that a single DNA strand is used as an template and the complementary strand is synthesized primed by random hexamer oligonucleotides. Priming and DNA synthesis is carried out by the Klenow fragment of the DNA polymerase. Unincorporated label was removed as described above. The specific activity of the probes synthesized were approximately $1\text{x}10^9$ cpm/μg.

Subcloning of DNA Fragments

Vectors used for subcloning include bluescript SKII (BSSK), pGEM 3Z+, and pGEM 7Z+. Vector plasmids containing compatible ends with the insert of interest, were obtained by restriction digestion. In order to prevent plasmid recombining, 5'-termini were dephosphorylated. This was effected by adding calf intestinal alkaline phosphatase (CIP) into the restriction enzyme digest. DNA was extracted with phenol/chloroform according to Maniatis et al (249), then chloroform and precipitated in 2.5 vol EtOH and 0.2M NaCl (final concentration) at -20^0 C for 16h or -70^0 C for 2h. The recombinant DNA was recovered by centrifugation at 4^0 C in a microfuge for 15min at 12,000 x g. The plasmid was washed in 70% EtOH, desiccated, and resuspended in TE pH7.5 to obtain a DNA concentration of about 100ng/ul.

For ligation reactions, gel purified insert DNA was mixed with 5'-dephosphorylated plasmid vector using 1-4u of T4 ligase in 1xligase buffer from BRL (5xligase buffer=0.25 M Tris-HCl pH 7.6, 50 mM MgCl2, 5mM ATP, 5mM DTT, 25%

(w/v) polyethylene glycol-8000) at room temperature (RT) or 15^0 C for 4h-16h. Aliquots of 4ul of the above ligation mix (up to 240ng) were used to transform 100µl of competent bacterial cells as described below. The transformed bacterial cells were spread on LB-ampicillin plates containing 100µg/ml ampicillin. Before plating the cells, 50µl of 2% X-gal, 20µl 2% isopropyl-thiogalactoside (IPTG) were added to distinguish recombinant plasmids from the nonrecombinant ones. If the lac-alpha peptide operon in the plasmid vector is interrupted by recombination, then IPTG is unable to induce the encoding of β-galactosidase that converts the X-gal (substrate) to a blue colored product. Thus, the recombinants appear as white colonies while the nonrecombinants give rise to blue colonies.

Plasmid DNA Amplification and Isolation

Competent bacterial cells were transformed by recombinant DNA plasmids using $CaCl_2$ dependent method (249). Briefly, 5ml of LB broth medium was inoculated and grown overnight in a shaking incubator at 37^0 C. One hundred ml of LB broth was then inoculated from the overnight culture and grown under the same conditions until OD_{600} was 0.4-0.5. The culture volume was split into two 50ml centrifuge tubes and cells were harvested by centrifugation at 1500 x g for 15 min. The cells were then resuspended in 40ml of cold (4 C) 50mM $CaCl_2$ and left on ice for 20-30 min. The cells were centrifuged again and resuspended in 5-10ml of cold 50mM $CaCl_2$, stored on ice in the cold room until use. The E. coli strains that were used in these studies were NM 522, JM 109, and

MV 1193.

Transformations were achieved by adding a maximum of 4µl of the plasmid DNA solution to 100µl of the competent cells on ice for 20-30 min. This mixture was heat shocked at 42^0 C for 3 min. Then 100µl of LB medium was added and the transformed cells were allowed to grow at 37^0 C for 30-40 min in a Brunswick shaker. Since all the plasmid vectors used in these studies were ampicillin-resistant, the transformed cells were spread on LB-ampicillin plates to select for ampicillin resistant colonies.

Plasmid amplification and recovery was essentially according to Maniatis et al (249); transformed cells were grown in 500 ml of LB medium with 100 µg/ml ampicillin. When the OD_{550} reached 0.6, chloramphenicol was added to a final concentration of 81 mg/500ml of the bacterial culture from a stock (chloramphenicol) of 34 mg/ml made in EtOH. Chloramphenicol is a bacteriostatic agent, which results in an increase in the plasmid DNA/bacterial chromosomal DNA ratio. Then the bacterial culture was grown for another 16-18h at 37^0 C in a Brunswick shaker. Cells were pelleted by centrifuging at 1,000 x g (4^0 C) for 10 min and resuspended in 10ml of ice cold lysis buffer (25mM Tris-HCl pH8.0, 10mM EDTA, 50mM glucose, and 1mg/ml lysozyme) on ice and transferred to 30ml Oakridge tubes (Nalgene Inc., Rochester, NY) maintained at 4^0 C for 10 min. Then, 10ml of freshly prepared lysis solution (0.2M NaOH/0.2% SDS) was added while still on ice and incubated for another 10 min. In order to seperate the bacterial chromosomal DNA from plasmid DNA, 10ml of 3M CH_3COONa pH 5.2 was added and left in ice for 15 min or more. The resulting viscous colloid was then centrifuged at 40,000 x g for 30min at 4^0 C to pellet the bacterial debris. The approximately 30ml

supernatant was transferred to 50 ml polypropylene centrifuge tubes and 0.6 volumes of isopropanol was added and left at RT for 30 min to precipitate the DNA. This was then pelleted by centrifuging at 9,000 x g for 20 min at RT. The resulting white colored pellet was desiccated and dissolved in 6ml sterile TE (10mM Tris-HCl pH 7.5, 1mM EDTA) and 6.6g of CsCl was added and dissolved before the addition of 400ul of 10mg/ml ethidium bromide (EtBr). Centrifugation was carried out at 3,000 rpm in an IEC model HN-S benchtop centrifuge (Needham Heights, Mass) to precipitate the protein-EtBr complexes. The supernatant was transferred to 12ml Ti 75 polyallomer centrifuge tubes, avoiding the pink precipitate. The void volume of the Ti 75 tubes were filled with mineral oil, balanced, sealed and centrifuged at 55,000 rpm in a Beckman Ti 75 rotor at 22^0 C for 16-18h.

Visualization of the lower plasmid band was under the long wave length UV light and recovery was effected via 18 gauge needle to a 3ml syringe. The EtBr was extracted with 8ml of isopentyl alcohol. Before the extraction of EtBr, the plasmid volume was adjusted to 4ml with H_2O to prevent the precipitation of CsCl. The EtBr extraction was done till the disappearance of the pink color which takes about three extractions. After these extractions, the volume of the plasmid containing solution was again adjusted to 4ml with sterile H_2O and DNA was precipitated by adding 2.5 volumes of EtOH and 0.04 volumes of 5M NaCl for several hours at -70^0 C or at -20 C for 16h. The plasmid then was recovered by centrifuging at 10,000 x g (4^0 C), washed with 70% EtOH and desiccated and finally dissolved in an appropriate volume (100-200μl) of TE pH 7.5 and stored at -20^0 C. The absorbance of the plasmid DNA was measured. Since, 1absorbance

unit at 260nm is equivalent to 0.05ug/ul of DNA, the concentration of the plasmid DNA was obtained.

Primer Extension Analysis

The 5' end of the IGFBP-1 mRNA (+1/RNA cap site) was determined by primer extended reverse-transcription (170). A 21-bp oligonucleotide (5'-CCACTTCCGCTACTATCTACC-3'), identical to the first 21 nucleotides of the CS15 sense-strand was end-labelled with T4 polynucleotide kinase (refer labelling of mobility-shift assay probes). The labelled primer was separated from unincorporated [γ-^{32}P]dATP by chromatography over a 5ml of sephadex G-25 column equilibrated with 0.05M ammonium bicarbonate. The peak fraction was dried in a Savant speed vac. For each primer extension reaction, 10ng of labeled oligonucleotide primer (1×10^6 cpm/ng) was annealed with 50μg of rat liver total RNA in a buffer containing 40mM piperazine-N,N'-bis (2-ethane sulphonic acid) (PIPES) pH6.4, 1mM EDTA pH8.0, 0.4M NaCl, and 80% formamide; heated to 70^{0}C and then cooled over 15min to 55^{0}C. The hybrids (DNA:RNA), after ethanol precipitation, were resuspended in 50mM Tris pH8.2 containing 50mM KCl, 8mM $MgCl_2$, 4mM dithiothreitol and 1mM of each dNTP in a final volume of 20μl; 1μl (1u/μl) placental RNase inhibitor and 40u of avian myeloblastosis virus (AMV) reverse transcriptase were added. After this mixture was incubated for 30min at 42^{0}C, 1μg of RNase A and 1μl of 0.5M EDTA were added and incubated for another 30min at 37^{0}C. The primer extended product was then extracted

with phenol-chloroform-isoamyl alcohol 25:24:1, followed by the precipitation with 300μl of ethanol. The precipitate was washed in 100% ethanol and then dried. The samples were resuspended in 10μl of 80% (vol/vol) foramide sequencing buffer, heated to 85^{0}C for 5min, quenched on ice and then analyzed on 6% polyacrylamide/8M urea sequencing gels.

Nuclear Extracts

Rats (4 animals/group) were divided into 3 groups : First, were sham operated, second were hypox rats and the third were hypox rats treated with human GH as outlined before (mRNA abundance study) for 8 days. From the livers of these three groups, nuclear extracts were prepared as follows.

Liver tissue (1-5g), was homogenized using a polytron for 3min in 4 volumes (20ml) of buffer I [10mM HEPES pH 7.9, 0.6 M sucrose, 25mM KCl, 1mM EDTA, 10% glycerol, 100u/ml trasylol (aproteinin), 1mM DTT, 1mM PMSF], then centrifuged for 10 min at 1000 x g and 4^{0}C till the hemoglobulin was removed (three times). The pellet was resuspended in 2 vol (10ml) of buffer I, and the pelleted cells were disrupted by homogenizing for 10 strokes in a Down's homogenizer with pestle A. The nuclei of the homogenate were pelleted by centrifuging for 25 min at 25,000 x g (4^{0}C) and resuspended (pellet) in 1 vol (5ml) of buffer C (buffer C= 20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl, 0.2mM EDTA, 1mM PMSF, 1mM EDTA and 1mM DTT) and Dounce homogenized with pestle B for 10 strokes; the nuclei were extracted by agitating end-over-end for 20 min at 4^{0}C. This procedure breaks open the nuclei and by

centrifuging at 25,000 x g for 30 min at 4^0C nuclear extracts were separated from the debris. The supernatant was carefully removed and dialysed in buffer D (20mM HEPES,20% glycerol, 0.1M KCl, 0.2 mM EDTA, 1mM PMSF, 1mM DTT), three changes within 3h in the cold room. All the manipulations were carried out in the a 4^0C cold room on ice. Aliquots were stored at -70^0C.

Labelling of Mobility Shift Assay Probes

Probes were all subcloned and labelled either with Klenow fragment of DNA polymerase (fragments with recessed 3'-ends) or with T4 polynucleotide kinase (PNK) which labelled blunt-ends or 5' recessed termini. In the Klenow labelling system sufficient plasmid was labelled with 1µl of 3,000Ci/mmol [α^{32}P]dATP to obtain 1-2µg of insert DNA. The labelled insert was gel purified then chloroform/phenol, and chloroform extracted, followed by precipitation of DNA as before. Finally the DNA was resuspended in TE pH 7.5 to adjust the volume to 1ng/µl and 10,000-20,000dpm/ng. Most restriction enzyme buffers were sufficient to effect the labelling by 4-5 u of Klenow fragment of the DNA polymerase I.

The case of PNK labelling was similar except the reaction was in the 1x PNK buffer (10 x PNK buffer=0.5 M Tris.Cl pH 7.6, 0.1 M $MgCl_2$, 50mM dithiothreitol, 1 mM spermidine, 1mM EDTA). All other details were as above.

Mobility Shift Assay Reactions

Reactions that allow interactions between the DNA and proteins of the nuclear extracts were set up as follows in 1.5ml Eppendorf tubes in a total reaction volume of 25μl: Poly dIdC (a synthetic heterodimeric polymer), 1μl from a stock of 2μg/μl, 1ng of labelled DNA probe (10,000-20,000cpm), 5μl of 5x binding buffer (5 x binding buffer= 50 mM Tris-HCl, pH 7.9 or 50mM HEPES-NaOH, pH 7.9, 250mM NaCl or KCl, 2.5mM EDTA, 50% glycerol, 5 mM DTT, 25 mM $MgCl_2$), and 5μg of the nuclear protein extract (refer protein assay below). In the probe only control reaction, all the constituents except for the nuclear extract were present. Finally in order to ascertain the DNA/nuclear factor interaction specificity, 50 molar excess unlabelled DNA (cold DNA) was added to the reaction mixture and all the above reactions were equilibrated at RT for 30 min. At the end of the incubation, 2.5μl of the loading buffer (same used in DNA analysis) added and analyzed.

Protein Assay

Protein concentration of the nuclear extracts were determined by Bio-Rad protein assay (Bio-Rad Laboratories Ltd., Mississauga, Ontario), according to the protocol supplied. This assay is based on the shifting of absorbance of acidic Coomassie brilliant blue G-250 from 465nm to 595nm when binding to protein occurs. Both the samples and standards were prepared according to the instructions of the supplier and the optical

density (OD) at 595nm was measured. The protein concentrations of the samples (nuclear extracts) were determined from the standard curve.

Mobility Shift Assay Gel Electrophoresis

Protein-DNA interactions were analyzed on 4% native polyacrylamide gels (5.33 ml 30% acrylamide , 4ml 2% bisacrylamide, 2ml 50% glycerol, 4ml 10 x modified TBE-1.29M Tris base, 25mM Na_2EDTA, 0.45M boric acid, and made to 40ml with ddH_2O and 300ul of 10% ammonium persulphate as well as 40μl TEMED were added). All the other details were as in Baldwin (255).

Electrophoresis was carried out at 15-25 mA at 4^0C (cold room) for an appropriate length of time. The progress was monitored by the migration of the glycerol/xylene cyanol/bromophenol blue mix (loading buffer) where the bromophenol blue has a migration equivalent to 70bp DNA. The gels were dried and autoradiographed.

RESULTS

1. ISOLATION AND CHARACTERIZATION OF COMPLEMENTARY DNA FOR THE RAT IGFBP-1

1.1. Isolation of a full length cDNA for the rat IGFBP-1

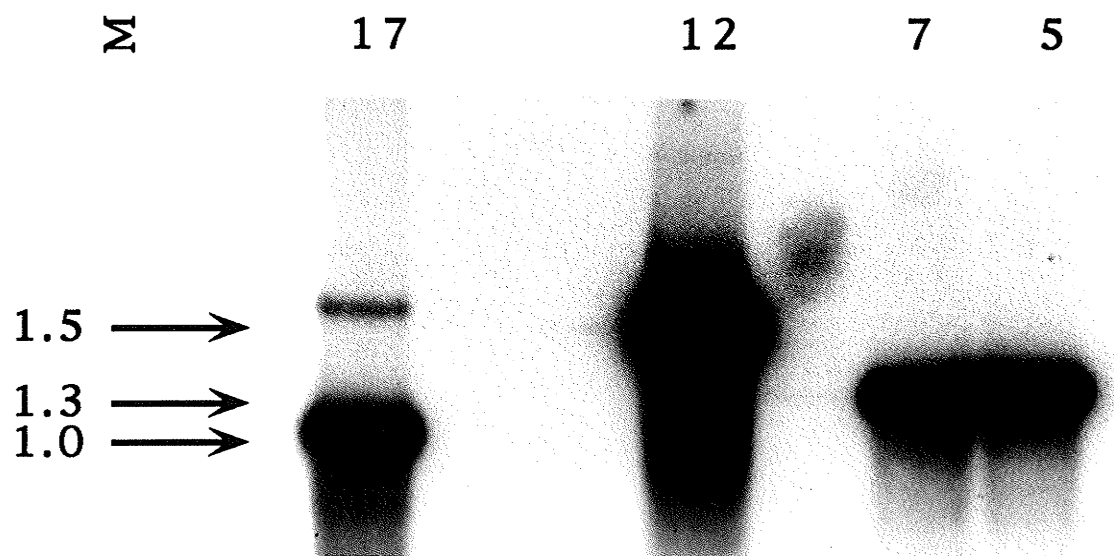
A rat liver λgt 11 cDNA library which kindly provided by Dr. Dakshinamurti, was screened. A 1.1 kb cDNA that was cloned in our laboratory from a rat decidual library (98) was used as a probe. A total of about 400,000 recombinants were screened and 17 plaques were positive on the primary screen; nine were false positives as they failed to hybridize in subsequent screenings. The rest of the positive plaques (eight in number), were purified by three screenings. Plaque DNA was isolated and the cloned cDNA inserts liberated by Eco R1 digestion.

Three types of clones were identified by size: (i) 1.5kb eg. phage 12 (ii) 1.3 kb eg. phages 5 and 7 and (iii) 1 kb eg. phage 17. As seen in Fig 3 which is a Southern blot probed with the partial rat IGFBP-1 cDNA (1.1kb) all three species hybridized to this above probe.

Since the rat liver mRNA transcript was estimated to be about 1.6 kb by Northern blot analysis, the objective was to clone a full length cDNA (containing the total protein encoding complement); the 1.5 kb EcoR1 insert of the phage 12 was sub-cloned

Fig 3. Clones of Rat IGFBP-1 cDNA with Different Lengths.

Positive plaques (as identified on top of each lane) were digested with EcoR1 to liberate the cloned rat hepatic cDNA inserts and electrophoresed on a 1.2% agarose gel, Southern transferred and probed with 1.1kb partial cDNA for IGFBP-1. Lengths in kb are depicted on the left-side panel. Phage 12 had the biggest insert with 1.5kb followed by phage inserts of 7 and 5 (1.3kb) while 1kb insert of phage 17 was the shortest.


M
17
12
7
5
1.5
1.3
1.0

into pGEM 3Z. The subclone #15-CS15 contained the 1.5 kb insert and subsequent DNA sequence analysis showed it to be a full length clone (see Sec.1.2.2).

1.2.1. Verification of clones obtained

In order to make sure that the clones obtained were not cloning artifacts, Northern blots with 10μg of fasted liver mRNA were probed with CS15 (1.5 kb cDNA insert of IGFBP-1) and also with the two other 1.3- and 1-kb cloned IGFBP-1 cDNA inserts. Fasted rat liver was chosen because the mRNA level of IGFBP-1 was shown to be about 10-fold elevated compared to that of normal liver (Sec. 2.1). All the three types of cloned inserts (1.5- 1.3- and 1-kb) hybridized with rat liver mRNA as seen in Fig 4, proving that these were clones related to IGFBP (98).

The length of the transcript in the rat liver detected by CS-15 (1.5kb/cDNA) was about 1.6 kb (Fig 5). The transcript for the rat IGFBP-2 (rIGFBP-2) was reported to be about the same size; IGFBP-2 is the main IGFBP in the fetus and the neonate as well as in the BRL-3A rat liver cell line (166, 167). The cloned rat IGFBP-1 (rIGFBP-1) cDNA here detected a transcript of 1.6 kb in 50ug of neonatal rat liver total RNA whereas it did not do so with either maternal liver RNA (50 ug) or with BRL-3A total RNA (Fig 5). This further suggested that the cDNA clone corresponded to the rat homolog of IGFBP-1. The IGFBP-3 transcript was reported to be about 2kb (46).

Fig 4. Northern blot of fasted rat liver RNA (10ug/lane) probed with cloned rat IGFBP-1 cDNA.

Number on top of each lane refers to the phage from which the cDNA originated; length of the hepatic transcript detected is shown on the left margin. Poly(A)$^+$ RNA was prepared from liver of rats after a 24h fast, electrophoresed then Northern blotted and probed with nick-translated cloned cDNA probes as described in MATERIALS AND METHODS.

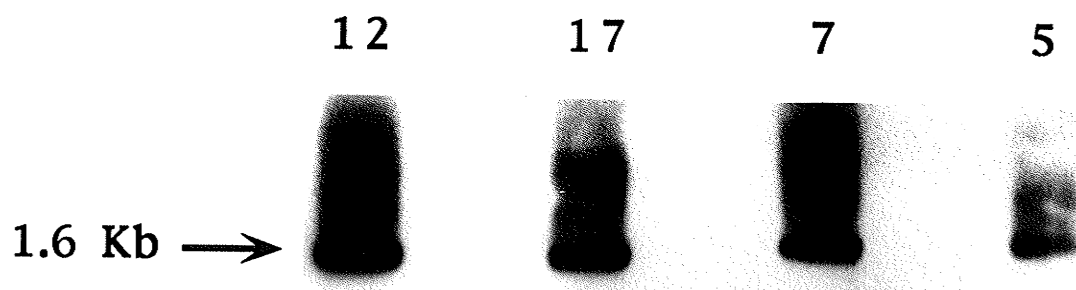
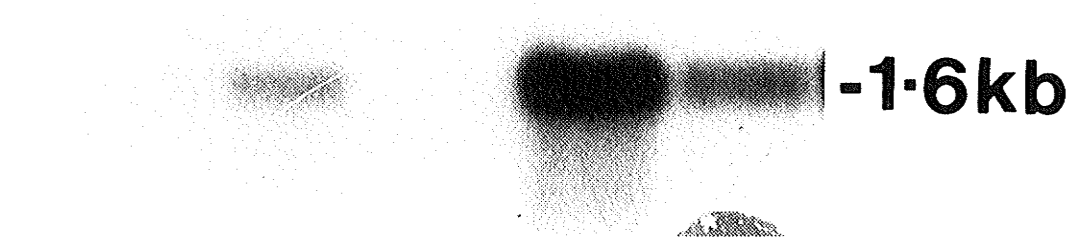

12
17
7
5
1.6 Kb

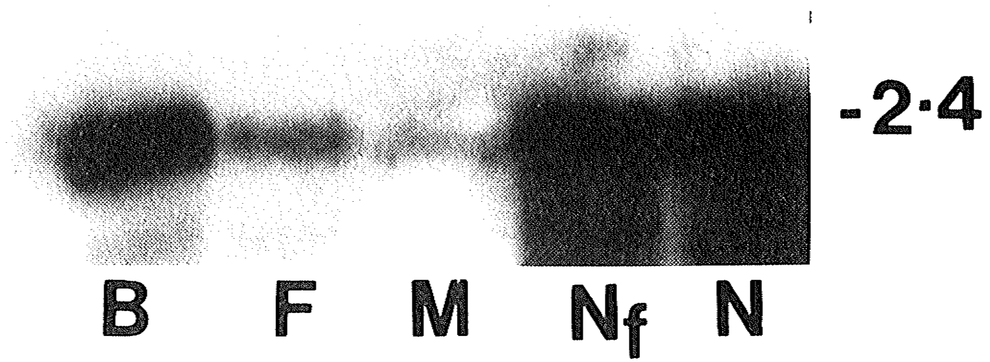
Fig 5. IGFBP-1 Expression in the Neonatal Rat Liver

A Northern blot with RNA from buffalo rat liver cells (B), neonatal rat liver (F), maternal rat liver (M), fasted adult rat liver (N_f), and hepatic RNA from nonfasted adult rat liver (N) are shown. Fifty micrograms of total RNA was loaded in lanes B,F,and M, whereas 10μg of poly(A)$^+$ RNA was loaded in lanes N_f and N. The upper panel shows the pattern of hybridization obtained with rIGFBP-1 cDNA, whereas in the lower panel the same filter has been hybridized with pNB29 to demonstrate that RNA has been loaded in each lane.

IGFBP-1



NB29



1.2.2. Sequence identification and characterization

The entire sequence information of clone CS15 is shown in Fig 6 (98) oved that it was indeed a full length clone because the entire coding complement for the rat IGFBP-1 was present. An open reading frame of 816 nucleotides was found with an in frame ATG site located 93 bp from the CS 15 start site. At the 3' end a poly $(A)^+$ tail of 56 nt, was found which was immediately preceded by the consensus polyadenylation signal-AATAAAA (98). The open reading frame for translation ended at one of the consensus stop codons- TGA -resulting in a 514 nt of 3'UT region.

The predicted amino acid (aa) sequence was 272 in length and M_r was 29.6 kilodalton (kD). The predicted peptide contained a 25-aa residue hydrophobic region at the amino terminal end. This region may represent the signal peptide characteristic of secretory proteins. Comparison with human IGFBP-1 revealed 66% aa homology (Fig 7) and conservative substitution enhanced it to 79% (98). The highest aa similarity with the human counterpart was found at NH2 and COOH terminal 1/3 of the mature protein while the presumptive signal peptide and middle 1/3 of the molecule differed the most (98). All the 18 cysteine residues present in the human IGFBP-1 were conserved in identical positions in the rat protein; this feature is analogous to hormone and growth factor receptors in which ligand binding domains frequently constitute Cys-rich regions, and may suggest an IGF binding domain in the case of IGFBPs (68).

Fig 6. Complete Nucleotide Sequence of the pCS15 Insert and the Predicted Amino acid Sequence of rIGFBP-1.

```
1     CCACTTCCGC TACTATCTAC CAGAAAGTCG TGACTACTGA GCCACTGCTG CCTGCCCAGA

61    TTCTCATCCA CCGCCTGCTG CGTCTGGTTG CG ATG CCG GAG TTC CTA ACT GTT GTT
                                          M   P   E   F   L   T   V   V

117   TCT TGG CCG TTC CTG ATC CTC CTG TCC TTC CAG GTT CGC GTA GTC GCT GGA
       S   W   P   F   L   I   L   L   S   F   Q   V   R   V   V   A   G

168   GCC CCC CAG CCA TGG CAC TGT GCT CCC TGC ACT GCT GAG AGG CTG GAG CTC
       A   P   Q   P   W   H   C   A   P   C   T   A   E   R   L   E   L

219   TGT CCA CCC GTG CCT GCT TCG TGC CCC GAG ATT TCT CGG CCT GCG GGC TGT
       C   P   P   V   P   A   S   C   P   E   I   S   R   P   A   G   C

270   GGC TGC TGC CCG ACA TGT GCC TTG CCA CTG GGT GCT GCC TGT GGT GTG GCC
       G   C   C   P   T   C   A   L   P   L   G   A   A   C   G   V   A

321   ACT GCG GCC TGC GCT CAG GGA CTC AGC TGC CGT GCG CTG CCA GGG GAG CCT
       T   A   A   C   A   Q   G   L   S   C   R   A   L   P   G   E   P

372   CGA CCT CTG CAT GCC CTC ACC CGT GGC CAG GGA GCC TGT GTA CTA GAA CCT
       R   P   L   H   A   L   T   R   G   Q   G   A   C   V   L   E   P

423   CCG CCA CCC GCC ACG AGC AGC TTG TCC GGT TCT CAG CAT GAA GAG GCA AAG
       P   P   P   A   T   S   S   L   S   G   S   Q   H   E   E   A   K

474   GCT GCT GTG GCC TCT GAG GAT GAG CTT GCC GAG AGC CCA GAG ATG ACA GAG
       A   A   V   A   S   E   D   E   L   A   E   S   P   E   M   T   E

525   GAA CAG CTG CTG GAT AGC TTC CAC CTC ATG GCC CCA TCC CGT GAG GAC CAG
       E   Q   L   L   D   S   F   H   L   M   A   P   S   R   E   D   Q

576   CCC ATC CTG TGG AAT GCC ATT AGC ACC TAC AGT AGC ATG CGG GCC CGG GAG
       P   I   L   W   N   A   I   S   T   Y   S   S   M   R   A   R   E

627   ATC ACT GAC CTC AAG AAA TGG AAG GAG CCC TGC CAA CGG GAA CTC TAT AAA
       I   T   D   L   K   K   W   K   E   P   C   Q   R   E   L   Y   K

678   GTG TTA GAG AGA TTA GCT GCC GCT CAA CAG AAA GCA GGA GAT GAG ATC TAC
       V   L   E   R   L   A   A   A   Q   Q   K   A   G   D   E   I   Y

729   AAA TTT TAT CTG CCA AAC TGC AAC AAG AAT GGA TTT TAT CAC AGC AAA CAG
       K   F   Y   L   P   N   C   N   K   N   G   F   Y   H   S   K   Q

780   TGC GAG ACA TCT CTG GAT GGA GAA GCT GGG CTC TGC TGG TGT GTC TAC CCA
       C   E   T   S   L   D   G   E   A   G   L   C   W   C   V   Y   P

831   TGG AGT GGG AAG AAG ATC CCT GGA TCT CTG GAG ACC AGA GGG GAC CCC AAC
       W   S   G   K   K   I   P   G   S   L   E   T   R   G   D   P   N

882   TGC CAC CAG TAT TTT AAT GTG CAA AAC TGA A AGTTGTTT CCTCCCTCCT
       C   N   Q   Y   F   N   V   Q   N   *

931   TCTTCACACA AAATATTTAA GTATATGTGT ATTTATACTC CGGAGCACAC CATTTTATAT

991   ATGTGTATAT GTATATATCC AGGAACTAGT TTTTATACTC CACATGCTGC TTGATGTACA

1050  AGTGGGTTGT ATTTATTCAC TCTAAGTTTA TTTTGTTCTA CCCTGTCCTT GTGCTGTATT

1110  AATTTATATA ACTGAAGCTT TTCTCATCTC CATACATGTA AATACTACCA TCTCAGCTCT

1170  CCAGAGTTCT GCTTTGAAAG GGCAGCGCGG TAGCTGCCTA GAACGAGCAC AAGTCAGTCT

1230  GAGGTAGGGG CCTTTCAGTG GGTTCAGGGA GGAAGTTAGC CCTGGCTCGG GGAGACTTCC

1290  TCATCGAATC CCACAGGTCT GTGTCTGATG CCTATTGGCT GGGAAGGTTC CGATGTTGGT

1350  TGTGTAATCA AAGCTAAACG TGGAAAGCTG CGTCCCATGC ACTGTTAAAC ACACGTCTGG

1410  AATAAAACAT TCTACCTGGA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA

1470  AAAAAAAAAA AAAAAA
```

Fig 7. Comparison of the predicted Amino Acid Sequence of rIGFBP-1 with Other Members of the IGFBP Gene Family

Regions of amino acid identity are boxed. The predicted amino acid sequences of hIGFBP-1, hIGFBP-3 and hIGFBP-2, are from refs. 89, 46, 166, respectively.

```
rIGFBP-1   M P E F L T V V S W     P F L I L L S F Q V R     V V A G
hIGFBP-1   M S E V P V A R V W     L V L L L L T V Q V G     V T A G
hIGFBP-3   M Q R A R P T L W A     A A L T L L V L L R G     P P V A
rIGFBP-2   M L P R L G G P A L P L L L P S L L L L L L G A G G C G P G V R A E

rIGFBP-1   A P Q P W H C A P C T A E R L E L C P P V P
hIGFBP-1   A P W Q C A P C S A E K L A L C P P V S
hIGFBP-3   R A G A S S G G L G P V V R C E P C D A R A L A Q C A P P P
rIGFBP-2   V L F R C P P C T P E R L A A C G P P P D

rIGFBP-1   A S C P E I S R P A G C G C C P T C A L P L G A A C G V A T A A C A Q
hIGFBP-1   A S C S E V T R S A G C G C C P M C A L P L G A A C G V A T A R C A R
hIGFBP-3   A V C A E L V R E P G C G C C L T C A L S E G Q P C G I Y T E R C G S
rIGFBP-2   A P C A E L V R E P G C G C C S V C A R Q E G E A C G V Y I P R C A Q

rIGFBP-1   G L S C R A L P G E P R P L H A L T R G Q G A C V L E P P P P A T S S L S
hIGFBP-1   G L S C R A L P G E Q Q P L H A L T R G Q G A C V Q
hIGFBP-3   G L R C Q P S P D E A R P L Q A L L D G R L C V V N
rIGFBP-2   T L R C Y P N P G S E L P L K A L V T G A G T C

rIGFBP-1   G S Q     H E E A K A A V A S E D E L A E S P
hIGFBP-1   E S D     A S A P H A A E A G S P E S P E S T
hIGFBP-3   A S A V S R L R A Y L L P A P P A P G N A S E S E E D R S A G S V E S P
rIGFBP-2   E K R R V     G A T P Q Q V A D

rIGFBP-1   E M T E E Q L L D S     F H L M A P S     R E D Q P I L W N A
hIGFBP-1   E M I E E E L L D N     F H L M A P S     E E D H S I L W D A
hIGFBP-3   S V S S T H R V S D P K F H P L H S K I I I I K K G H A K D S Q R
rIGFBP-2   S E D D H S E G G L V E     N H V D G T M     N M L G G S S A G R

rIGFBP-1   I S T Y S S M R A R E I
hIGFBP-1   I S T Y D G S K R L H V
hIGFBP-3   Y K V D Y E S Q S T D T Q                                            N
rIGFBP-2   K P P K S G M K E L A V F R E K V N E Q H R Q M G K G A K H L S L E

rIGFBP-1   T D L K K W K E P C Q R E L Y K V L E R L A A A Q K A C D E I
hIGFBP-1   T N I K K W K E P C R I E L Y R V L E S L A K A Q E T S G E I
hIGFBP-3   F S S E S K R E T E Y G P C R R E M E D T L N H L K F L N V L S P R G V
rIGFBP-2   E P K K L R P P P A P T P C Q Q E L D Q V L E R I S T M R L P D D R G P

rIGFBP-1   P N C N K N G F Y H S K Q C E T S L D G E A G L C W C
hIGFBP-1   P N C N K N G F Y H S R Q C E T S M D G E A G L C W C
hIGFBP-3   P N C D K K G F Y K K K Q C R P S K G R K R G F C W C
rIGFBP-2   L E H L Y S L H I P N C D K H G L Y N L K Q C K M S L N G Q R G E C W C

rIGFBP-1   V Y P W S G K K I P G S L E T R G D P N C H Q Y F N V Q N
hIGFBP-1   V Y P W N G K R I P G S P E I R G D P N C Q I Y F N V Q N
hIGFBP-3   V D K Y G Q P L P G Y T T K G K E D V H C Y S H Q S K
rIGFBP-2   V N P N T G K P I Q G A P T I R G D P E C H L F Y N E Q Q E N D G V H A

rIGFBP-2   Q R V Q
```

1.2.3. Transient transfections

Further proof that CS15 clone encoded the rIGFBP-1 was provided by transient transfection studies. The construct which was made by Dr. LJ Murphy contained the CS15 inserted in an expression vector pSVLJ in both forward and reverse orientations, and driven by an SV40 late promoter. COS-1 cells were transiently transfected for 24h and serum free media was conditioned for another 48h period, and analysed by ligand blotting. The tracer used was [^{125}I] IGF-I . The mock transfected cells secreted three proteins with apparent molecular masses of 47-, 45- and 40-kD, presumably endogenous binding proteins of the cells. A unique band of about 29 kD was apparent in conditioned media of cells transfected with pSV-CS15 (F) construct which contains CS15 in the forward orientation. This band was absent in cells transfected either with pSVLJ (vector only) or with pSV-CS15 (R) which is the plasmid containing CS15 in the reverse orientation as seen in Fig 8 (98).

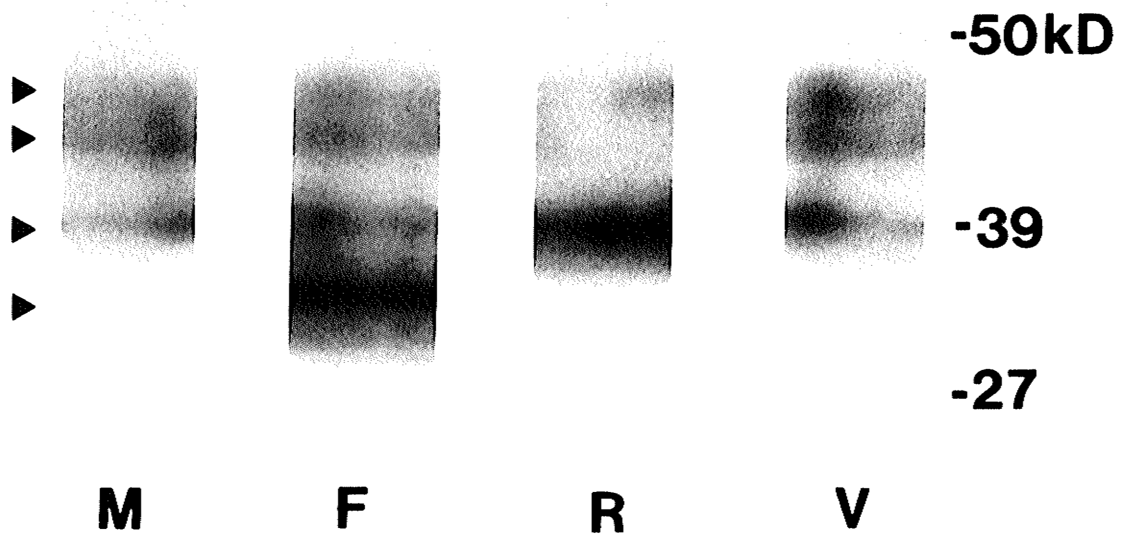
2. In vivo Regulation of rIGFBP-1 Expression

2.1. Fasting and Refeeding experiments

Under growth retarding conditions such as fasting, IGF levels decrease in vivo (71 and references therein). How the IGFBPs respond to such circumstances may provide clues to their regulation.

Fig 8. Transient Expression of rIGFBP-1 in COS-1 Cells

Conditioned media from mock-transfected cells (M), cells transfected with a plasmid containing the IGFBP-1 cDNA in the forward orientation (F), cells transfected with a plasmid containing the cDNA in the reverse orientation (R), and cells transfected with the vector only (V) was analyzed by polyacrylamide gel electrophoresis and ligand blotting with (^{125}I)IGF-1. The positions of the protein mol wt markers are shown. Arrow heads on the left margin represent bands corresponding to endogenous IGFBPs secreted by the COS-1 cells.


-50kD
-39
-27
M
F
R
V

To this end, groups of 4-5 rats were fasted for 24- and 48-h periods. Another two sets of rats were given free access to food for a 24- and 48-h periods respectively, after a 24h fast. Northern analysis of hepatic mRNA from these groups indicated that fasting resulted in a 10-fold increase in the steady state levels of IGFBP-1 mRNA compared to non-fasted controls. This marked elevation of IGFBP-1 occurred within 24h and remained elevated at 48h of fasting. A 24h fast followed by 24h of refeeding resulted in a dramatic reduction in the IGFBP-1 mRNA levels below that of controls. This reduced steady state levels of IGFBP-1 was maintained up to 48h after refeeding as seen in Fig 9 (98). To determine the time course of the effect of refeeding on IGFBP-1 mRNA levels, fasted rats were refed and sacrificed at various time points as seen in Fig 10. In this experiment, refeeding reduced the level of IGFBP-1 mRNA below the levels of detection by Northern blot within 1h. The expression of IGFBP-I was suppressed for up to 24h as shown in Fig 10 (100).

Progression of the fasting/refeeding time course experiment was monitored by the measurement of the serum glucose levels as shown in Table 1B. After a 24h fast, the serum glucose levels depressed significantly compared to the non-fasted controls but recovered to normal levels within 1h of refeeding and remained at or above control levels during rest of the refeeding time points.

2.2. Growth Hormone Regulation of rIGFBP-1 Expression

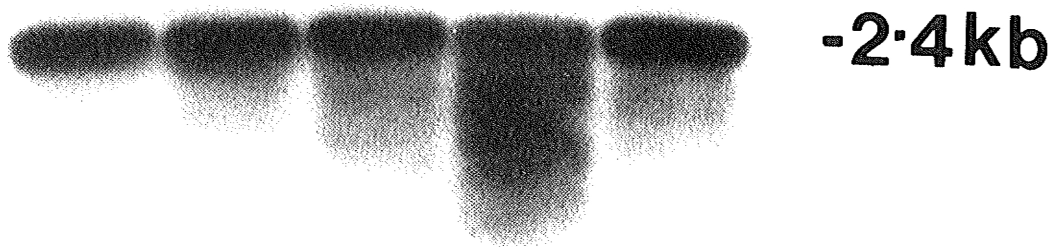
To assess the potential role of pituitary hormone, especially growth hormone,

Fig 9. The Effect of Food Deprivation on Hepatic IGFBP-1 Expression

A Northern blot of hepatic poly(A)$^+$ RNA from food-deprived and refed rats is shown. The upper panel shows the pattern of hybridization obtained with the rIGFBP-1 cDNA, whereas in the lower panel the same filter has been hybridized with pNB29 as a loading control. C, control rat; lanes that correspond to 24h, 48h fasted and 24h, 48h refed rats are represented. On the right panel, RNA size markers are shown.

IGFBP-1

NB29



C 24 48 24 48h

fasted refed

Fig 10. The effect of fasting and refeeding on hepatic IGFBP-1 expression.

Rats were fasted and sacrificed at various times after access to chow, as indicated. The abundance of IGFBP-1 mRNA was determined by densitometry of autoradiograms and has been expressed relative to the hybridization signal obtained in nonfasted control rats (C). The data represent the mean±SEM for four or five rats per time point. The insert shows the representative Northern blot of hepatic RNA from nonfasted (C), fasted (time zero), and refed rats. In the upper panel the pattern of hybridization obtained when probed with the rat IGFBP-1 cDNA, while in the lower panel, the same blot probed with pNB29 as a loading control where it can be seen that approximately equal amounts of RNA were loaded per lane. The size of the transcript was determined by comparison with the position of 18S and 28S ribosomal RNA.

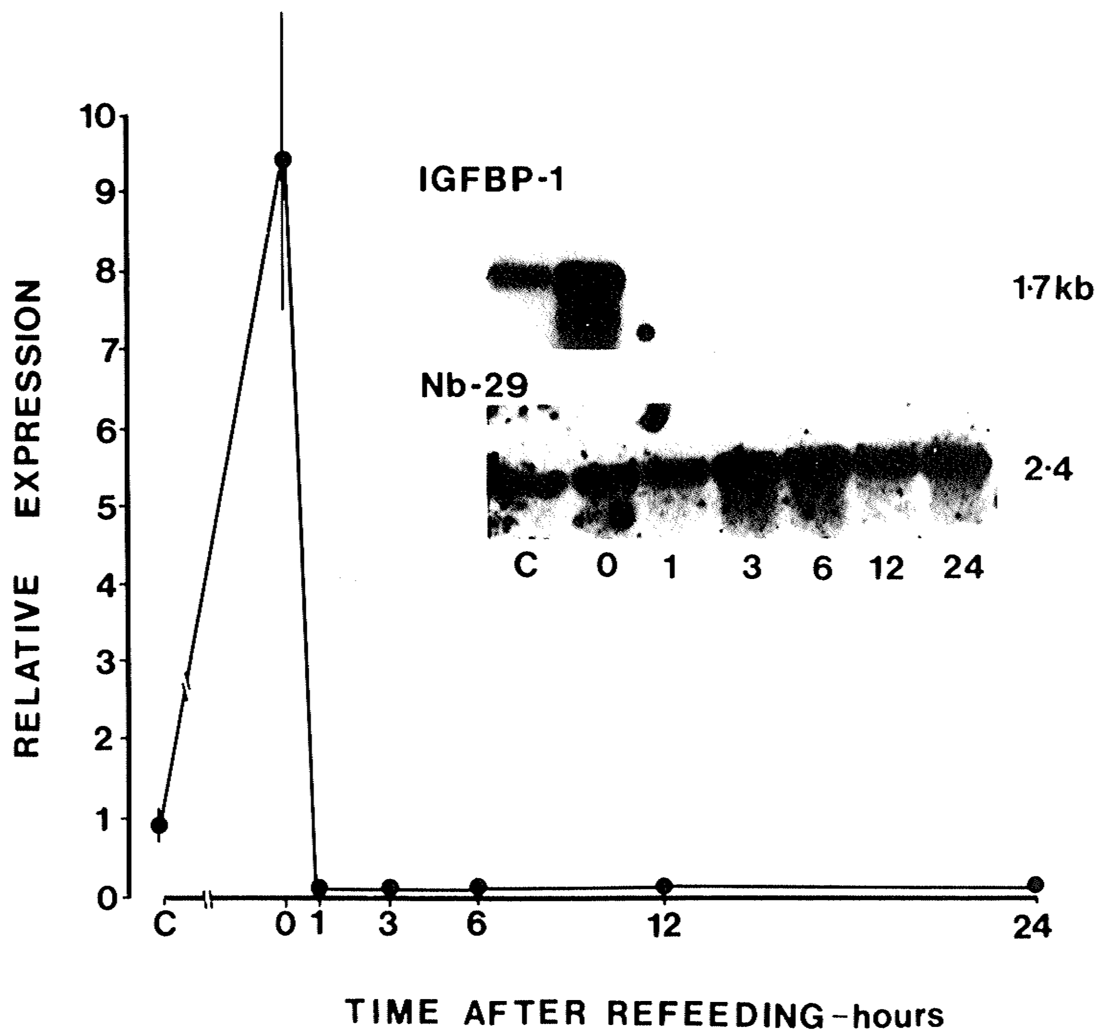

IGFBP-1
1·7kb
Nb-29
2·4
C 0 1 3 6 12 24
RELATIVE EXPRESSION
10
9
8
7
6
5
4
3
2
1
0
C 0 1 3 6 12 24
TIME AFTER REFEEDING-hours

Table 1B. The effect of refeeding on glucose concentration

GROUP	BASAL	Time after refeeding (h)				
		1	3	6	12	24
Glucose(mg/dL)						
Control rats (n=12)	125±5					
Fasted rats (n=12)	75±4[a]					
Refed rats (n=6)		140±9[b]	150±6[a,b]	145±6[b]	150±5[a,b]	118±4[b]

Data represent the mean ± SEM for the number of animals indicated,

[a] P<0.01 for the significant difference between control group and other treatment groups, as determined by analysis of variance and Dunnett's t test.

[b]P<0.01 for the difference between the fasted rat group and other treatment groups.

in the regulation of IGFBP-1 expression, experiments were performed in hypox rats. The hepatic IGFBP-1 transcript abundance was elevated significantly in hypox rats compared to sham operated controls as seen in Fig 11 (6.5 ± 1.7-fold; n=9 vs. sham-operated controls, $p<.05$). An age dependent depletion of IGFBP-1 levels has been reported (275). But in older rats, hypophysectomized at nine weeks/200-250gm, a significant increase in mRNA levels was also observed as seen in Fig 11 (17.9 ± 3.7; n=4 vs. sham-operated controls, $p<0.05$), suggesting the age independence of this of this phenomenon.

Chronic GH treatment [100ug per 100gm of body weight (BW)] for 8 days resulted in a significant drop in hepatic IGFBP-1 mRNA levels, both in young and old rats (Fig 12); GH-treated rats gained significantly more weight compared to untreated hypox controls (12.1 ± 2.4 vs. 2.93gm 0.05; $p<0.05$ and 4.7 ± 2.5 vs. 1.8 ± 1.3gm; $p<0.05$ for young and old rats respectively) as reported before (99).

2.3. Insulin regulation of rIGFBP-1

Insulin has been shown to regulate rIGFBP-1 mRNA levels both in vitro and in vivo as reviewed in the introduction. After a overnight fast, plasma insulin levels decrease by 50% in man and 30-60 min. after a meal it goes up 3-10 fold (118). The question that arose was whether the regulation of IGFBP-1 expression by nutritional status is mediated by insulin. In order to answer this question, 24h fasted rats were given 4 IU of insulin at various time points as shown in Fig 13 and hepatic mRNA levels measured by northern blotting. Insulin did not have an effect on IGFBP-1 message up to 3h but had a marked

Fig 11. The Effect of Hypophysectomy on Hepatic IGFBP-1 mRNA abundance.

A northern blot of poly(A^+) RNA (10μg/lane) from hypox rats (H) or rats subjected to sham operation (S) is shown. The upper panel shows the pattern of hybridization obtained with the IGFBP-1 cDNA. The filters were subsequently probed with NB29 as a control for gel loading.

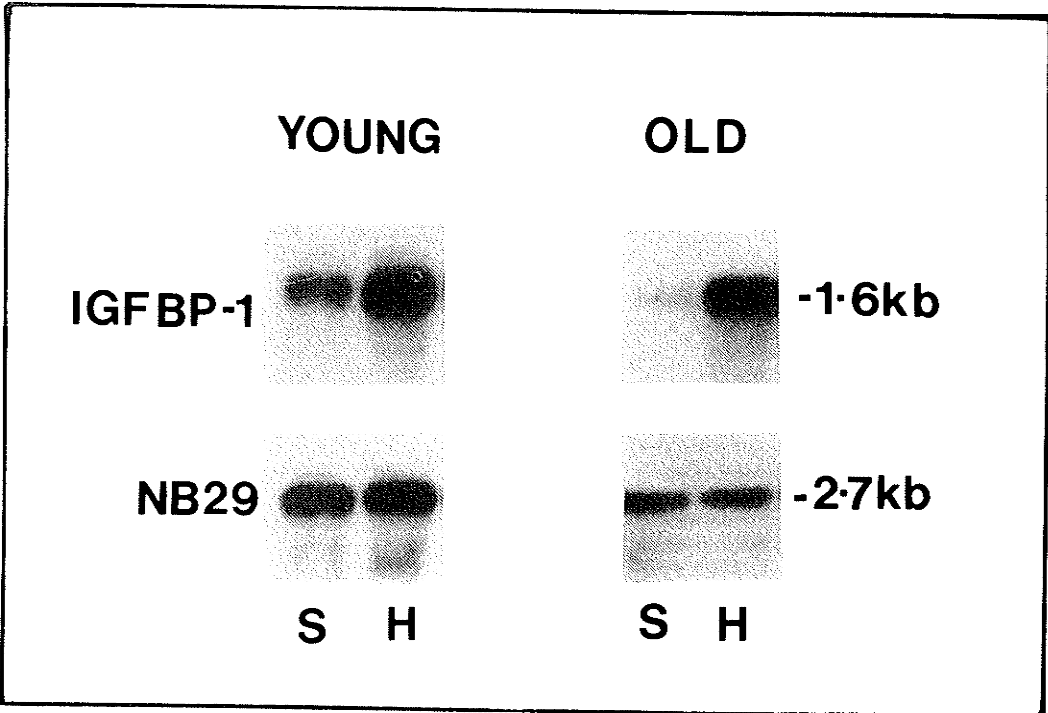
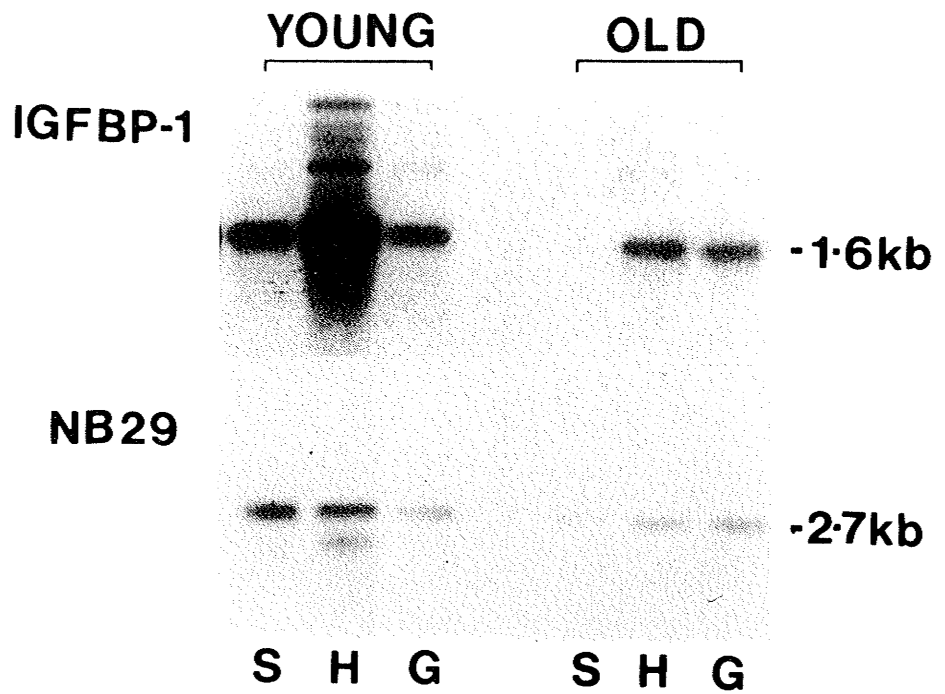
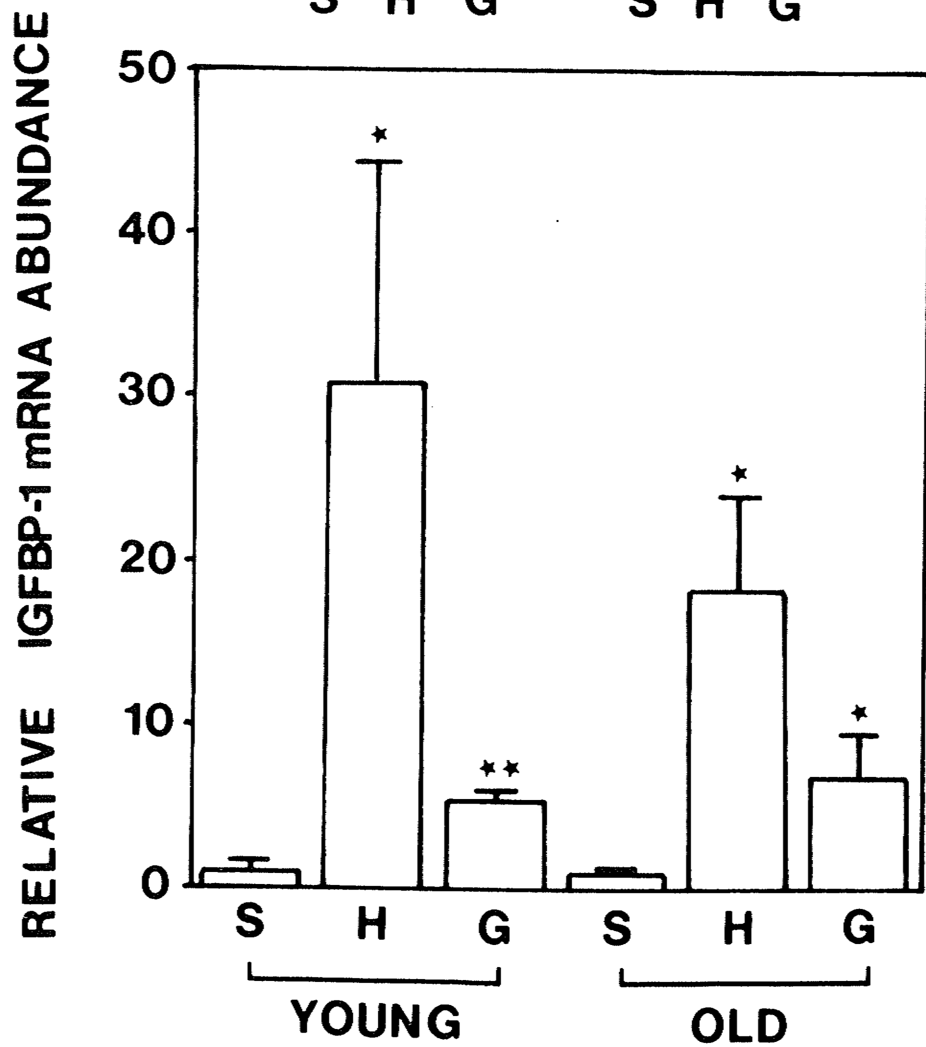

YOUNG
OLD
IGFBP-1
-1·6kb
NB29
-2·7kb
S
H
S
H

Fig 12. The Effect of Chronic GH Administration on Hepatic IGFBP-1 mRNA Abundance in Hypox Rats

The upper panel depicts a representative experiment while the data plotted below are the results from four individual rats per treatment group. S, sham operated; H, hypox; G, GH treated hypox rats. Data have been normalized with the loading control and expressed relatively to the sham-operated control in each group. *, $p<0.05$; **, $p\leq0.005$ (for the difference between GH-treated and untreated hypox rats, respectively).


YOUNG
OLD
IGFBP-1
NB29
-1·6kb
-2·7kb
S H G
S H G


RELATIVE IGFBP-1 mRNA ABUNDANCE
50
40
30
20
10
0
S H G
S H G
YOUNG
OLD

hypoglycemic effect as shown in Table 2 (100).

3. Cloning of the rat IGFBP-1 gene

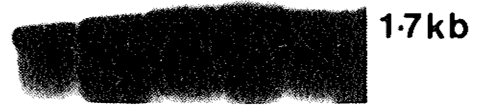
3.1. Coding sequences

In order to examine the molecular regulation of the IGFBP-1 gene, cloning of the genomic DNA, including the 5'FL region was undertaken. Mammalian genomes are about $3x10^9$bp in size and assuming the rat IGFBP-1 gene is similar in size to the human, which is 5,000bp (170), and is one copy per haploid genome, the probability of encountering a positive clone in a genomic library is 1 in 10^6. Initially three such libraries were screened; $2x10^6$ recombinants per library in duplicate were probed with the full length cDNA probe-CS15, and two positive clones were identified; the first one was found in the Kasper's library (Fig 14) while the second was from the HaeIII library. Further work with this library (Hae III) was discontinued since it contained only a smaller genomic insert compared to that of the Kasper's library.

The recombinant clone isolated from the Kasper's library, K-1 was subjected to restriction enzyme mapping including Southern blot analysis. This library was constructed by inserting rat liver genomic DNA partially digested by Sau3A into the BamH1 site of the charon 35 vector. This vector has a very useful polylinker with many unique sites for six base pair cutters (Fig 15). An oligonucleotide that contained the first 21 nt of CS15 was used as a probe in K-1 genomic Southern blots in order to identify the

Fig 13. The Effect of Insulin Administration on Hepatic IGFBP-1 mRNA Abundance in Fasted Rats.

The relative expression of IGFBP-1 mRNA was determined as described in Fig 12. The data represents the mean±SEM for five rats per time point. A representative Northern blot of RNA from control rats (C), rats fasted for 24h (time zero), and fasted rats injected with 4U of insulin and sacrificed at various times after insulin injection is shown. The upper panel shows the pattern obtained when the nitrocellulose filter was hybridized with a rat IGFBP-1 cDNA, whereas the pattern obtained with the NB-29 cDNA is shown in the lower panel.


IGFBP-1
1·7kb

Nb-29
2·4
C 0 0·25 0·5 1 3

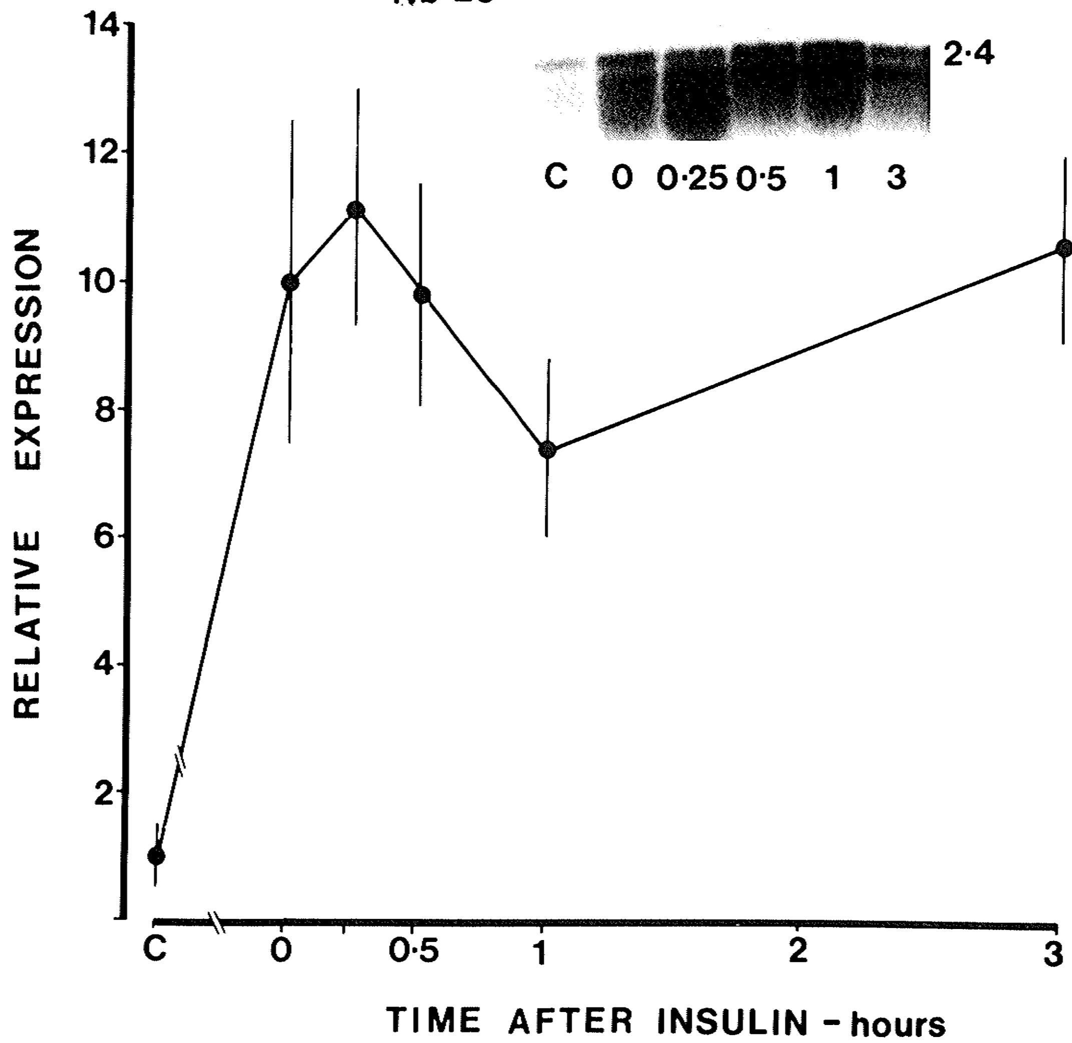

RELATIVE EXPRESSION
14
12
10
8
6
4
2
C
0
0·5
1
2
3
TIME AFTER INSULIN - hours

Table 2. The effect of insulin administration on serum glucose concentration

Treatment group	Time after insulin (min)					
	0	15	30	60	180	
Glucose(mg/dL)						
Control rats (n=12)	125±5					
Fasted rats (n=12)	75±4[a]					
Fasted rats+insulin		14±2[a,b]	25±6[a,b]	25±3[a,b]	5±1[a,b]	(n=6)

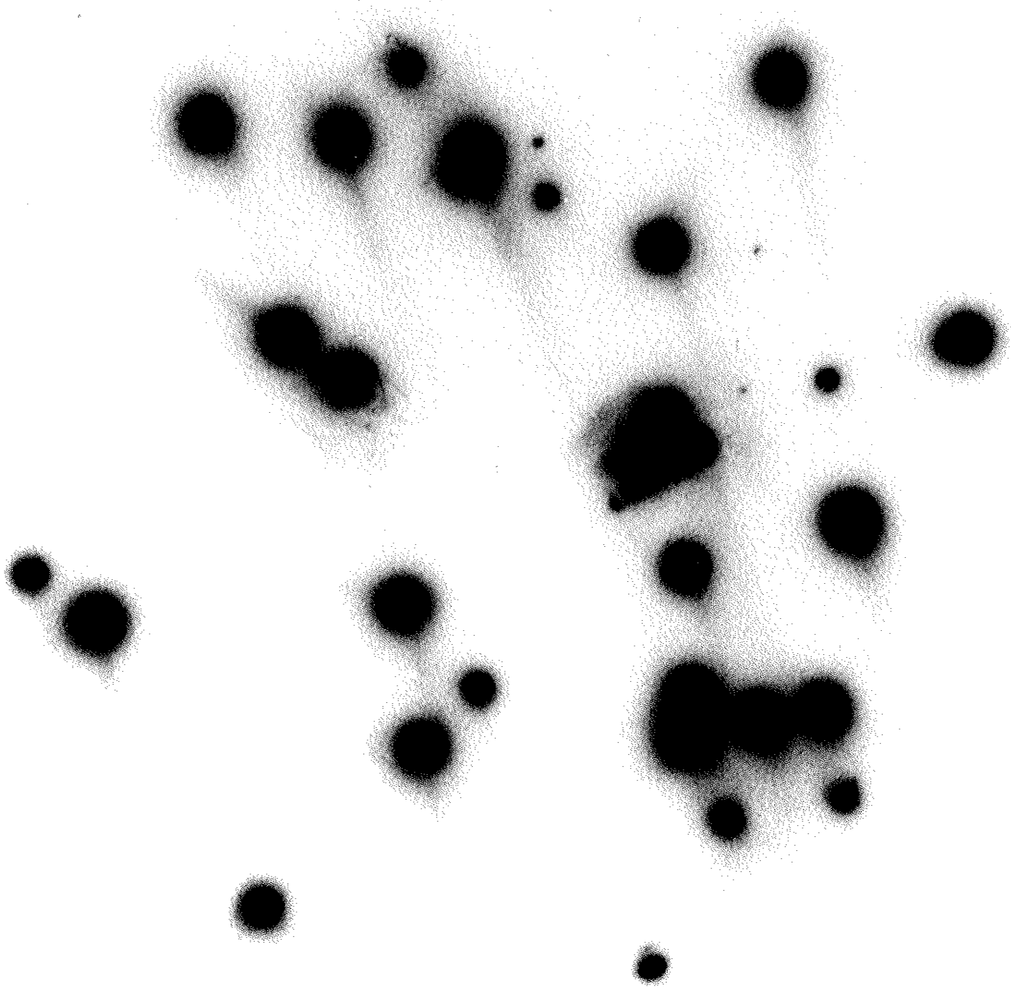
Data represent the mean ± SEM for the number of animals indicated,

[a] $P<0.01$ for the significant difference between control group and other treatment groups, as determined by analysis of variance and Dunnett's t test.

[b] $P<0.01$ for the difference between the fasted rat group and other treatment groups.

Fig 14. Isolation of Recombinant Genomic Phage K-1

Kasper's genomic phage library was plated and recombinant phage-containing plaques were identified by filter hybridization using [^{32}P]-labeled 5' genomic fragment-PS400 as a probe; results of such a hybridization where filter with plaques containing recombinant genomic phage K-1, after three such screenings, are shown.



5'end. A 1.3 kb Pst1 fragment (P1.3) that hybridized to this 21-mer oligonucleotide was subcloned into pGEM3Z as described in MATERIALS AND METHODS. Further subcloning of P1.3, was carried-out into Pst1/Sac1-400, and Sac1/Pst1-900bp fragments. The vector used was bluescript SKII (BSSK II).

The dideoxy chain-termination method of Sanger (241) was employed to sequence these clones. The DNA sequence of the P1.3 was obtained from clones PS-400 and SP-900. The sequencing strategy is shown in Fig 16. At the 5'-end of the clone, from Pst1 to BamH1 sites, charon 35 vector-arm sequence was present. The recreation of a BamH1 site is only possible if such a site (BamH1) is present in the genomic insert of the phage recombinant, i.e. in the rIGFBP-1 gene. As mentioned before (MATERIALS AND METHODS), genomic DNA was digested with Sau3A and ligated to the BamH1 site of the vector which will only re-create a BamH1 site if the genomic end is BamH1 and not merely a Sau3A site.

In order to elucidate the mRNA cap site, primer extension analysis (Fig 17) was performed using the 21-mer CS15-oligonucleotide as the primer and 50μg of rat liver total RNA as the template (for details see MATERIALS AND METHODS). The results suggested the presence of a the mRNA cap site to be at an Adenosine (A) site, 81 nt 5' to CS15 start site (Fig 16). In the cDNA (CS15) no Pst1 site was found. Thus the 3' Pst1 site of P1.3 or that of SP900, is likely located in the 1st intron. This suggests the presence of the 3'-boundary of the 1st exon and intron-1 information in SP900. Comparing the sequence of CS15 (Fig 6) with that of P1.3, the 3'-boundary of exon-1 was located to 545 bp down-stream from the mRNA cap site (Fig 16).

Fig 15. Structure of the Charon 35 PhageVector

In the Kasper's library, the genomic DNA fragments were cloned into the BamH1 site of the polylinker where they replace the E. coli stuffer fragment (stuffer); the position of the stuffer is depicted. Polylinkers/Multiple cloning sites (MCS), located on either end of the stuffer fragment, are represented; all but Pst1 and Sma1 restriction sites in the polylinker are unique.

Reproduced from Sambrook et al (249).

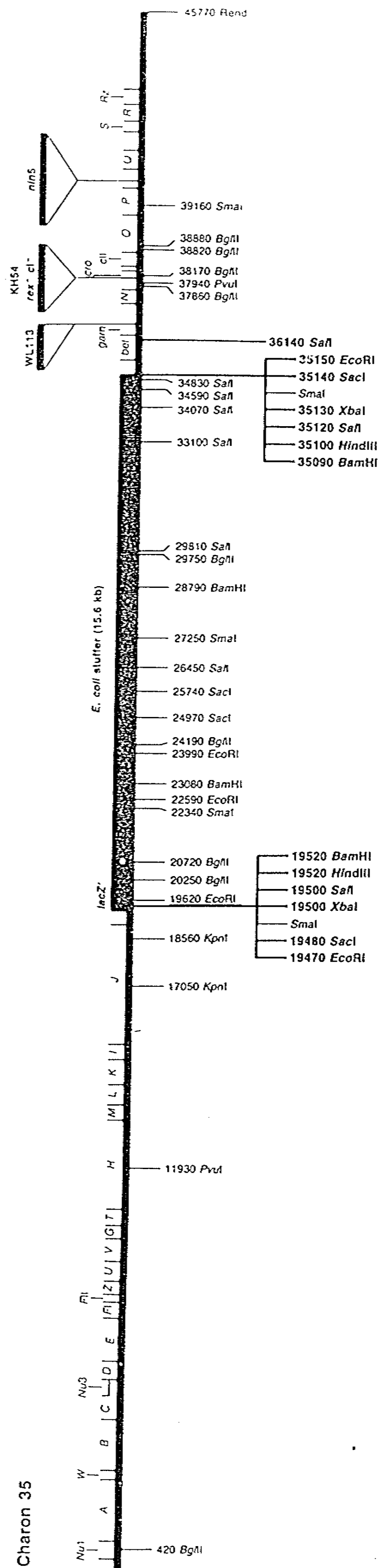

45770 Rend
39160 SmaI
38880 BglII
38820 BglII
38170 BglII
37940 PvuI
37860 BglII
36140 SalI
35150 EcoRI
35140 SacI
SmaI
35130 XbaI
35120 SalI
35100 HindIII
35090 BamHI
34830 SalI
34590 SalI
34070 SalI
33100 SalI
29810 SalI
29750 BglII
28790 BamHI
27250 SmaI
26450 SalI
25740 SacI
24970 SacI
24190 BglII
23990 EcoRI
23080 BamHI
22590 EcoRI
22340 SmaI
20720 BglII
20250 BglII
19620 EcoRI
19520 BamHI
19520 HindIII
19500 SalI
19500 XbaI
SmaI
19480 SacI
19470 EcoRI
18560 KpnI
17050 KpnI
11930 PvuI
420 BglII
nin5
KH54
rex⁻ cI⁻
WL113
E. coli stuffer (15.6 kb)
lacZ'
Charon 35

Fig 16. Structure and Sequencing Strategy of Exon 1

Transcription start site is assigned the +1 position and 5' to the +1 site are negative numbers while those 3' are positive numbers according to convention. The BamH1 site at -83, 5'- and 3'-boundaries of the exon at +1 and +545 respectively are depicted. CS15 cDNA clone start-site, translation start site (ATG) and Hind III and Pst1 sites of intron 1 are shown. Sequencing strategy including the subclones, direction of sequencing and the primers used are represented. Ava1(Av); BamH1(B); Sac1(S); Hind111(H); Pst1(P). Universal reverse primer (RP), T3, T7, SP6, and 15-mer oligonucleotide of the first 15nt of CS15 (CS15P) were used to prime appropriate sequencing reactions.

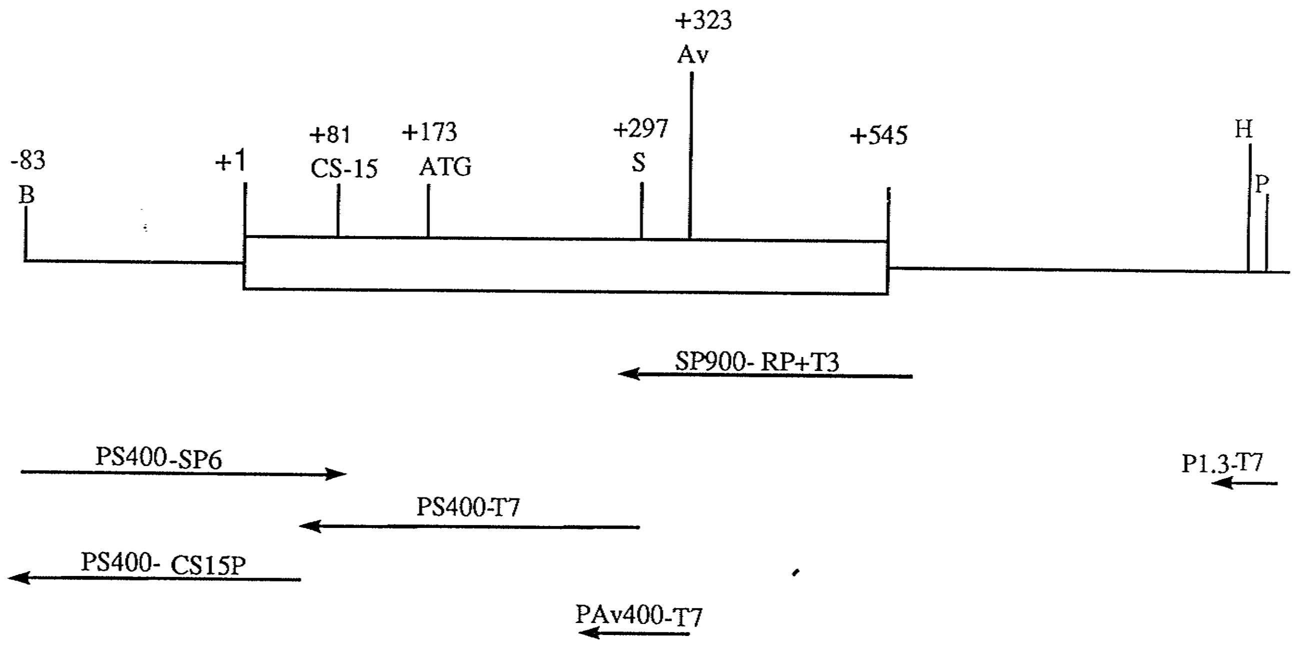
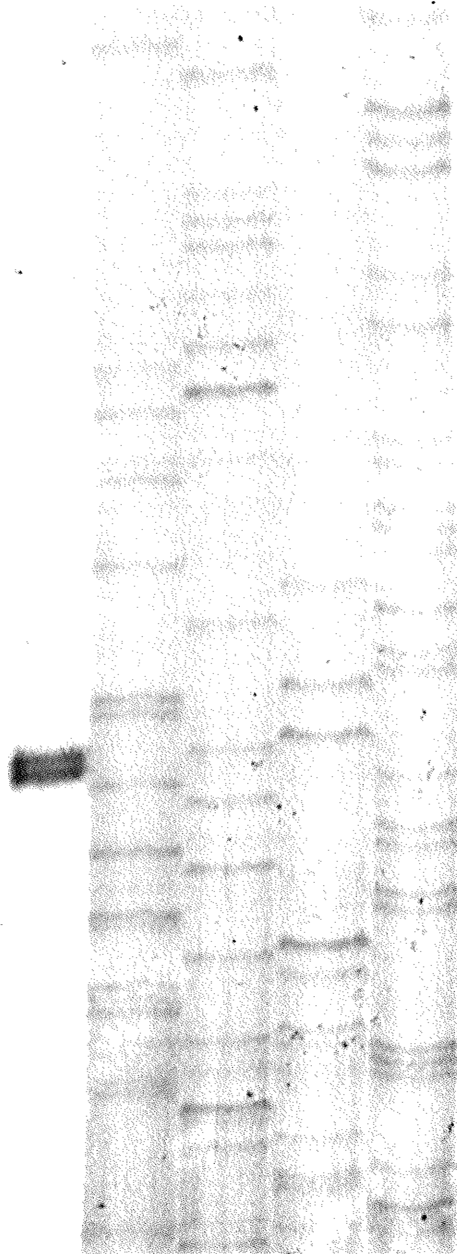

+323
Av
+81
CS-15
+173
ATG
+297
S
+545
H
P
-83
B
+1
SP900- RP+T3
PS400-SP6
P1.3-T7
PS400-T7
PS400- CS15P
PAv400-T7

Fig 17. Primer Extension Analysis of pPS400 to Locate mRNA cap site.

Lane depicted as Prim Extd Pdt is the primer extended product obtained after extension of a ^{32}P-labeled 21-mer oligonucleotide which contains the first 21 nt of the CS15 (cDNA) sense-strand. This was used as the template to anneal 50μg of rat liver total RNA and extended via reverse transcription reaction to the mRNA cap site (transcription start) of the gene as described in MATERIALS AND METHODS. A DNA sequencing reaction was performed on the corresponding genomic plasmid (pPS400) with the same primer (21-mer CS15) and all products were analyzed in parallel on a 6% polyacrylamide sequencing gel. The sequencing reactions containing 2',3'-dideoxy nucleosides of guanosine (G), adenylate (A), thymidine (T), and cytidine (C) are represented. Primer extended product appers as a doublet. The slower migrating band corresponds to the transcription start site Adenosine containg nucleotide while the faster migrating band is unknown.

Prim Extd Pdt G A T C



Since the P1.3 was sequenced from subclones PS-400 and SP-900, it was necessary to prove the contiguity across the +297 Sac1 site. For this purpose the fragment spanning from the Pst1 (vector arm) to the +323/Ava1 site was subcloned in vector-pGEM 3Z. Sequencing of this clone-PAv400- revealed the contiguity of the P1.3 subclones- PS400 and SP900 (Fig 16).

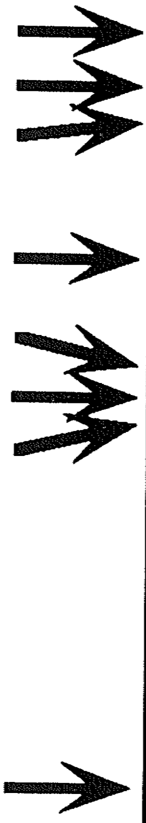
In order to elucidate the structure of the gene in terms of exon/intron boundaries, a restriction enzyme map of genomic phage DNA is imperative.

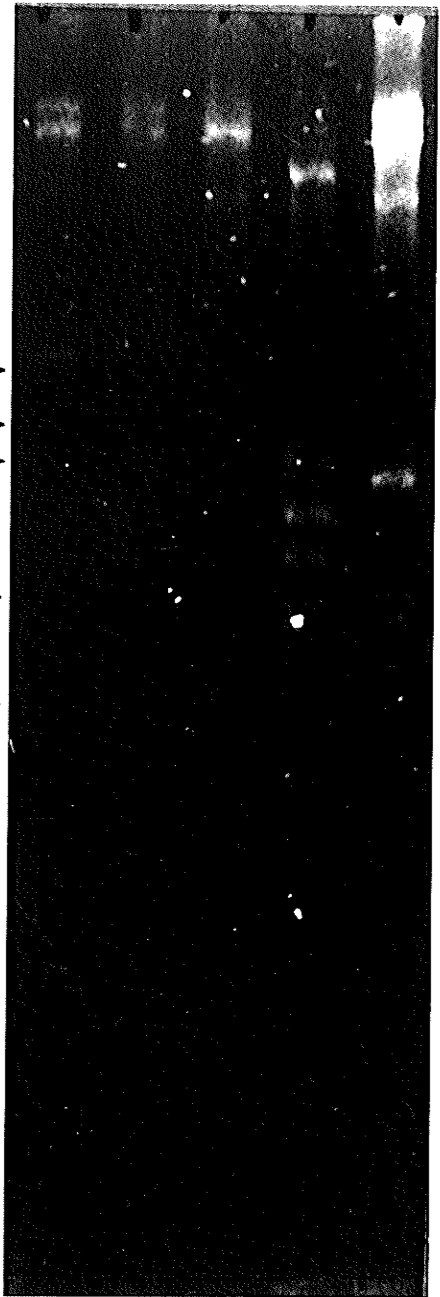
The K-1 phage DNA was first subjected to single enzyme digestions followed by Southern blot analyses. Digestion with Hind III resulted in 5.8-, 3.5-, 1.4-, and 1.2-kb fragments and, except for the 5.8kb, the fragments hybridized with CS15-full length cDNA probe (Fig 20). Only the 1.2kb fragment hybridized with the 5' probe-PS400 (Fig 21-II). The genomic insert of the K-1 phage is 11.9kb and the most 5' Hind III fragment is the 1.2kb one. There is a Hind III site in intron-1 which is 115bp upstream from the 3'-Pst 1 site of the P1.3 fragment (Fig 16). Therefore the Hind III-1.2kb fragment (H1.2) must span from polylinker Hind III site to this Hind III site -1.2 downstream (intron 1 Hind III) (Fig 19). The 5.8-,3.5-, and 1.4-kb fragments must be 3' to the 1.2kb fragment. Digestion with Sac 1 resulted in fragments of 3.8-, 3.4-, 2.5-, 1.8-, and 0.4-kb in lengths (Fig 21-I). The 0.4kb fragment hybridized both with PS400 (Fig 21-II) and CS15 (Fig 18-II) while the 3.8- and 3.4-kb fragments (Figs 18-II and 21-II) only did so with CS15. The 2.5- and 1.8-kb fragments did not hybridize with either PS400 or CS15 probes (Figs 18 and 21). The 0.4kb must extend from the Sac1 polylinker site of K-1 to the +297/Sac 1 site in the exon 1 (Fig 16).

Figs 18-I and -II. Restriction Endonuclease Analysis of Recombinant Genomic Phage (K-1) DNA.

The K-1 recombinant phage DNA was subjected to restriction single enzyme digestion with EcoR1 (E), HindIII (H), Sac1 (Sc), Pst1(P), or Xba1(X); resulting DNA fragments were analyzed on 0.6% agarose gels then blotted onto nitrocellulose filters and probed with ^{32}P-labeled CS15 as detailed in MATERIALS AND METHODS. The ethidium bromide (EtBr) stain (I) and autoradiogram of the Southern blot (II) is shown; on the left margin the mol wt markers in kb are depicted while on top of each lane, the restriction enzyme used is represented.

I





II

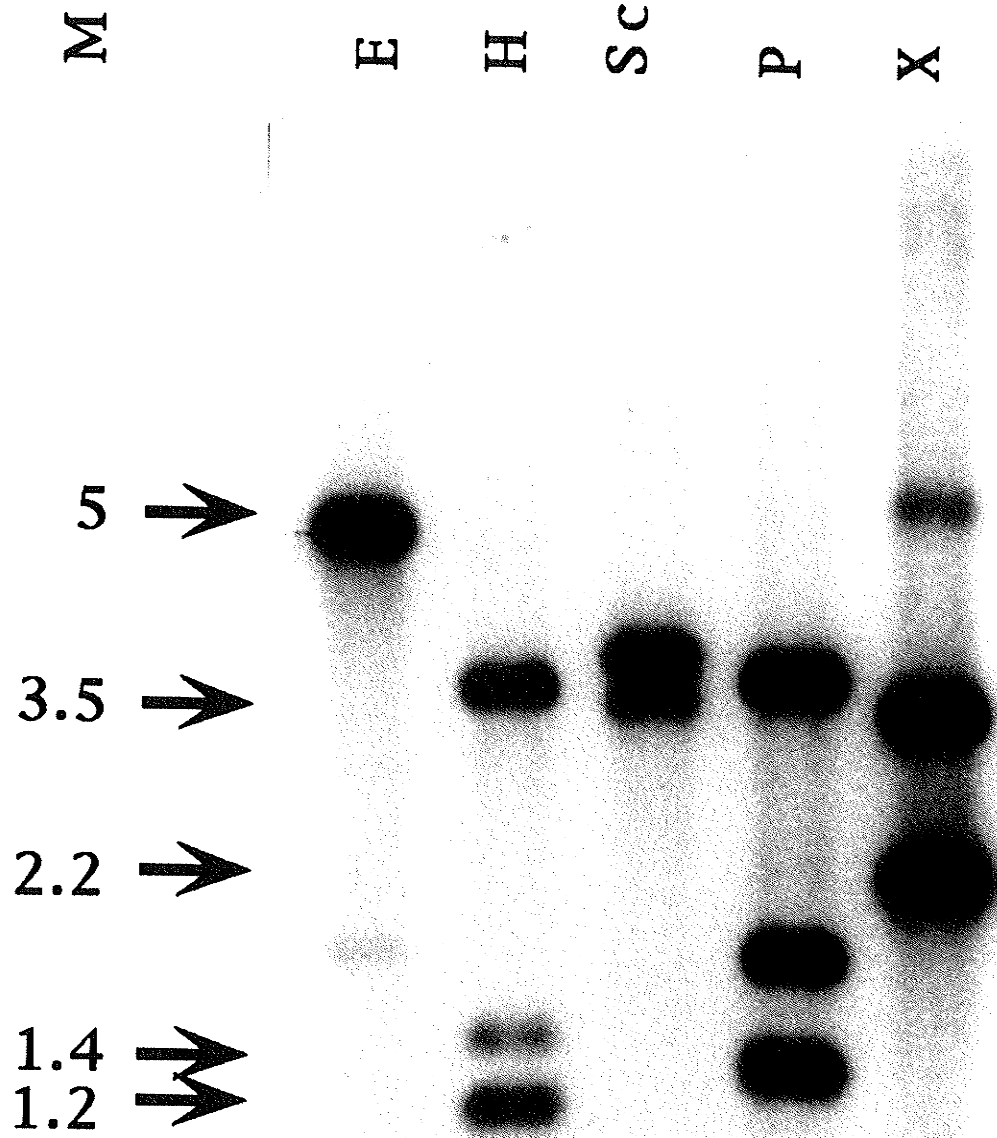


Fig 19. Partial Restriction Endonuclease Map (RE) of Recombinant K-1 Genomic Phage

The multiple cloning site (MCS) and restriction enzyme sites EcoR1(E), Sac1(Sc), Xba1(X), Sal1(Sl), Pst1(P), BamH1(B), HindIII(H), and distances in kb are shown.

H
Sl
Sc
E X P
B
MCS

P
.1

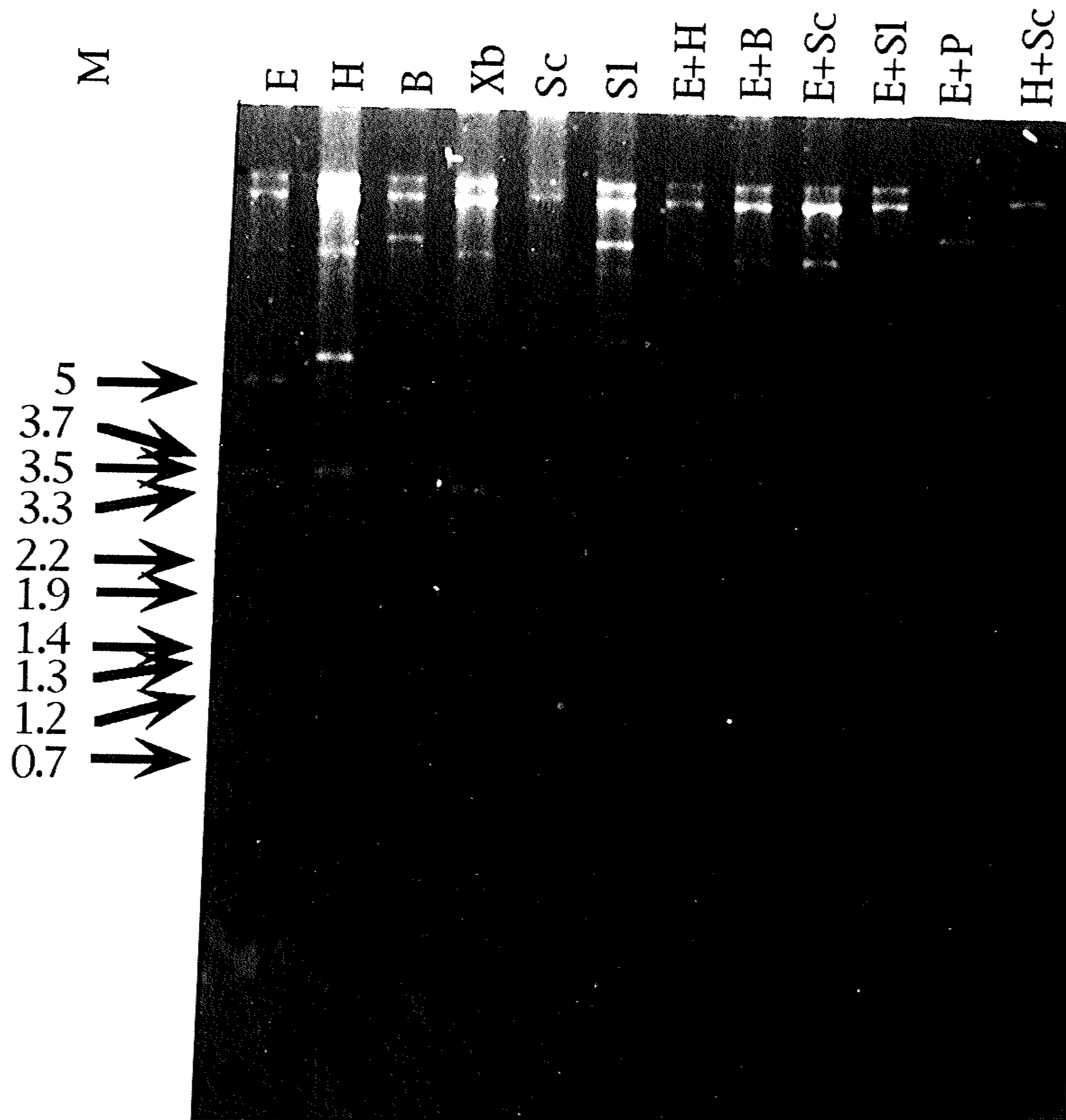
Sc H X Sc H E X E Sc

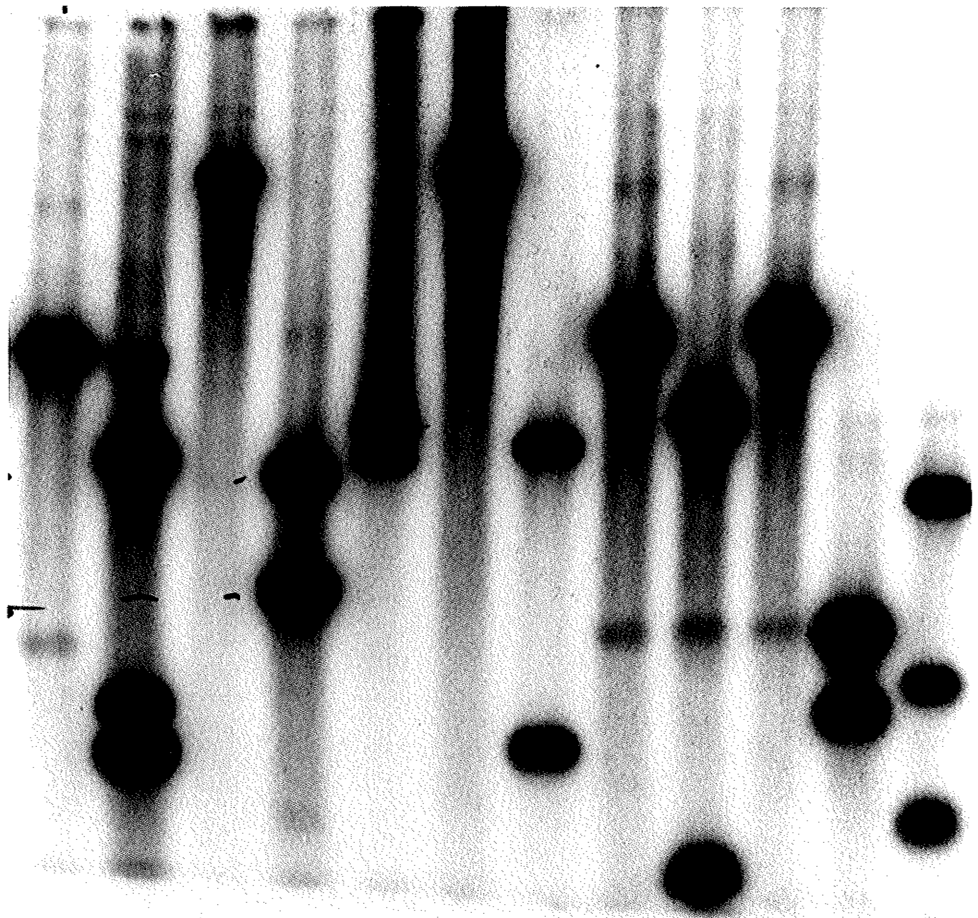
.4 .8 .9 2.0 .5 .3 .5

Figs 20-I, -II, 21-I, -II and -III. Restriction Enzyme Analysis of K-1 Genomic Recombinant

The restriction enzyme(s) used is shown on top of each lane and the mol wt markers in the left margin; abbreviations and other details as before (Figs 18 and 19). The gel (20-I) was tranfered to nitrocellulose and the corresponding Southern blot probed with CS15 is shown (Fig 20-II). The gel shown in Fig 21-I, was probed first with PS400 (Fig 21-II) followed by EH300 (Fig 21-III). All probes were ^{32}P labelled as described in MATERIALS AND METHODS.

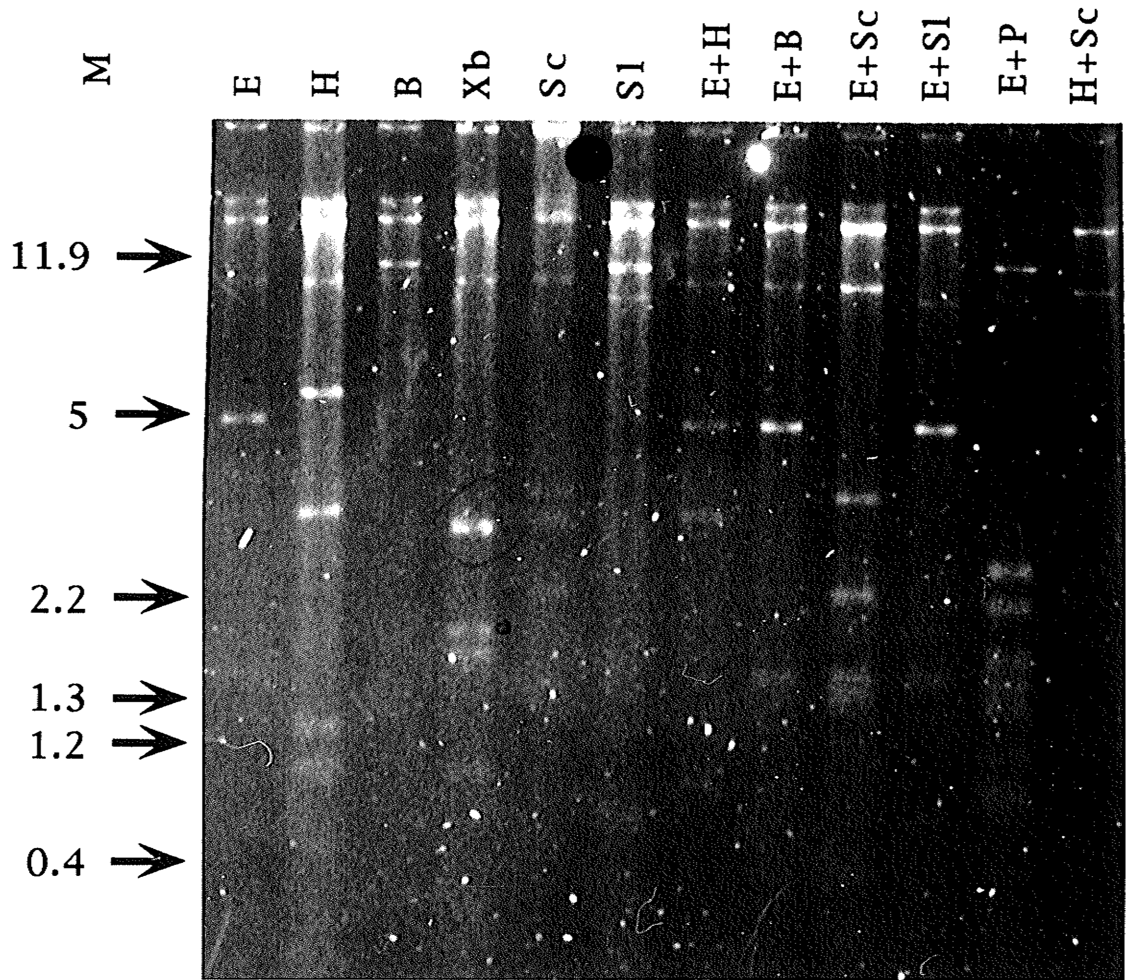
I

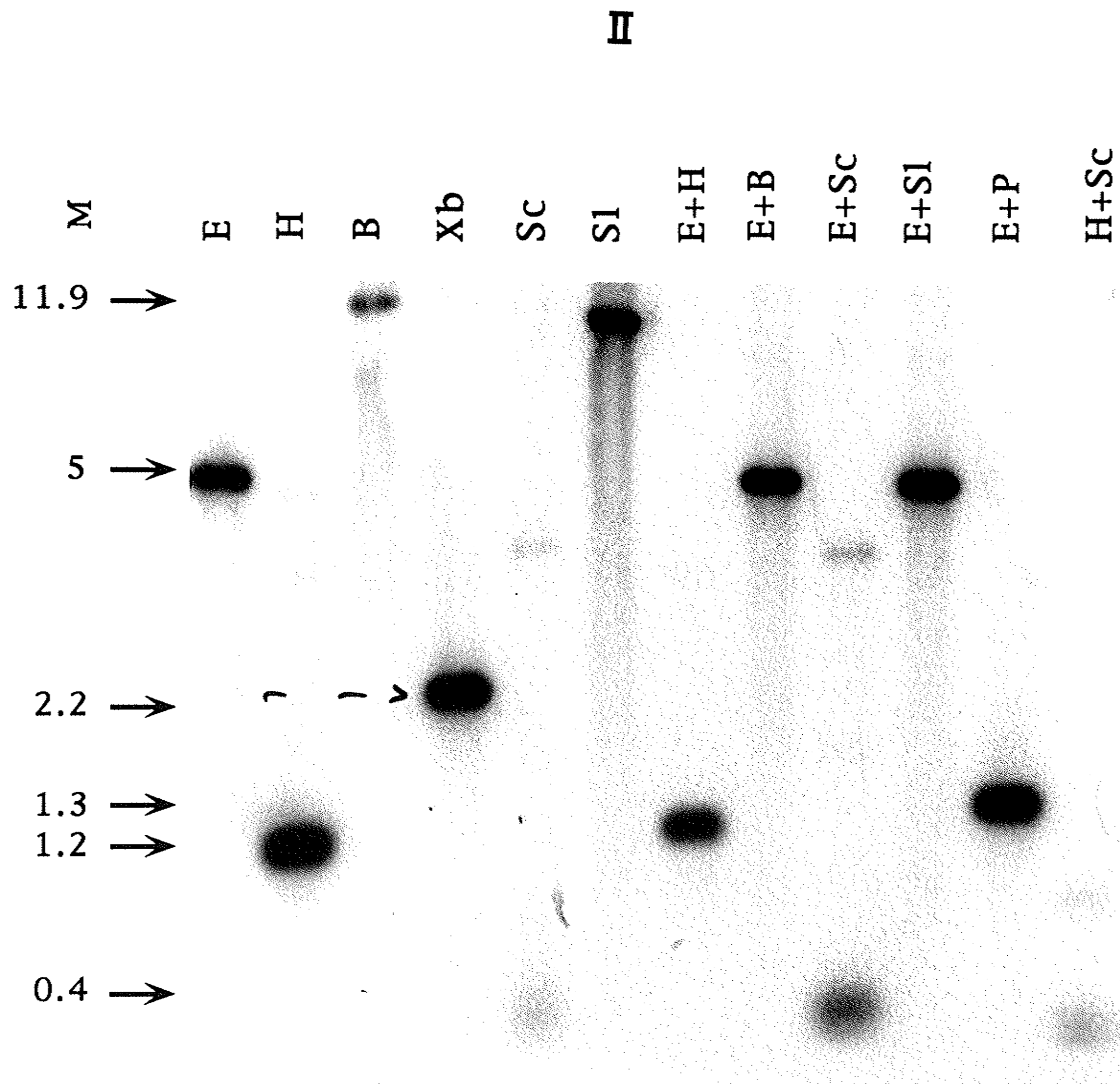




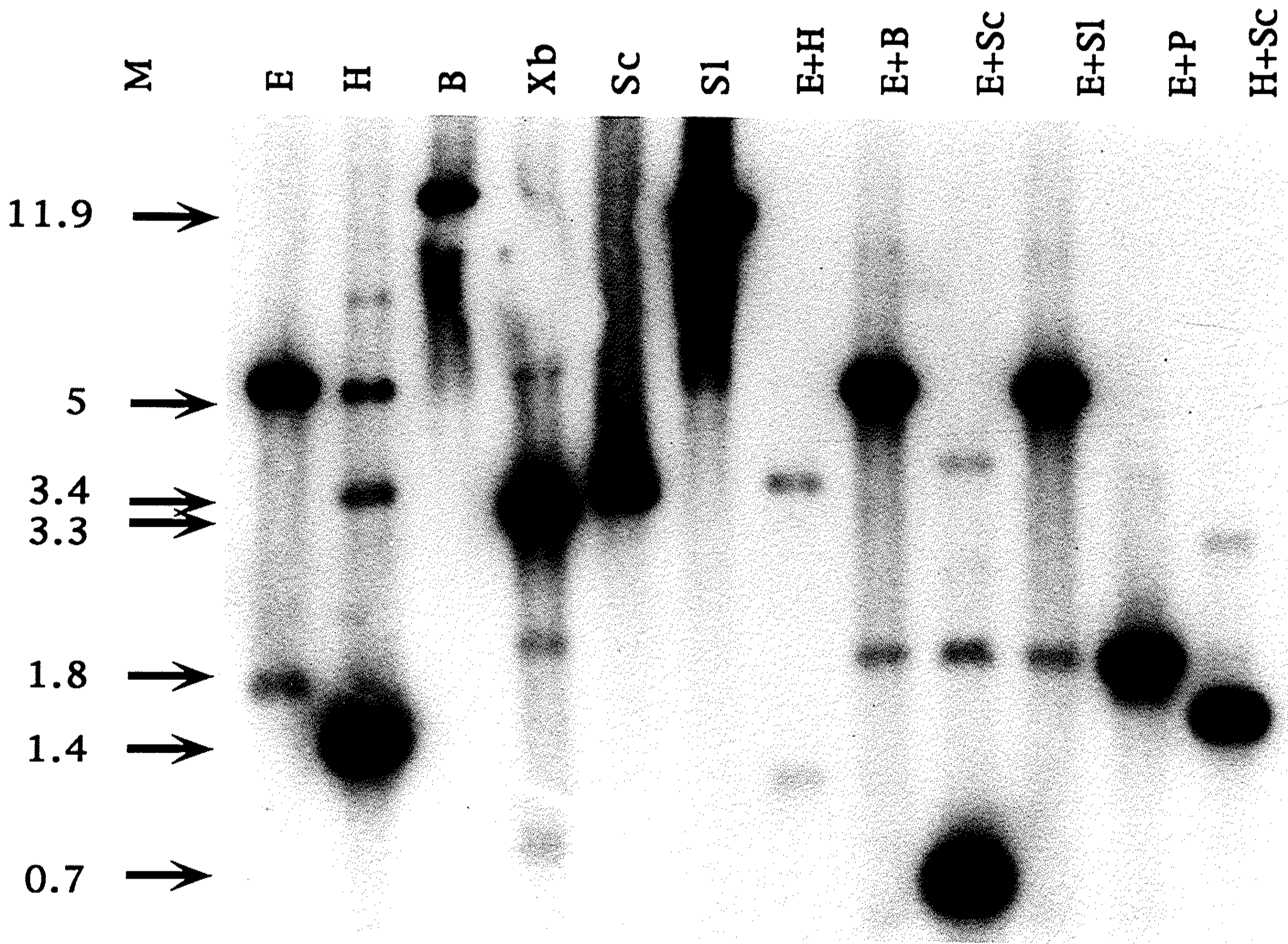
II
M
E
H
B
Xb
Sc
Sl
E+H
E+B
E+Sc
E+Sl
E+P
H+Sc
11.9
5.0
3.7
3.5
3.3
2.2
1.9
1.4
1.3
1.2
0.7

I





II
M
E
H
B
Xb
Sc
Sl
E+H
E+B
E+Sc
E+Sl
E+P
H+Sc
11.9
5
2.2
1.3
1.2
0.4


III
M
E
H
B
Xb
Sc
Sl
E+H
E+B
E+Sc
E+Sl
E+P
H+Sc
11.9
5
3.4
3.3
1.8
1.4
0.7

The two 3.8- and 3.4-kb fragments must be 3' to the 0.4-kb fragment. The 2.5- and the 1.8-kb fragments must be further 3' to the above two (3.8-, 3.4-kb) fragments, outside the transcribed sequences.

The Xba 1 digestion gave rise to 2.2- and 3.3- kb fragments which hybridized to CS15 (Fig 20). Only the 2.2 kb fragment hybridized to PS400 (Fig 21-II), thus making it the more 5' fragment, spanning from the polylinker (phage arm) Xba 1 site to a downstream Xba 1 site (Fig 19).

Restriction enzyme Pst 1, digestion resulted in many fragments because of the 25 Pst1 sites in the vector-charon 35 (242). But only 3 fragments of the lengths 3.5- 1.9- and 1.3- kb hybridized to CS15 (Fig 18). The exon 1-containing Pst 1.3 kb (P1.3) fragment which was subcloned and sequenced before, is the most 5' of the three. As mentioned before, P1.3 spans from the multiple cloning site (MCS) Pst 1 to the Pst 1 site in intron 1.

Digestion with BamH1 or Sal1, both resulted in 11.9 kb fragments which hybridized with both the PS400 and CS15 probes (Figs 20 and 21). The polylinker of the vector contains both BamH1 and Sal1 sites (Fig 15) and the genomic insert in K-1 phage is 11.9 kb. Therefore the absence of either BamH1 or Sal1 sites in the in K-1 genomic insert is confirmed.

The tentative RE map deduced from single restriction enzyme digests is shown in Fig 19.

Further refinement of the RE map was achieved by double digests with restriction enzymes. Double digests with EcoR1 and Hind III resulted in 5-, 3.5-, 1.2-, 1.1-, 0.8- and

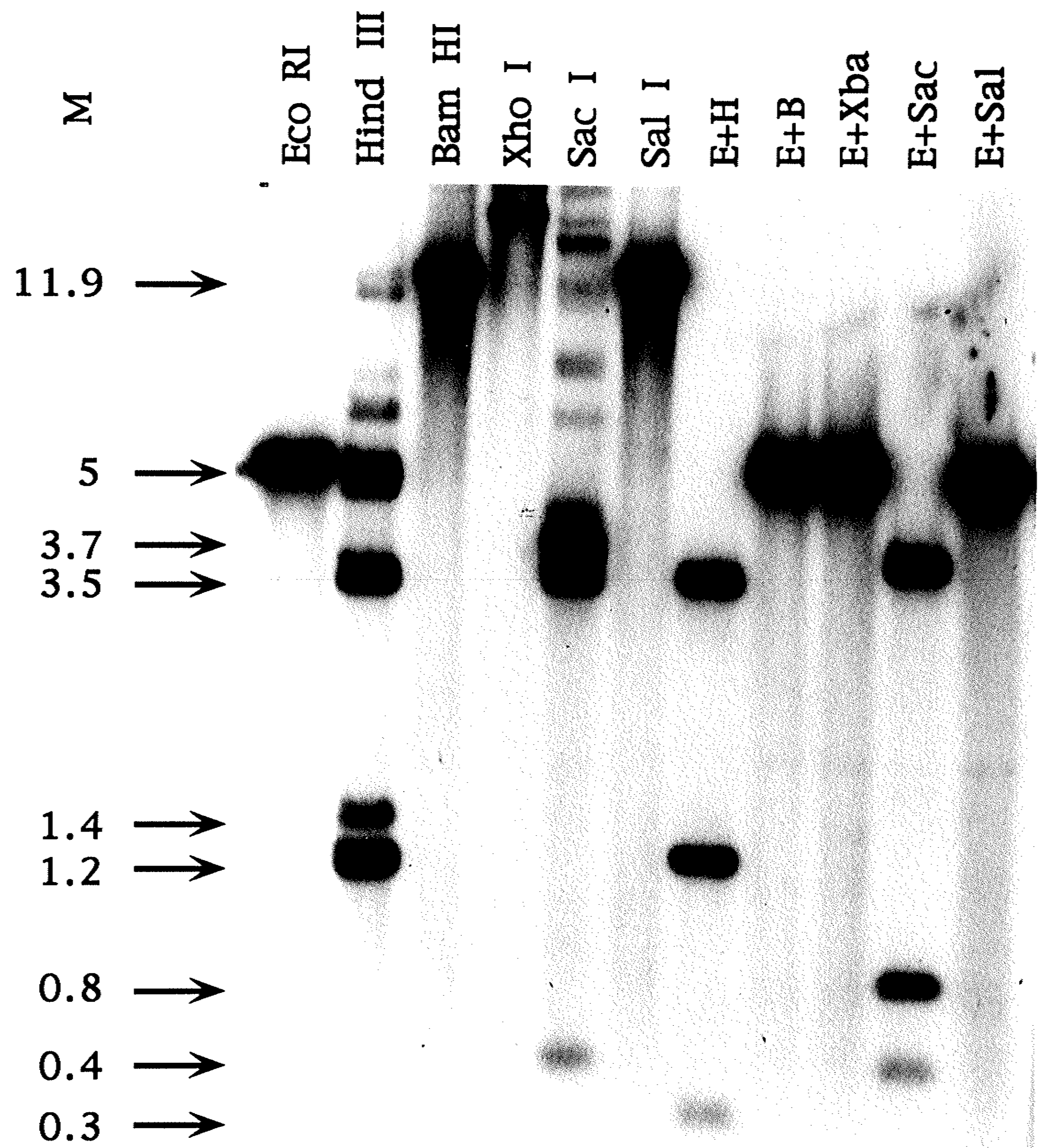
0.3-kb fragments (Fig 21-I), of which only the 3.5-, 1.2- and 0.3- kb fragments hybridized with CS15 (Fig 22), and in addition, the 1.2kb fragment also hybridized with PS 400 (Fig 21-II); the 0.3 kb fragment was only seen in the Southern blot and not in ethidium bromide (EtBr) stained gel (Fig 22). Combining these results with that of single digests, suggests the positions of Eco R1 and Hind III sites in K-1 genomic insert as seen in Fig 23.

The smallest Eco R1/Hind 111-0.3 kb (EH 0.3) fragment which hybridized to CS 15, was sub-cloned in both pGEM-3Z and BSSK vectors. The DNA sequence revealed the presence of 25 bp 3' flanking (3'FL) information as well as 306bp of last exonic information (Fig 25).

The EH 0.3kb fragment was used as a probe in Southern blots and out of the two Sac 1 fragments that hybridized with CS15: 3.4- (S3.4), 3.8-kb (S3.8), only the 3.4 kb fragment hybridized with the EH 0.3 (Fig 21-III). These results order the three Sac 1 fragments from the most 5', S0.4 followed by the S3.8 fragment and to most 3' S3.4 (Fig 23). Restriction enzyme EcoR1 gave rise to a 5 kb CS15 hybridizing fragment and a non-hybridizing 1.9- kb fragment (Fig 18). The 5 kb EcoR1 fragment (Eco-5), also hybridized with PS 400 whereas the 1.9 kb did not (Fig 21-II). These results are compatible with Eco-5 spanning from the polylinker EcoR1 site to a 3' EcoR1 site in the gene; 1.9 kb fragment must be 3' to the Eco-5 fragment. In order to account for the 11.9 kb genomic insert of the K-1 phage, there must also be another 5 kb EcoR1 fragment (Eco-2) 3' to Eco-5 fragment, which does not hybridize with CS15 and because it comigrates with Eco-5, can not be distinguished from the latter (Eco-5). Double digestion of K-1 phage DNA

Fig 22. Restriction Enzyme Analysis of Genomic K-1 Clone.

Southern blot of K-1 genomic recombinant probed with CS 15. Restriction enzymes used for digestion are shown at the top end of each lane. DNA molecular weight markers in kb (M) are represented in the left panel. Other details are as in Fig 18.

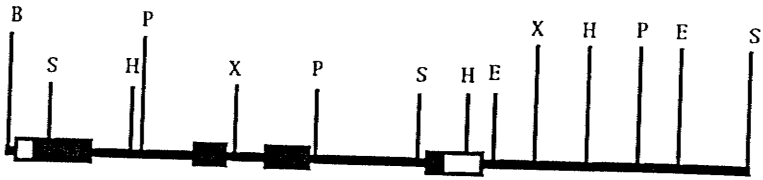


M
Eco RI
Hind III
Bam HI
Xho I
Sac I
Sal I
E+H
E+B
E+Xba
E+Sac
E+Sal
11.9
5
3.7
3.5
1.4
1.2
0.8
0.4
0.3

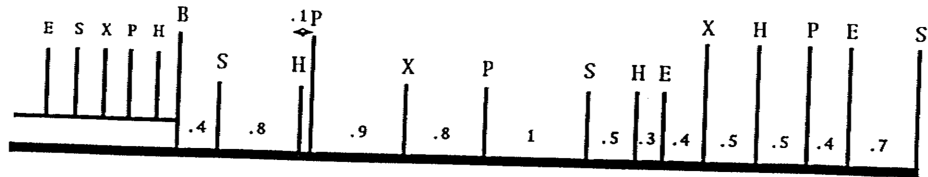
Fig 23. Restriction Enzyme Map of rat IGFBP-1 Genomic Recombinant K-1

I: Structure of the rat IGFBP-1 contained in recombinant phage K-1. Exons 1 to 4, from left to right are represented in boxes and the translated part depicted as solid boxes. Restriction enzyme sites abbreviated as before (Fig 19) except Sac1 (S), are shown. II: Restriction enzyme map of the K-1 phage; part of the phage arm with its multiple cloning site is represented as a double line. Fragment lengths are represented in kb. III: Representation of the restriction enzyme fragments that are produced when K-1 genomic phage was subjected to EcoR1 (E), Hind111 (H), Pst1 (P), Xba1(X), Sac1 (S), Hind111 (H), as well as E+H and S+ H double digests.

I



II



III

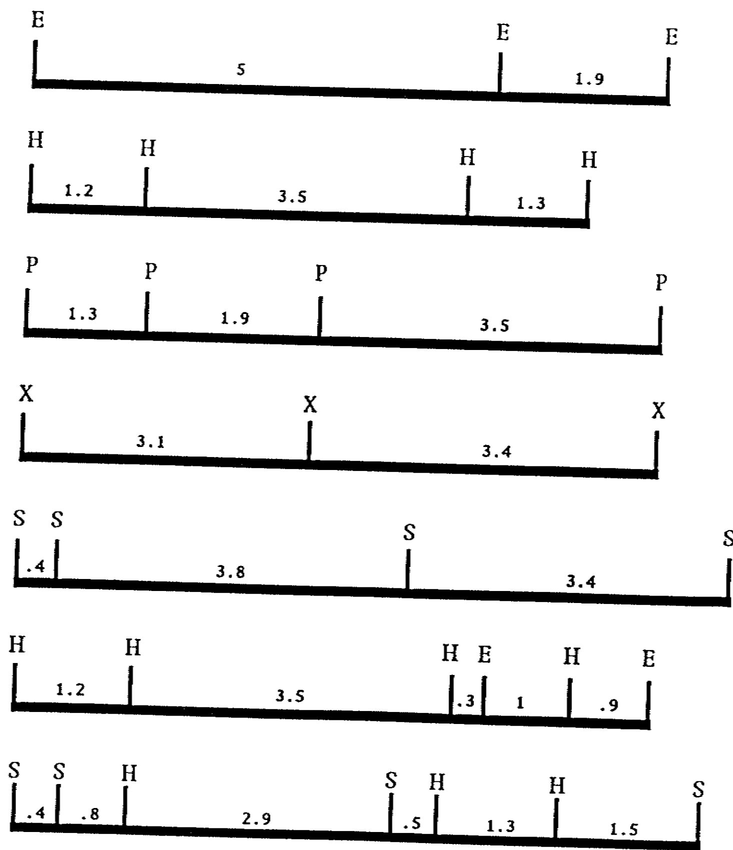
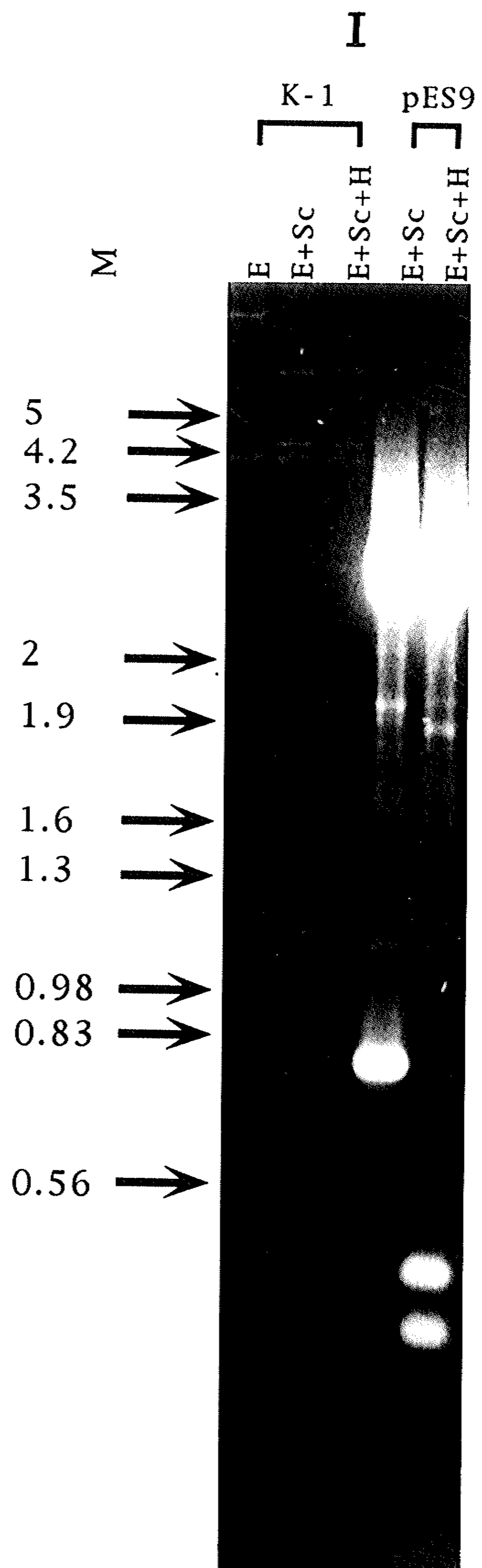


Fig 24. Restriction Enzyme Analysis of pES9

EtBr stain (I) and Southern blot probed with CS15 (II) are shown. Lanes containing K-1 phage DNA run parallely as a control are depicted in a bracket and so is the pES9 DNA containing lanes; restriction enzymes are abbreviated as before (Fig 19) and mol wt markers (M) are in kb in the left margin.


I
K-1
pES9
M
E
E+Sc
E+Sc+H
E+Sc
E+Sc+H
5
4.2
3.5
2
1.9
1.6
1.3
0.98
0.83
0.56

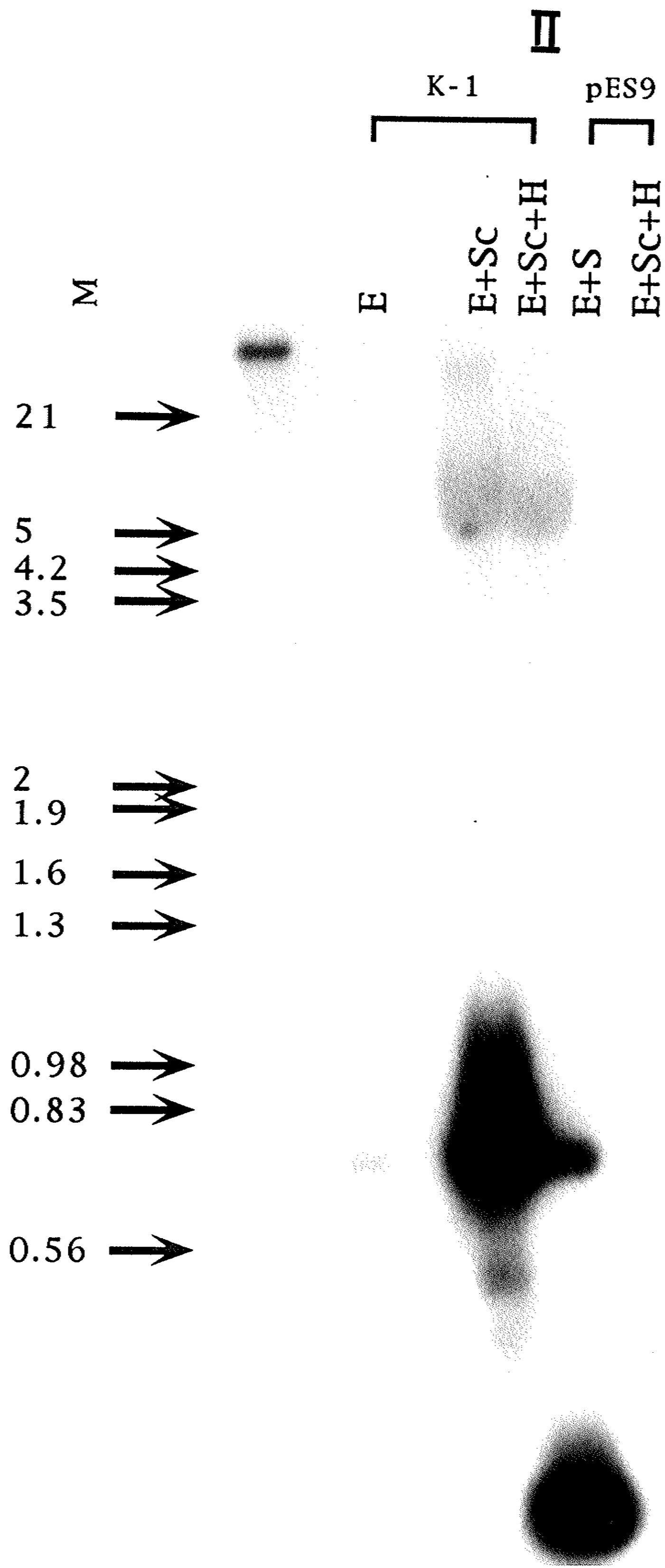
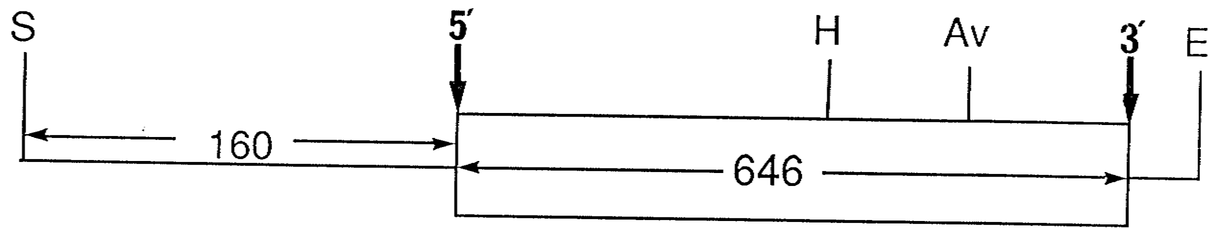

II
K-1
pES9
M
E
E+Sc
E+Sc+H
E+S
E+Sc+H
21
5
4.2
3.5
2
1.9
1.6
1.3
0.98
0.83
0.56

Fig 25. Structure and Sequencing Strategy of Exon 4

The Sac1 site (S) of intron 3, and the estimated 160bp distance between S and the 5'-boundary of the exon are shown. The boxed area depicts the exon and 646 is the length of exon in nt; 5'- and 3'-boundaries are represented. Restriction sites Hind III(H), Ava 1(Av), and EcoR1(E) which were used to generate subclones, are shown. Subclones HS19, EH300, EH20, SAv .6 and the primers (KS, T3, T7, and SP6), used to sequence the exon as well as the direction of sequencing are also shown.


S
5′
H
Av
3′
E
160
646

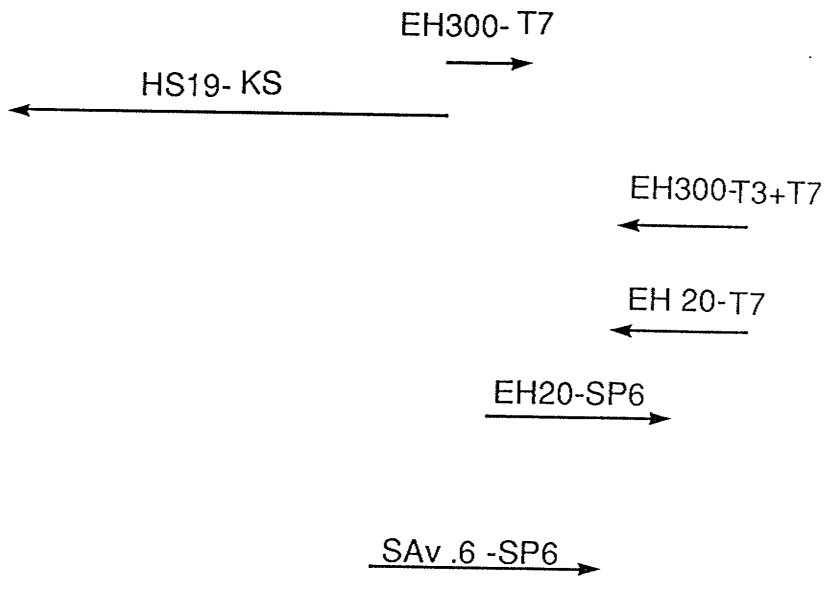

EH300- T7
HS19- KS
EH300-T3+T7
EH 20-T7
EH20-SP6
SAv .6 -SP6

Fig 26. Partial Sequence of the Rat IGFBP-1 Gene

Complete 1627bp sequence of the 5' flanking region is shown. Negative numerals depict the positions 5' to the transcription start site (+1); downstream of the transcription start site is represented in positive numbers relative to the cDNA. Consensus sequences of octamer binding site (OTF), insulin response element (IRE), API binding site (API), AP2 binding site (AP2), glucocorticoid response element (GRE)', CAAT box (CCAAT), and TATA box (TATA) are shown on the sense strand (>). Sequence, 5'- and 3'-boundaries of the four exons are represented; both 5' flanking and exon sequences are depicted in upper case letters while lower case letters represent the intron sequences and estimated lengths of introns are given in brackets. Important restriction sites with their relative positions within brackets are depicted. Other details are as in the text.

5′ FL

Pst1(-1623)

-1627

CTGCAGAATCCTCTAGTAGGGCCACGGGCTGTCGAGTGGCAGTACCAGAA

>IRE-(5)

-1577

GCTGAGCCAGGACAAGTCCCTGTCCCTCTCTGGAGACAAGAAAGGTCGTT

-1527

CTGTCATCAGATCGTAATGGTCAGCCTGCACCTGTTGGCTTTTCGATTGT

EcoR1(-1460)

-1477

GATATAACTGAGTTCCTGAATTCTCTACCCCTATGGGACTCTTCCTCAGC

-1427

CTTTACCTGATATAGCCTACCTCCCCACTCCCATTCCTCAACTCTCCTGG

-1377

CATATGTCTGTTTCCTGTGGCCCACTGGCCCATGTCTCTGATCCTTGGTA

Rsa1(-1324)

-1327

TTGTACTCCTTATCTGTTAGGGCTTTATTGTAACGCAAATTAAAAAAAGA

>OTF(2)

-1277

AAAGTCAGTCAGTCACTATTTACATACCTGTTCTGTGCTGGGCATCTATA

-1227

CTGGTTTCCTTGAACGAGAAAACCAATGTTGGTAGGCGATACACTGTTGC

-1177

CCATGCGTTTAAACTGCTTTAGTCAAGTCACCTGAGAAGCCATTTCCCAA

-1127

GTCTCAACTACAACGTTCATCACTGCTGGGTCACAGGGATGTGAGTTTTT

>AP1

-1077

GTCCAGGGTGTTCTAGGGCAGTCTCTGTGAACTTGCCCTAAAAACCCCTA

>GRE-2

Xba1(-1013)

-1027

CAACCTTTGCCTTCTCTAGAGCCAGGGGCTAAAGTTGAGATCTCCAAGGC

-977

TCTGAAAATGCCATGTCGTAAGACACTGAAGGGCTGGACAGGCATGCACC

Rsa1(-920)

-927

ACTGTGGTACAGTAGCTTTCAGGGTTCTACCCCACTCCAAACCAATACCT

-877

CAAAGGCCAGACAAGTGGCTCCAAGCTCTTGATGGAGGTTGGTCGCTCTG

-827

AGCTAGCACCCAGGTCAGGTAGGAGACAAAAATCTACACCTGGGGACATA

>AP2-(4)

-777

GGAGTTATTCATAGACCGGGGGGGGGGGGGCATCCCTGATTAGTTTTCTC

>AP2-

(3)

-727

CGGGAGGTTTTGGGGAGATTCCTGAGTGAGTCCCACTTCTGATTGTCCTT

-677

AAACATTTCTGAAGTTTTCCTTGCAATATAAAAGAATGAAAAAACAATGC

-627

AGAGAAAGCTGTCAGACTTGATAGCCATCTCAGTTAAACTAGACTGTTAG

-577

ACCGTCAGAGACAGCTTGGGTCCGGGGATAGAGGCTAGGCTAGGCTCCTG

>AP2-

(2)

-527

GGGTCCTGGACTTCTCTCCTCTTTGGAGGTATAACTGTTTCTTTTGGAAT

-477

GGGGTGAACAATGAATTTCTTCACTTGATGGCTAGAGGCTTCTTGGGGAG

-427

GAGAGGAAACAACTGTGGGTAGTATCACAAAGAACCCCACTTCTTCCCCT

-377

CCCTTTGGGGCCTGAAGCCTTCTTGGTCTAATCTGTTCTTGCTTTTCCTT

>AP2(1) >IRE-(3) >GRE-(1)

-327

AGTCCCTTAGGTATTCCTTGAGTTCGGTTGAAGAGCCTGGCGGGCTAGGC

>IRE-(1)

-277

CTTTGATTTCTCCCTGAACAAAAACAAGTGCCCTCCCCCATCTGACTACC

-227

AGCTCGCCGTTAGAATTGGACTTTAGCTCCTGTCCCAATCCATCACAACA

-177

GGACAAACATAGTAGGAAACCTGATCCATTTACGCCCCTTCCACCCACGG

-127

TTTGTGCGGAGCTCACAAGCAAAACAAACTTATTTGAACACGGGGATCC

>OTF(1)

-77

TAGCACGCTGCCCTGACAATCATTAACCCGTGCTGCCGAGCCAGCCCTTC

>CCAAT

-27 ATAAGGCCCTGGGTATGGCCAGCCAGC

>TATA

EXON 1

+1 ATGGTCCACTGCCCGCCGAGACACAAACCCAGCGAGCATTGAACA

+46

CTGCACACGGCCATCTGCCCAGAGAGCTGTGACCACCACTTCCGC

+91 TACTATCTACCAGAAAGTCGTGACTACTGAGCCACTGCTGCCTGC

*

+136 CCAGATTCTCATCCACCCGCCTGCTGCGTCTGGTTGCGATGCCGGA

+181

GTTCCTAACTGTTGTTTCTTGGCCGTTCCTGAT CCTCCTGTCCTT

+226

CCAGGTTCGCGTAGTCGCTGGAGCCCCCAGCCATGGCACTGTGC

+271

TCCCTGCACTGCTGAGAGGCTGGAGCTCTGTCCACCCGTGCCTGC

+316

TTCGTGCCCCGAGATTTCTCGGCCTGCGGGCTGTGGCTGCTGCCC

+361

GACATGTGCCTTGCCACTGGGTG CTGCCTGTGGTGTGGCCACTGC

+406

GGCCTGCGCTCAGGGACTCAGCTGCCGTGCGCTGCCAGGGGAGCC

+451

TCGACCTCTGCATGCCCTCACCCGTGGCCAGGGAGCCTGTGTACT

+496

AGAACCTCCGCCACCCGCCACGAGCAGCTTGTCCGGTTCTCAGCA

TGAAGgtactacaaccctctctg -------------INTRON- 1(1.2Kb)--

----- cctcagggct catgcgtctgtgtctgtggggcttgcttttcag

EXON 2

+546

AGGCAAAGGCTGCTGTGGCCTCTGAGGATGAGCTTGCCGAGAGCC

+591 CAGAGATGACAGAGGAACAGCTGCTGGATAGCTTCCACCTCATGG

+636 CCCCATCCCGTGAGGACCAGCCCATCCTGTGGAATGCCATTAGCA

+681

CCTACAGTAGCATGCGGGCCCGGGAGATCACTGACCTCAAGAAA

+726

TGGAAGgtgagaccctgcactcagaccttcaggtttagctatcta cgtgaagaggtttgtctaga----------

INTRON2(0.8kb)----------------

taatttttgtctcttgtactcatgctaatataaaattatcctttag

EXON 3

+732 GAGCCCTGCCAACGGGAACTCTATAAAGTGTTAGAGAGATTAGCT

+777 GCCGCTCAACAGAAAGCAGGAGATGAGATCTACAAATTTTATCTG

+822

CCAAACTGCAACAAGAATGGATTTTATCACAGCAAACAGgtaggtggctttgct

catccagatccttgtaaaacttcatgatttttttttttaaa

tcaaatgattcacaggcccaatacacatcatgggtagctttcttaggtgagatccagccctgcag--------------------

INTRON-3(1.4kb)---------- ----caga

EXON 4

+860

TGCGAGAGACATCTCTGGATGGAGAAGCTGGGCTCTGCTGGTGT

+905 **GTCTACCCATGGAGTGGGAAGAAGATCCCTGGATCTCTGG**

+945 AGACCAGAGGGGACCCCAACTGCCACCAGTATTTTAATGTGCAAA
+990 ACTGAAAGTTGTTTCCTCCCTCCTTCTTCACACAAAATATTTAAG

+1035 TATATGTGTATTTATACTCCGGAGCACACCATTTTATATATGTGT

+1080 ATATGTCCAGGAACTAGTTTTTATACTCCACATGCTGCTTGATGT

+1125 ACAAGTGGGTTGTATTTATTCACTCTAAGTTTATTTTTTTCTACC

+1170 TGTCCTTGTGCTGTATTAACCCATATAACTGAAGCTTTTCTCATC

+1215 TCCATACATGTAAATACTACCATCTCAGCTCTCCAGGTTCTGCTT

+1260 TGAAAGGGCAGCGCGGTAGCTGCCTAGAACGAGCACAAGTCAGTC

+1305

TGAGGTAGGGGCCTTTCAGTGGGTTCAGGGAGGAAGGTTAGCCCT

+1350 GGCTCGGGGAGACTTCCTCATCGAATCCCACAGGTCTGTGTCTGA

+1395

TGCCTATTGGCTGGGAAGGTTCCGATGTTGGTTGTGTAATCAAAG

+1440 CTAAACGTGGAAAGCTGCGTCCCATGCACTGTTAAACACACGTCT

+1485 EcoR1

GGAATAAAACATTCTACCTGG aaacactgctgtctctgtggaattc

with EcoRI and Sac 1 generated a 0.8 kb fragment which hybridized both with CS 15 (Fig 22) and EH 0.3 probes (Figs 21-III). Thus this was most probably the EcoRI/Sac1 0.8 kb (ES 0.8) fragment that contained the last exonic information (Fig 23). Restriction endonuclease (RE) analysis of ES 0.8 (subclone pES9) with Hind III resulted in 0.5 kb Sac 1/Hind III (HS 0.5) fragment and the Hind III/Eco RI- 0.3 (EH 0.3) fragment (Fig 24) as predicted from the RE map of K-1 (Fig 23).

The subcloning of HS 0.5 kb fragment in BSSK from ES 0.8 made it possible to sequence it. This DNA sequence (HS 0.5) enabled the localization of the 5' boundary of exon 4 (Fig 25). Furthermore, the ES 0.8 fragment contains about 160 bp of intron information followed by 646 bp of the last exon (exon 4) and 25 bp of 3' flanking sequence ending at the EcoRI site (Fig 25). Comparing the full lengh cDNA sequence (Fig 6) with that of the two exons identified- first and last, and by analogy to the human gene, the rat IGFBP-I last exon is the 4th exon. Characterization of the genomic clones has led to the identification of exon 1 and exon 4. Therefore only the exon 2 and exon 3 await cloning and sequencing.

Cloning and Sequencing of Exon 3

The 3.3 kb Xba1 fragment (Fig 28), was sub-cloned into BSSK (pX3.3). Restriction enzyme analysis and Southern blot hybridization of pX3.3 (Fig 27) suggested a Pst1 site about 1.1 kb down-stream from its (pX3.3) 5' end and a Sac1 site about 0.9 kb from this Pst1 site whereas the 3'- Xba site was located about 1.2-1.3 kb 3' to the

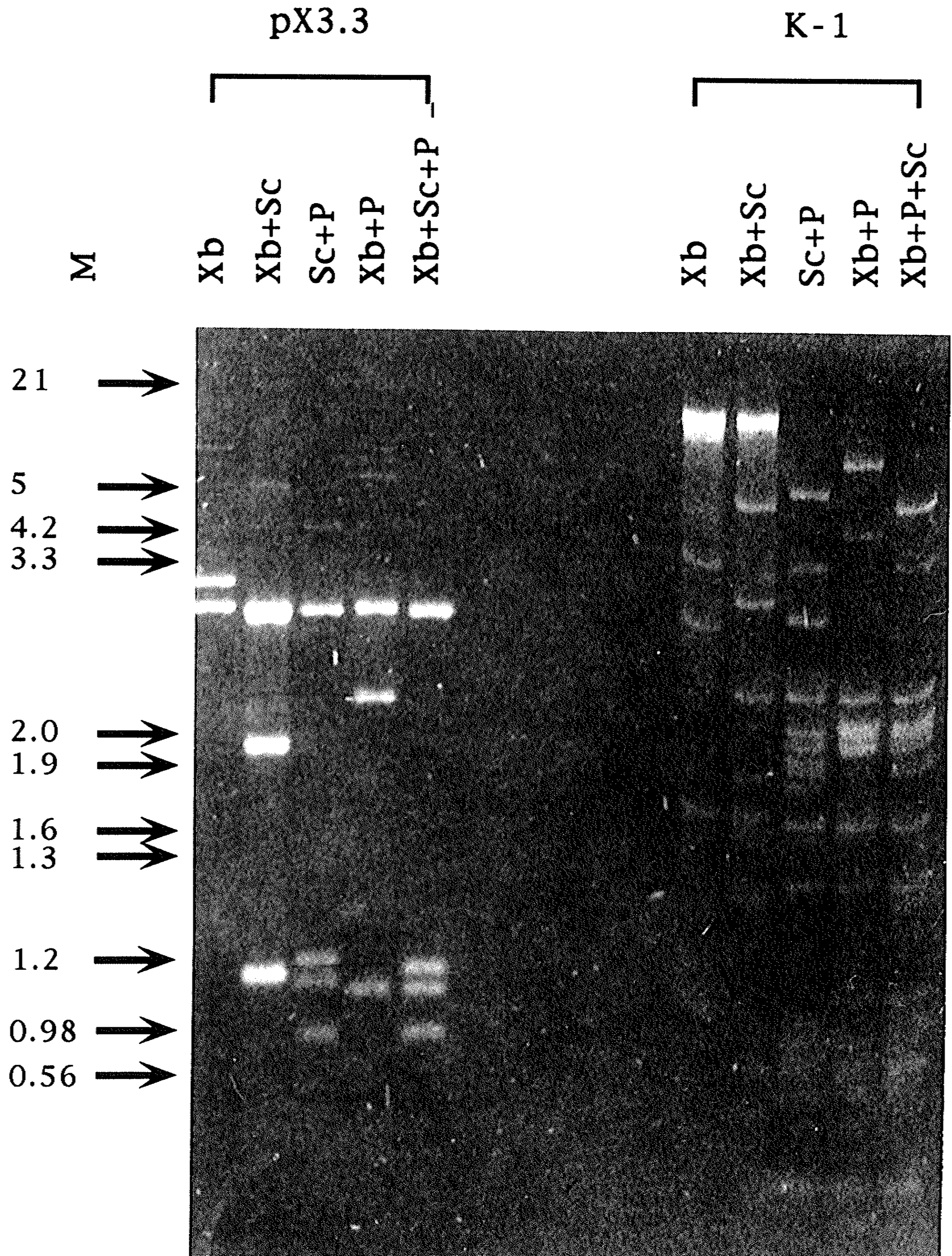
aforementioned Sac1 site (Fig 28). The RE map of X3.3 was constructed as follows: First the subcloning of Sac1/Xba1 1.2-1.3 kb (SX1.2) fragment in BSSK (pSX 1.2) and then restriction mapping it as shown in Fig 29 suggested the presence of Sac1/HindIII 0.5kb (SH0.5) and Hind III/Xba 0.7kb (HX 0.7) fragments which hybridized to CS15 in Southern analysis. Thus the SX 1.2kb fragment includes the Sac1/EcoR1 0.8 kb fragment where the exon 4 is located (Fig 25). Alignment of the X 3.3 fragment with that of the K-1 genomic clone is shown in Fig 28. This alignment suggests a Pst1 site, 1.1 kb down-stream from a Xba 1 site, which would explain a 1.9 kb- Pst1/Pst1 fragment (P1.9) observed in Pst1 single digests K-1 genomic DNA. Furthermore this fragment (P1.9) hybridized with CS15, suggesting the presence of exonic information (Fig 18-II). The SX1.2 hybridized to CS15 as expected (Fig 27). The Xba 1/Sac 1 fragment of 2.1 kb in length (XS 2.1) of the X3.3 also hybridized CS15 (Fig 27-II). Further subcloning this fragment (XS 2.1) into Xba1/Pst1- 1.1kb fragment (XP1.1) and Pst 1/Sac1 1kb fragment (PS1) in BSSK vector, was achieved. Southern analysis with CS15 as a probe revealed that only the XP 1.1 hybridized to CS15.

The restriction enzyme analysis of X3.3 and its sub-clones therefore revealed two main pieces of information: First, the map position of two more Pst1 sites in the K-1 genomic insert. A single digest of genomic K-1 with Pst1 results in three CS15 hybridizing fragments of 1.3-, 2- and 3.5-kb in lengths (Fig 18-II). Also the 1.3kb fragment contains the exon 1 (Fig 23). Thus, length between above intron 1-Pst 1 and the Pst 1 in X3.3, is 2kb accounting for the 2kb-Pst1 fragment. Second, exon sequences are present in XP1.1 fragment (Fig 28). The sequencing of DNA as shown in Fig 30

Fig 27. Restriction Enzyme Analysis of Subclone X3.3

The X3.3 fragment from the K-1 genomic recombinant was subcloned in vector BSSK; pX3.3 was subjected to restriction analysis as indicated at the beginning of each lane. The EtBr stain (I) and Southern hybridization (II) with CS15 as the probe are shown. Rest of the details are as before (Fig 18).

I



II

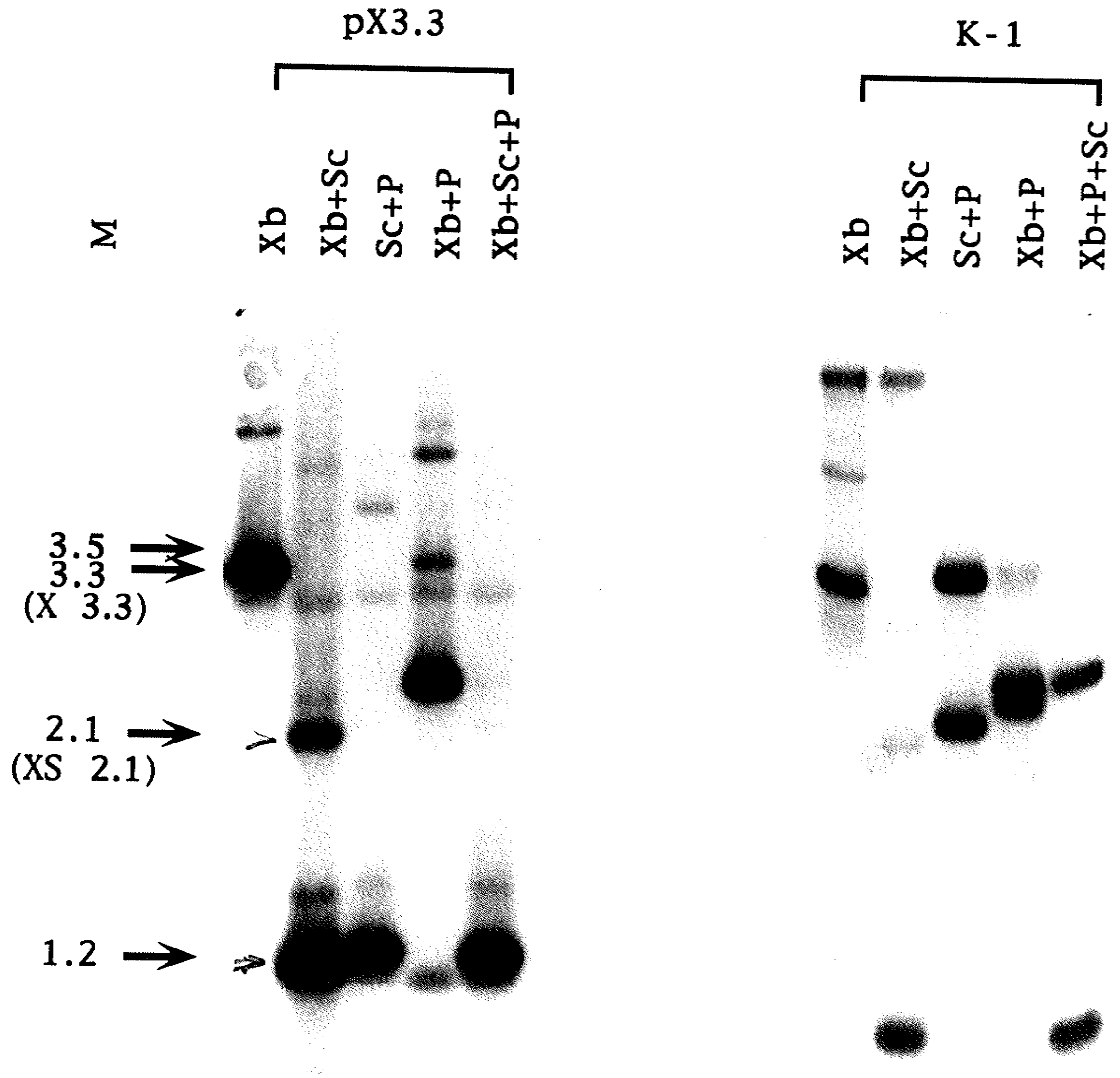
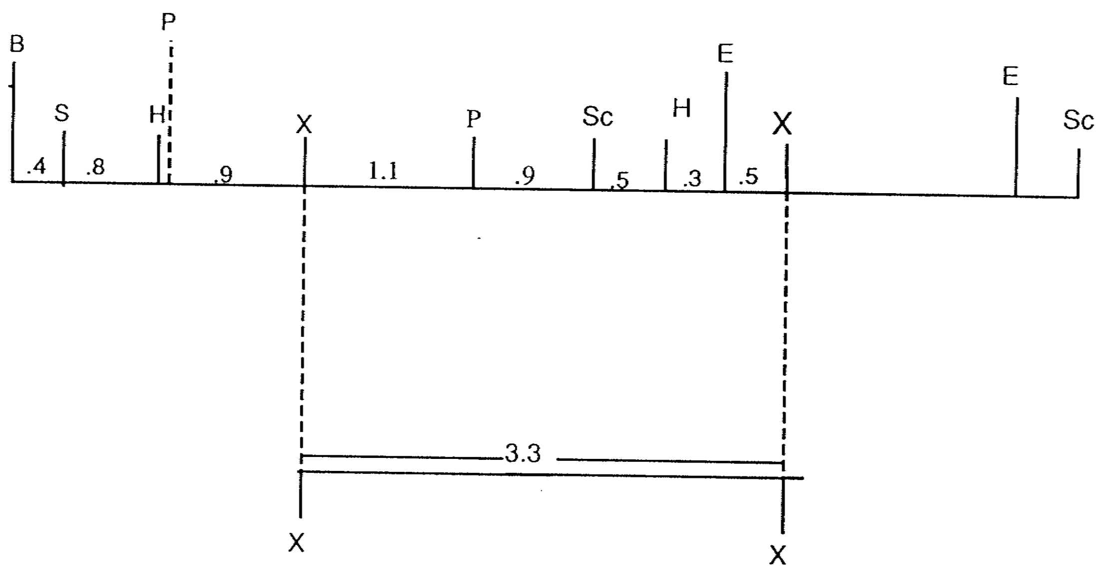


Fig 28. Alignment of Subcloned Fragment Xba1/Xba1-3.3kb (X3.3) with the Restriction Enzyme Map of K-1 Recombinant DNA

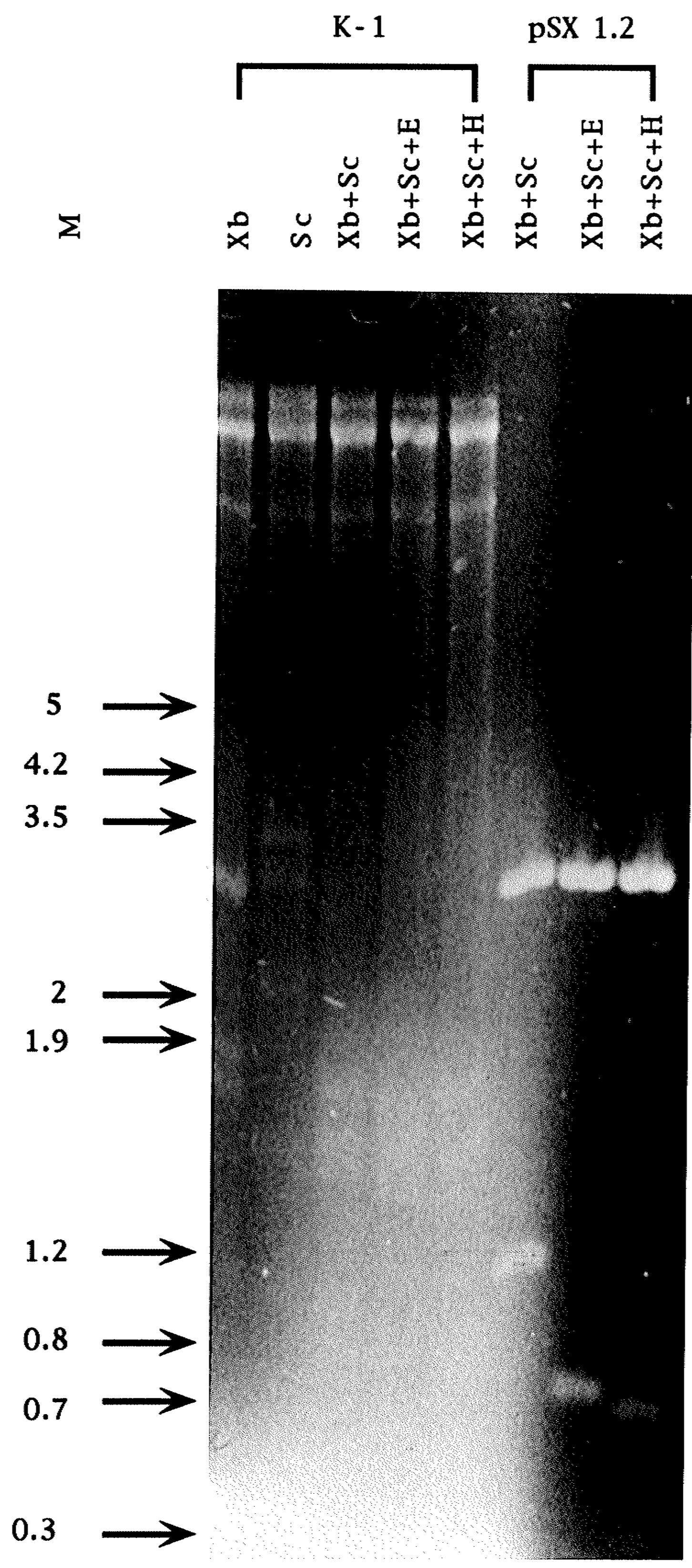
Lengths are represented in kb. The fragments Xba1/Pst1 1.1kb and Pst1/Sac1 0.9kb are depicted. Abbreviations for restriction enzymes are as in Fig 19.

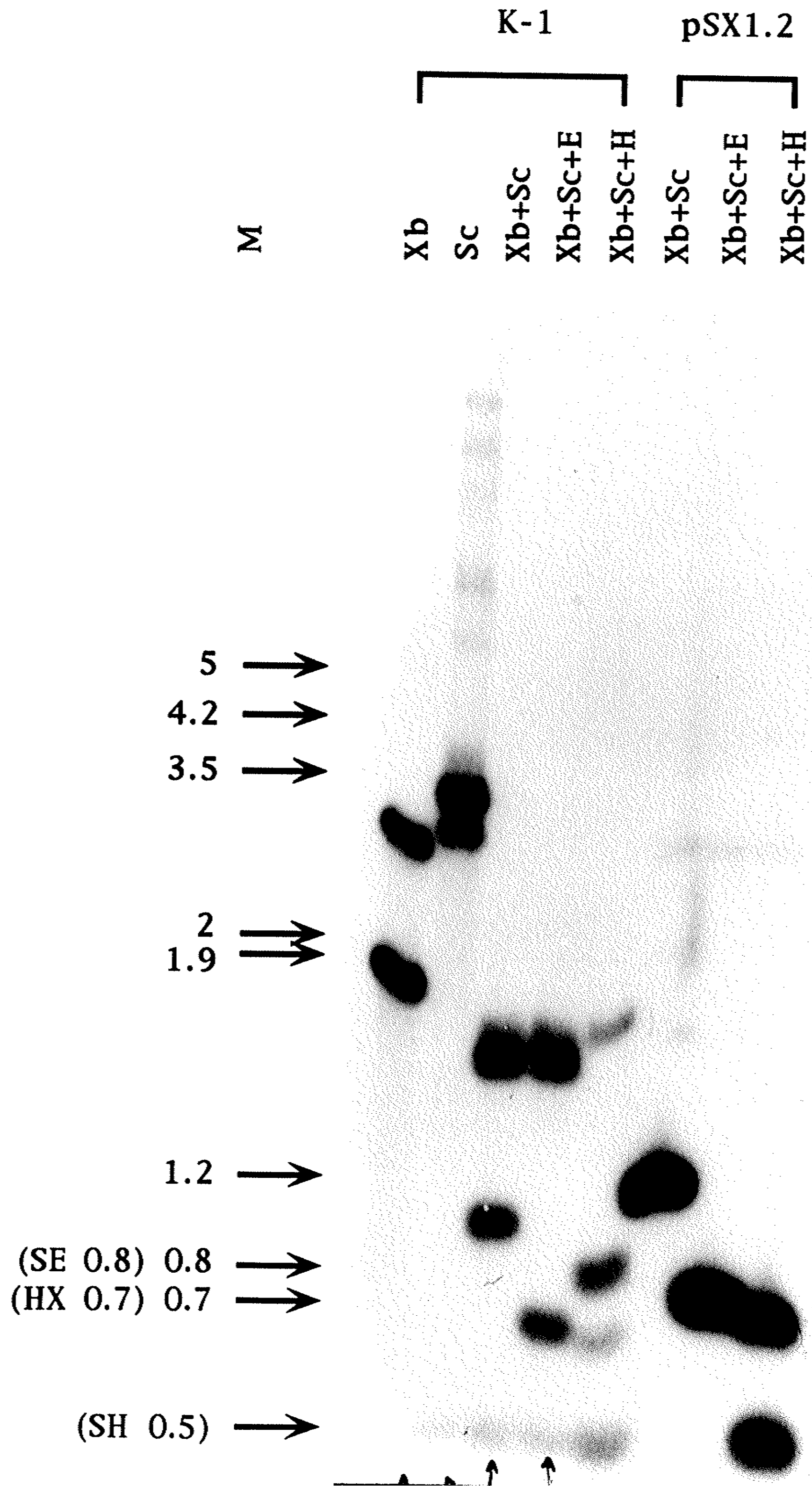


B
S
H
P
X
P
Sc
H
E
X
E
Sc
.4
.8
.9
1.1
.9
.5
.3
.5
3.3
X
X

Fig 29. Restriction Enzyme Analysis of subclone pSX1.2

Subclone contains the 1.2kb Sac1/Xba1 fragment in vector BSSK. Both EtBr stained gel (I) and Southern blot probed with CS15 (II) are shown. At the top end of diagrams, enzyme digestion that both pSX1.2 and K-1 DNA was subjected to are indicated; K-1 DNA was analyzed in parallel as a control; mol wt markers in kb (M) are shown on the left margin; relative positions of restriction fragments Sac1/EcoR1 0.8kb (SE0.8), Hind111/Xba1 0.7kb (HX0.7) and Sac1/Hind111 0.5kb (SH0.5) in the Southern blot (II) are shown. All other details are as before (Fig 18).


I
K-1
pSX 1.2
M
Xb
Sc
Xb+Sc
Xb+Sc+E
Xb+Sc+H
Xb+Sc
Xb+Sc+E
Xb+Sc+H
5
4.2
3.5
2
1.9
1.2
0.8
0.7
0.3



II
K-1
pSX1.2
M
Xb
Sc
Xb+Sc
Xb+Sc+E
Xb+Sc+H
Xb+Sc
Xb+Sc+E
Xb+Sc+H
5
4.2
3.5
2
1.9
1.2
(SE 0.8) 0.8
(HX 0.7) 0.7
(SH 0.5)

revealed the presence of the entire exon 3 in this fragment XP 1.1.

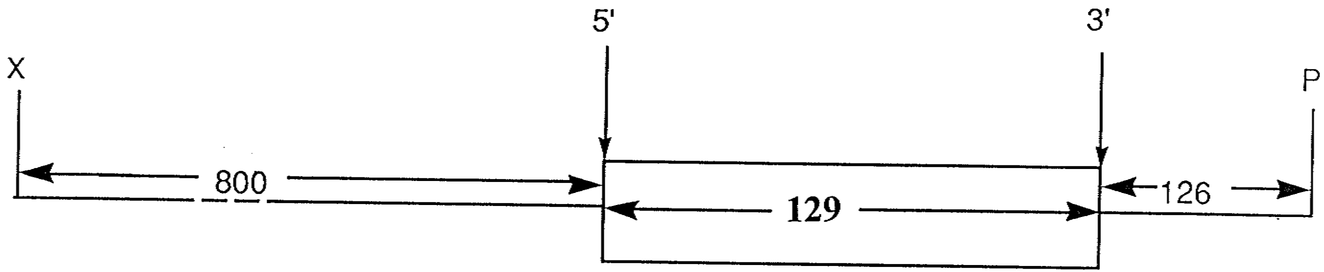
The 3'-boundary of exon 3 localized to 126 nt 5' to the Pst1 site of the XP1.1. Exon 3 was 129 nt in length and the Xba 1 site was estimated to be localized about 0.8kb upstream from the 5' boundary of the exon (Fig 30).

Structure and Sequence of Exon 2

Now only the structure and sequence of the second exon had to be elucidated. The 3.5kb Hind 111 fragment (H3.5) spanning from intron 1 Hind III in the 5'-end to the Hind 111 site in exon 4 (Fig 31), was subcloned into BSSK. Sequencing from either end showed that at the 5'-end there was overlap with the sequence downstream of the Hind111 site of intron 1. The 3' end of the H3.5 fragment is the Hind111 site of 4th exon, as predicted for the correct H3.5 fragment (Fig 23). Restriction endonuclease digestion of this H3.5 with Hind 111 and Xba 1 resulted in 1 kb fragment of Hind 111/Xba 1 (HX1) and Xba 1/Hind 111-2.5 kb fragment (XH2.5) as expected (Fig 32). The XH2.5 which contained the exon 3 and part of the exon 4 (346 nt), hybridized to CS15 as predicted (Fig 32-II). Hybridization between CS15 and HX1 also occurred in Southern analysis as predicted (Fig 32-II). The HX1 was subcloned in BSSK. The exon 2 sequence identified from the CS15 (Fig 6) that occurs between 3'- boundary of exon 1 and the 5' boundary of exon 3, contained an Apa1 restriction site, 31 nt upstream (5') to the predicted 3'-boundary of exon 2. Restriction enzyme digestion of H3.5 with Hind III and Apa1 resulted in a 0.9 kb HindIII/Apa1 fragment which hybridized with CS 15 (Fig 32).

Fig 30. Structure and Sequencing Strategy of Exon 3

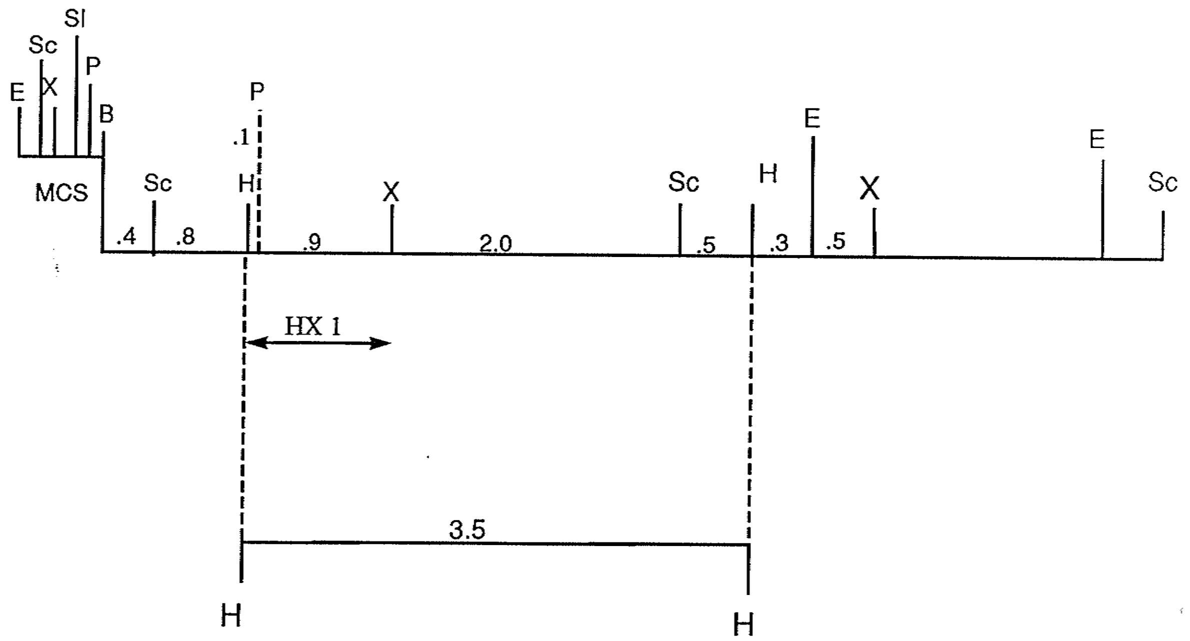
The Xba1(X) site of intron 2 and the estimated length of 800bp(800) to the 5'-boundary of the exon 3 is shown. The boxed area represents the exon and is 129nt in length. The 5' boundary of the exon(3') and the distance of 126nt to the Pst1 site(P) of intron 3 are represented. The subclone Xba1/Pst1-1.1kb (XP1.1) used to sequence the exon and the primer KS are depicted along with the direction of sequencing.



XP1.1 -KS

Fig 31. Alignment of H3.5 Subcloned Fragment with the RE Map of K-1.

Multiple cloning site (MCS) of the K-1 phage arm, lengths in kb and the fragment Hind111/Xba1 1kb (HX1) are represented. Restriction enzyme abbreviations are as in Fig 19.



Sl
Sc
X
P
E
B
P
.1
MCS
Sc
H
X
Sc
H
E
X
E
Sc
.4
.8
.9
2.0
.5
.3
.5
HX 1
3.5
H
H

This suggested the presence of the 3' boundary of exon 2 within about 200 nt 5' to Xba 1 of HX1 (Fig 31). The total exon 2 sequencing strategy is shown in Fig 33 where both Hind III/Apa1-0.9kb fragment (HA0.9) containing plasmid and HX1 subclones were utilized. The structure of exon-2 elucidated using above subclones i.e. HA 0.9 and HX1 is also shown in Fig 33. The 3' boundary of the exon is located 59 nt upstream from the Xba 1 site (relative to pHX 1) and exon 2 is 186 base pairs in length. The Hind III site of HX was estimated to be located at about 0.75 kb 5' to exon-2 (Fig 33).

A complete restriction endonuclease map of the genomic phage K-1 including the four exons, is shown in Fig 23. Also the single and double restriction enzyme digested fragments and ensuing Southern hybridization analyses agreed with this RE map.

3.2. Structure of the rIGFBP-1 Gene

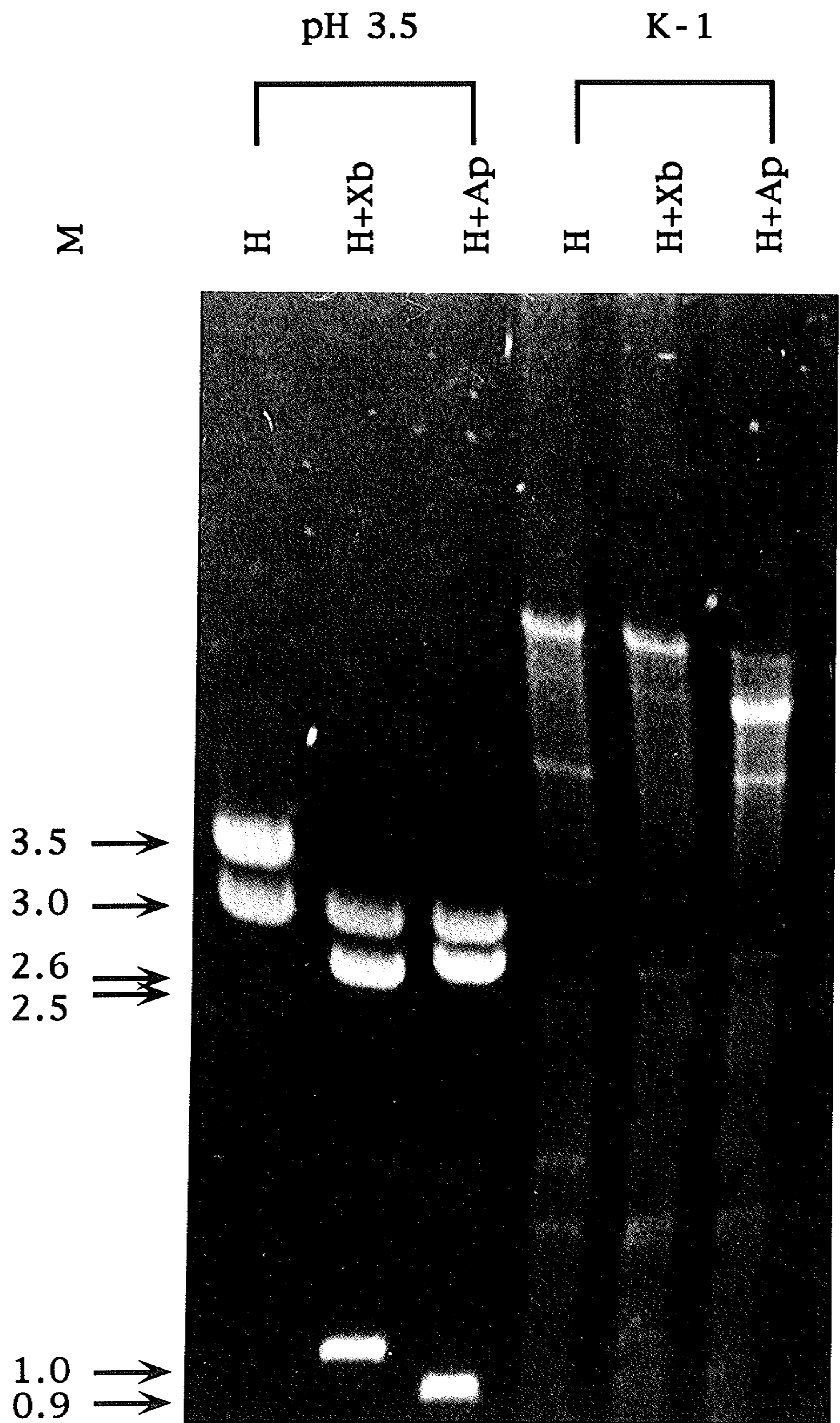
3.2.1. Transcribed region.

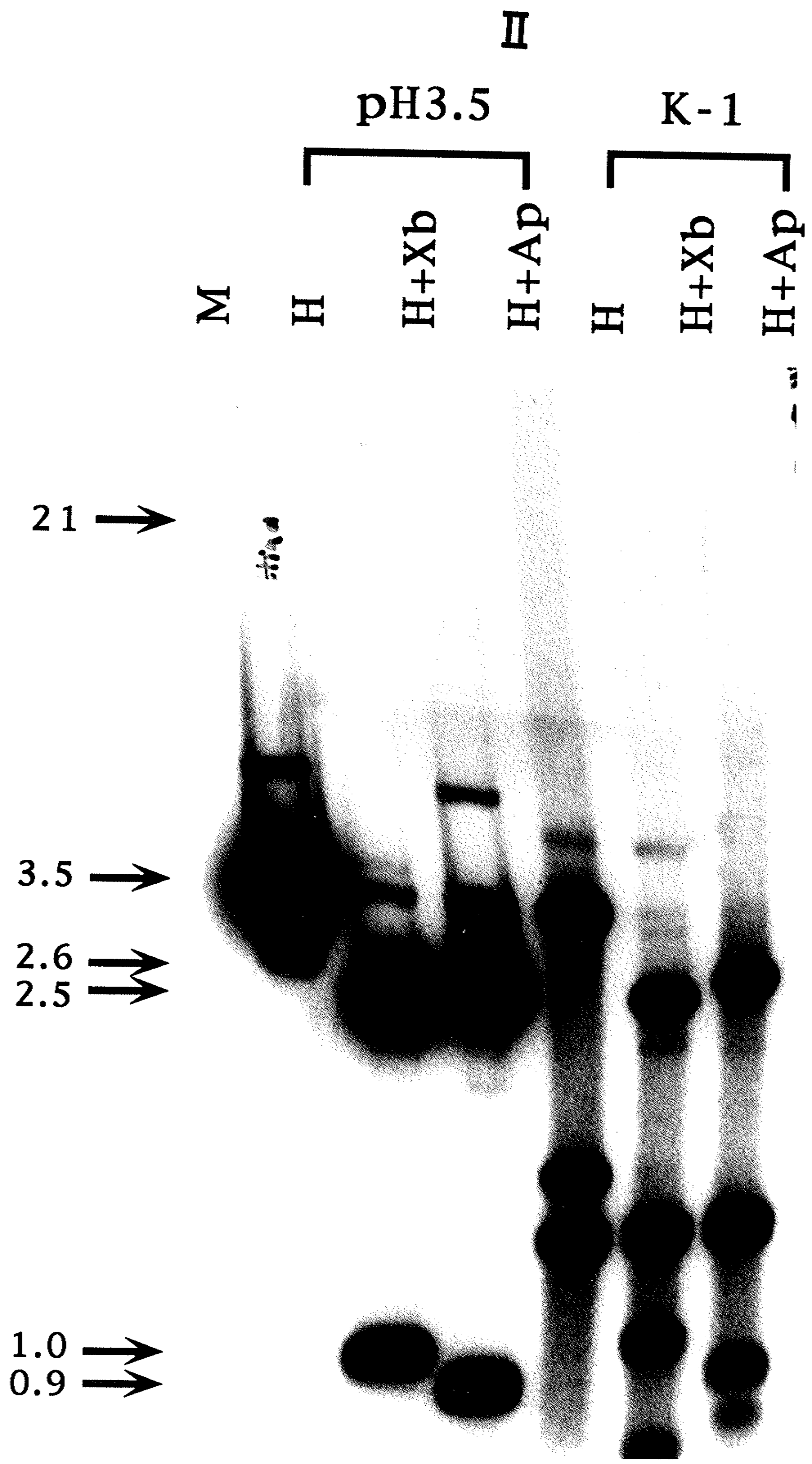
This region spans from the mRNA cap site (+1/5'-boundary of exon 1) to the 3'-boundary of the 4th exon .

Exon 1: The length is 545 nt while the intron that follows is estimated at 1200 bp (Fig 26). The phase of the intron is I, akin to the human situation; in both cases the intron interrupts a glutamic acid encoding GAG triple codon at the first nucleotide-G (Table 3). The translation initiation site is located at +173, resulting in a 173 nt 5' untranslated region (5' UTR).

Fig 32. Restriction Endonuclease Analysis of Subclone pH3.5.

EtBr stained gel (I) and the Southern blot (II) with CS15 as a probe are shown. On the left margin positions of the mol wt markers in kb and the bands that correspond to HindIII/HindIII-3.5kb (H3.5), HindIII/ApaI-2.6kb (HA2.6), Xba1/HindIII-2.5kb (XH2.5), HindIII/Xba1-1kb (HX1), and HindIII/ApaI 0.9kb (HA0.9) in the Southern blot are depicted. Restriction enzyme digested K-1 DNA was run side by side as a control to ensure that the fragment originates from K-1 and is not a cloning artifact. Other details are as in Fig 18.

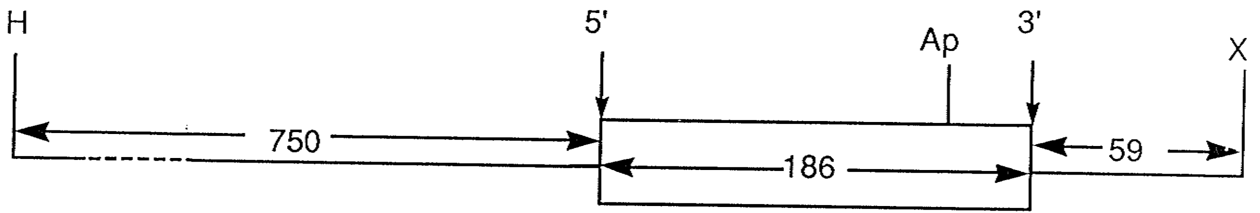

pH 3.5
K-1
M
H
H+Xb
H+Ap
H
H+Xb
H+Ap
3.5
3.0
2.6
2.5
1.0
0.9



II
pH3.5
K-1
M
H
H+Xb
H+Ap
H
H+Xb
H+Ap
21
3.5
2.6
2.5
1.0
0.9

Fig 33. Structure and the Sequencing Strategy of Exon 2

HindIII(H)/Xba1(X) fragment containing exon 2 is shown. Numbers represent the length in nt. Boxed area depicts the exon and the length of which is shown within the box. Subclones (HX 0.9, and HX 1), primers (T3 and T7) and the direction of sequencing are also shown.



HX1-T3

HA0.9 -T7

Exon 2: The length of this exon is 186nt, followed by intron 2 which is estimated at 0.8kb in size. This intron, unlike intron-1, is phase 0 type because it occurs between two triple codons encoding lysine and glutamine residues and, again is identical to the human gene (Table 3).

Exon 3: The smallest of the four exons is 129nt in length. Intron 3 is estimated at 1.4kb by Southern blot analysis and is a 0-phase intron (Table 3). The amino acid residues interrupted by this intron were Gln and Cys.

Exon 4: The largest of the exons both in rat and man IGFBP-1 genes, is exon 4 with 646bp in rat and 706bp in man (Table 3). Only 131 nt are translated in the rat gene thus giving rise to a 514nt 3'-untranslated region (3'-UTR). Translation ends at the Asn residue, immediately followed by a consensus "non-sense" codon TGA as well as the consensus poly adenylation signal AATAAAA.

All three exon/intron boundaries of the rIGFBP-1 gene conform to the classical AG/GT rule as in the case of the human gene. Deviations from the larger consensus sequence for vertebrate splice sites are also conserved for the most part between rat and human IGFBP-I genes (Table 3).

Table 3. Intron-Exon boundaries of the Rat and Human IGFBP-1 Genes

Exon[d] Number	Length	5'-Splice site	Intron N[a]	L[b]	3'-Splice site	P[c]
#Consensus:		**AG/gtaagt**			**(Py)10 n cag/G**	
Rat 1	545	**G**[e]**/gtacta**----	I	1200	---**ctttt cag/A**G	1
		g-			-lu	
Man* 1	514	**G/gtacca**---	I	1600	--- **cctttccag/A**G	1
		g-			-lu	
Rat 2	186	AA**G/gtgaga**ccc-	II	800	--**atcctttag/G**AG	0
		lys			glu	
Man 2	170	AA**G/gtgagg**ccca-	II	1240	-**attctcttag/G**AG	0
		lys			glu	
Rat 3	129	CA**G/gtaggtgg**cttt-	III	1400	- ct**gtcttcag/TG**C	0
		gln			cys	
Man 3	129	CA**G/gtaggtgg**cc---	III	1240	- **gtctttgcag/TG**T	0
		gln			cys	
Rat 4	646					
Man 4	706					

a: Intron number b: Intron length in bp c: Phase of the intron

d: Exon length in bp * Cubbage et al. (170)

e: amino acid residue- encoded and -interrupted are shown. Lower case letters, intron- and upper case letters are exon- sequences.

#Consensus vertebrate 3'- and 5'-splice acceptor sequence (221). Deviation from the consensus sequence is underlined;bold letters indicate identity between Rat and Man.

3.2.2. The 5' Flanking Region

3.2.2.I. Cloning

In order to better understand the molecular regulation of the rIGFBP-1 gene, cloning of the 5' FL region was imperative.

The K-1 genomic phage contained only 83 nt of 5' flanking sequence (Fig 16). Therefore, to isolate and clone more 5' FL information, the following rat genomic libraries were screened: (i) EcoR1- A rat genomic library where female rat liver genomic DNA has been partially digested with EcoR1 and subcloned into the same site (EcoR1) of charon 4a phage vector.

(ii) Hae III: In this library the vector is the same charon 4a but the male SD rat liver genomic DNA was partially digested by HaeIII and subcloned in the EcoR1 site.

(iii) Kasper's genomic library: Third library screened was the Kasper's genomic library; resulted in the isolation of the K-1 genomic phage .

(iv) Dash II- from Strategene (La jolla CA), was a new generation library with a 22kb genomic insert whereas the former three contained only 8 to 12 kb inserts.

About $2x10^6$ recombinant phages in duplicate were screened from each of the four libraries where PS400 was used as a probe and 2 positive clones were identified. The first one was from Kasper's genomic library (K13) and the second clone D-3 was from the Dash II library (Fig 34). Restriction enzyme analysis of K-13 demonstrated that of this clone was identical to K-1 and thus it (K-13) was not pursued further. Alignment of the

restriction map of D-3 phage with that of K-1, suggested the presence of at least 7 kb of 5' FL information in the former (Fig 35) compared to the latter.

Restriction enzyme analysis of D-3 with EcoR1, resulted in a 6.8 kb fragment which hybridized both CS15 and PS400 probes (Figs 36-I and -II); Hind III gave rise to 3.5- and 1.4-kb fragments which hybridized only to CS15 while the most 5' fragment which is 8.2 kb fragment hybridized with both PS-400 and CS15 as predicted (Figs 36-I and -II). The corresponding 5' Hind III fragment of K-1, was 1.2 kb in size (Fig 35), suggesting the presence of at least 7 kb of additional 5' FL information in D-3. The Pst1 digestion of D-3 resulted in 3.5- and 1.9-kb fragments as predicted by the RE map (Fig 35) and both these fragments hybridized only to CS15 while the most 5'-2.9 kb fragment did so with CS15 and PS400 (Figs 36-I and -II). Digestion products of Xba1 also were predicted correctly from the RE map (Fig 35) and resulted in a 3.1 kb fragment that hybridized both to CS15 and PS400 (Figs 36-I and -II). The following fragments: 2.1 kb Xba1/Hind III (XH2.1), EcoR1/Hind III-2.6 kb (EH2.6), and Pst1/Hind III-2.8 kb (PH2.8), all of which hybridize PS400, were subcloned into BSSK (Fig 37). Since it was known from previous work that the mRNA cap site occurred about 1.1 kb upstream from the Hind III site (H) of intron 1 (Fig 26), then XH2.1, EH2.6, and PH2.8, possess 1- 1.5- and 1.7-kb of 5' FL information respectively. In order to ensure the cloned rIGFBP-1 gene from recombinant phages (K-1 and D-3) is the endogenous rat gene, the rat genomic DNA was restriction endonuclease mapped. To this end, genomic DNA from the rat was subjected to restriction enzyme digestion and the resulting Southern blots were probed with PS400 (5' probe) and CS15 (cDNA probe); the results are shown in Figs 38-I (PS400) and -II

Fig 34. D-3 Phage DNA probed with PS400

Phage DNA was blotted onto nitrocellulose filter after lysing the phage as described in MATERIALS AND METHODS. Positive plaques containing recombinant D-3 phage clones were purified after three screenings with filter hybridization using PS400 as the probe which was [^{32}P] labelled. The resulting autoradiogram is shown.

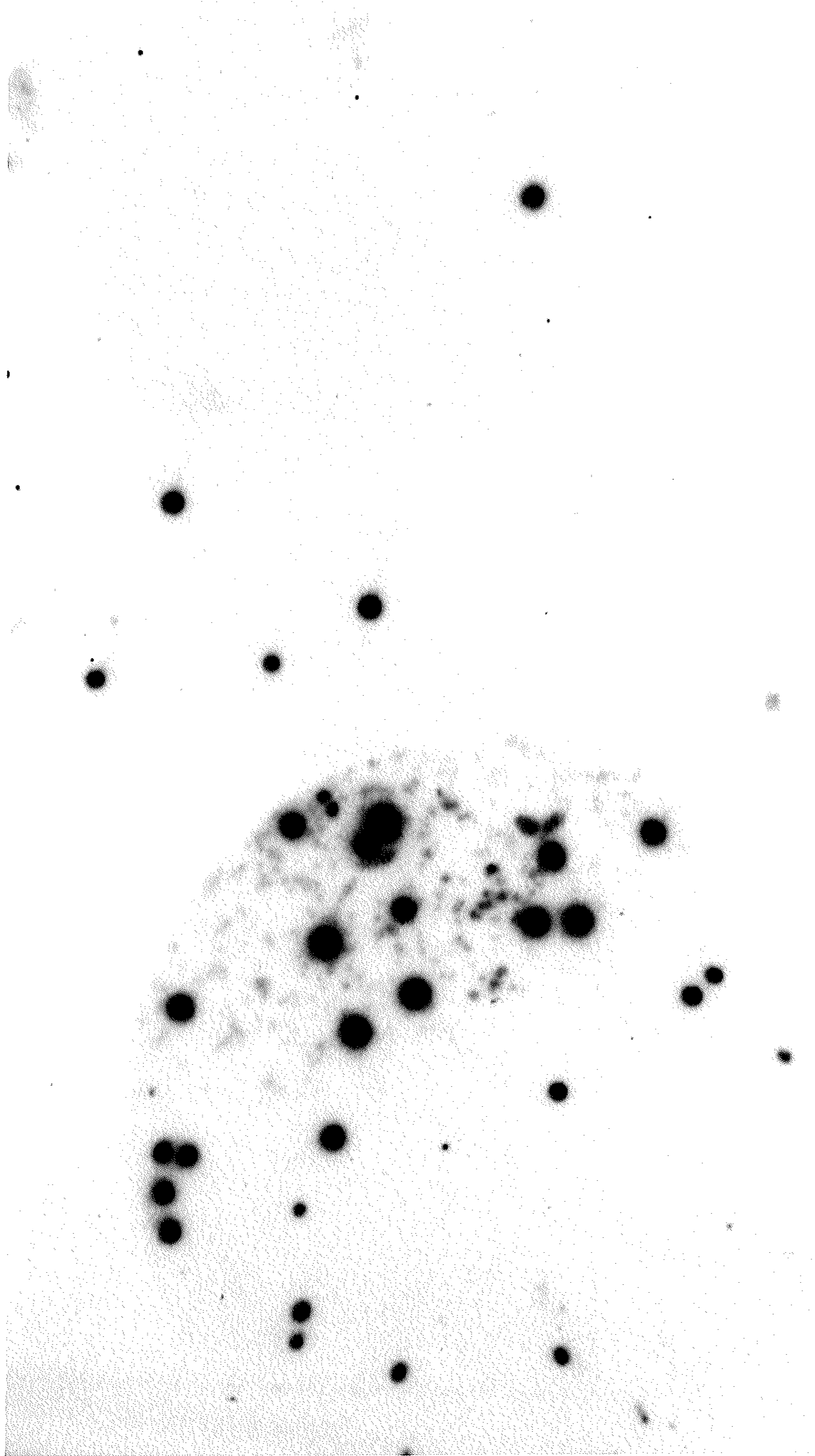
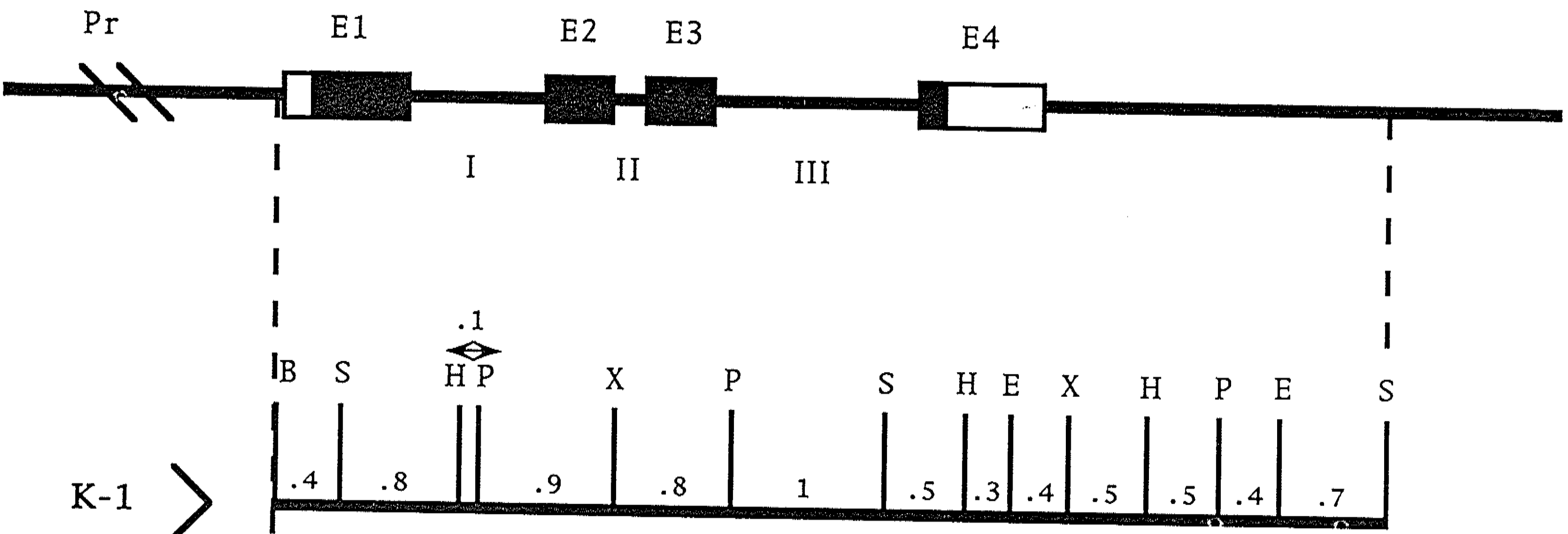
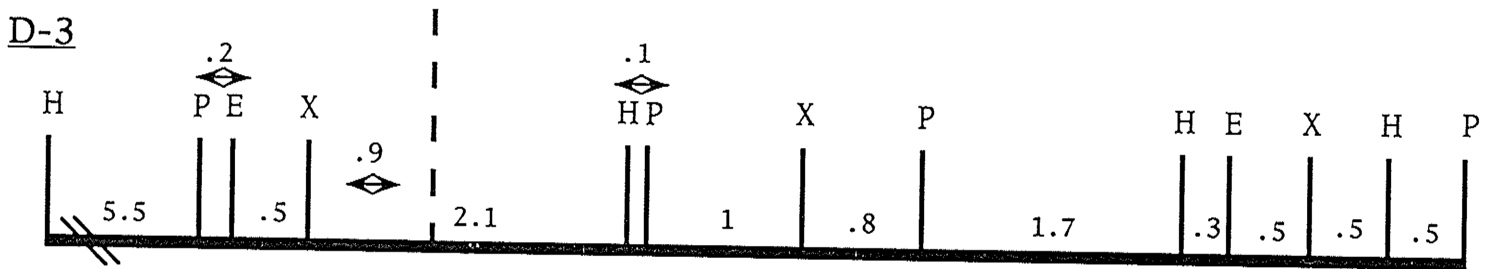


Fig 35. Alignment of rIGFBP-1 Recombinant Phages K-1 and D-3

The structure of the rIGFBP-1 is shown at the uppermost end. Promoter (Pr), exon 1 (E1), exon 2 (E2), exon 3 (E3), and exon 4 (E4) are represented as boxed areas. The solid area in the boxes depicts the translated region; open area in E1 and E4 are 5'- and 3-untranslated regions respectively. Restriction endonucleases are abbreviated as in Fig 18 except Sac1 represented here as S. Restriction enzyme maps of recombinants K-1 and D-3 aligned with each other and the rIGFBP-1 gene are depicted. The fragment lengths are in kb.



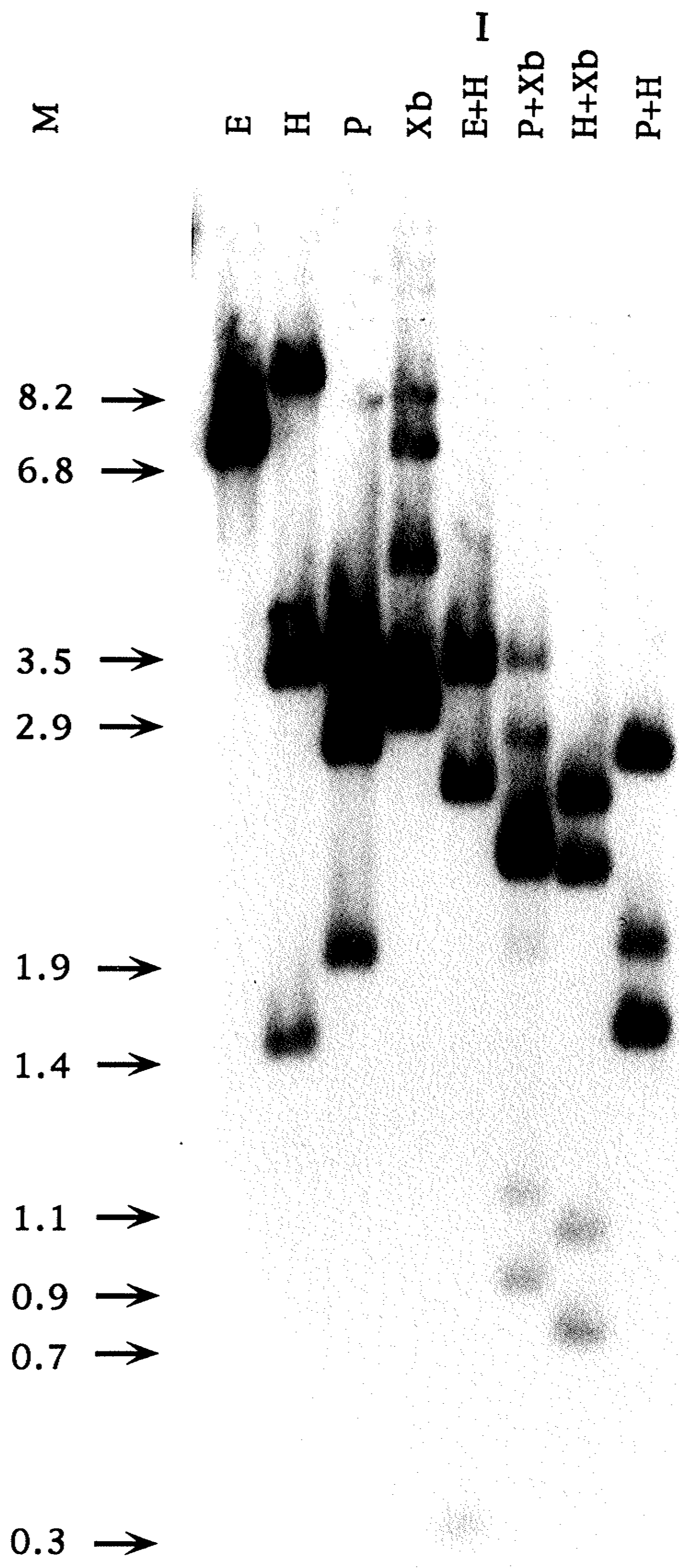
Pr
E1
E2
E3
E4
I
II
III
K-1
B S H P X P S H E X H P E S
.1
.4 .8 .9 .8 1 .5 .3 .4 .5 .5 .4 .7

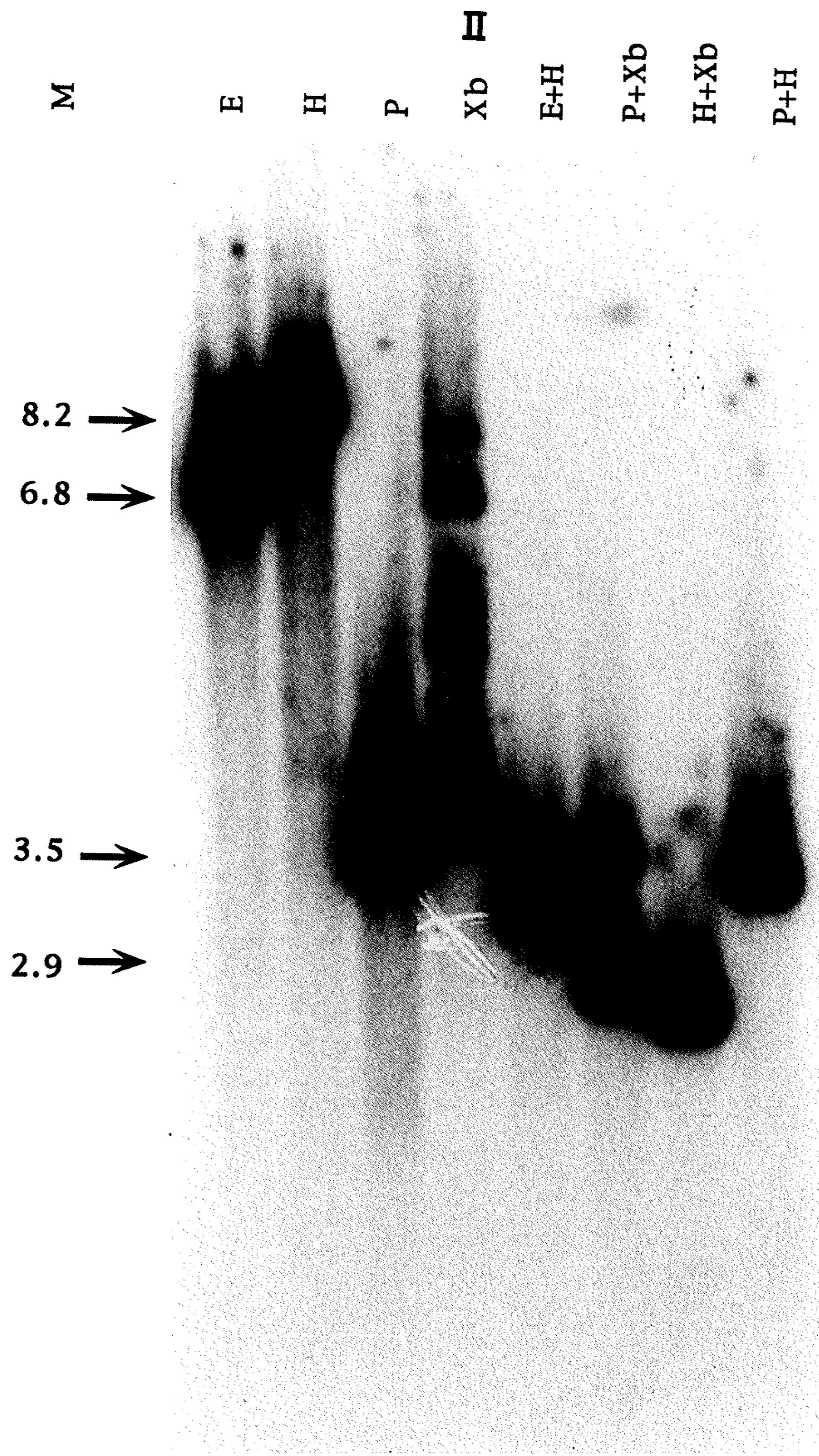


D-3
H P E X H P X P H E X H P
.2
.9
.1
5.5 .5 2.1 1 .8 1.7 .3 .5 .5 .5

Figs 36-I and -II. **Southern blots of D-3 phage DNA**

D-3 phage DNA was restriction endonuclease digested and analyzed as detailed before (Fig 18). The resulting Southern blots were probed either with CS15 (I) or with PS400 (II). Left margin depicts the mol wt markers (M) in kb and at the top end of each lane represents the restriction enzyme used in each digestion. Abbreviations for restriction enzymes are as in Fig 18.


M
E
H
P
Xb
E+H
I
P+Xb
H+Xb
P+H
8.2
6.8
3.5
2.9
1.9
1.4
1.1
0.9
0.7
0.3


II
M
E
H
P
Xb
E+H
P+Xb
H+Xb
P+H
8.2
6.8
3.5
2.9

(CS15). The restriction fragments produced by each restriction enzyme digestion are exhibited in Fig 39B-II and the deduced restriction enzyme map is represented in Fig 39B-I which overlapped with the restriction enzyme map of the cloned rIGFBP-1 gene from the recombinant phages D-3 and K-1 (Fig 39A), suggesting the cloned rat IGFBP-1 gene is identical to the endogenous gene.

3.2.2.II. Sequencing of 5' FL

Further sub-cloning of the PH 2.8, EH 2.6 and XH 2.1 fragments to Pst1/Bam HI-1.7kb (BP 1.7) EcoR1/ BamH1-1.5kb (EB 1.5) and Xba1/BamH1-1kb (XB1) was carried out, in the cloning vector-BSSK. In all three sub-clones, the 3' end was the BamHI site at -83 of the rat IGFBP-1 gene 5' flanking. The EcoR1/Xba1 0.5 kb fragment (Ex0.5) also was subcloned (Fig 40).

These subclones: PB 1.7, EB 1.5, EX 0.5 and XB 1 were sequenced on either end as described in MATERIALS AND METHODS. The sequencing strategies are shown in Figs 40 and 41A. In the PB 1.7 clone, 164 bp down-stream from the Pst1 site in the 5' flanking region, sequence is an EcoR1 site. As well, sequence overlap with EB 1.5 was found which suggested contiguity across this EcoR1 site (Fig 26). The EB 0.5 kb fragment was sequenced completely.

Restriction endonuclease mapping suggested the presence of two useful HpaII sites in the XB1 fragment. These two fragments: HpaII 173bp (Hp173) between HpaII sites at -728 and -555, and HpaII/ BamHI 474 bp (HpBm474) fragments which extend from -

555/HpaII site to -83/BamH1 site, were subcloned (Fig 41A). Since Cla1 has HpaII compatible ends, HpB474 was sub-cloned into C1a1/BamHI and Hp174 into Cla1 sites respectively of the cloning vector, BSSK.

In order to elucidate the contiguity of EX 0.5 and XB1 fragments over the Xba1 site, cloning and sequencing a subclone encompassing it (Xba1) was required. The Rsa 404 sub-clone fits the description because it contains the fragment spanning from Rsa1 at -1324 to that (Rsa1) at -920 (Fig 26) which was subcloned into Sma1 site of pGEM-7Z. The sequence obtained for Rsa404, overlapped across Xba1 the site, with that obtained from EX0.5 and XB1 sub-clones hence proving that they (EX0.5 and XB1) are in fact contiguous across Xba1 (Fig 40).

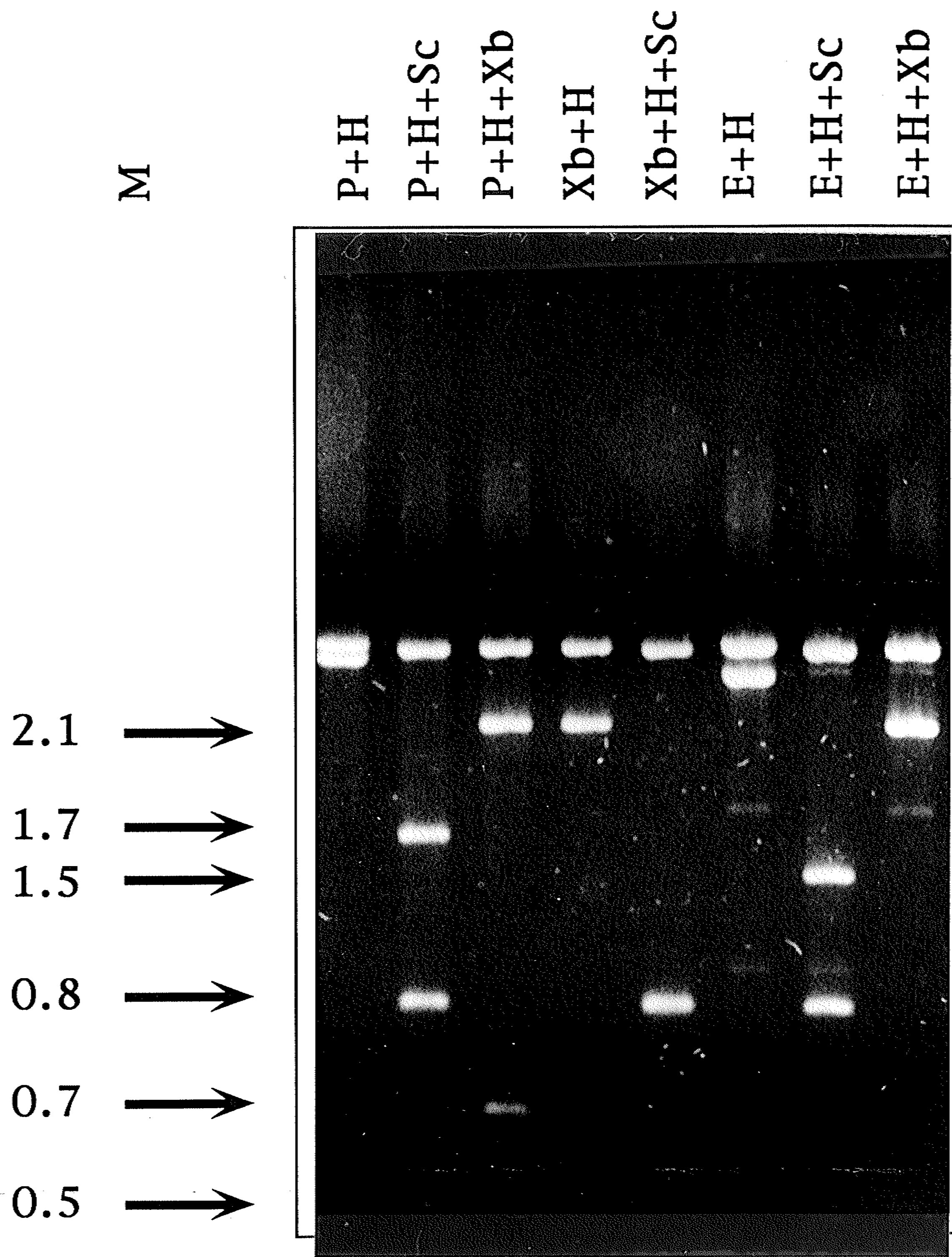
The sequencing strategy of the entire rIGFBP-1 gene that is sequenced already including 1627bp of 5' region up to the Pst1 site, is shown in Fig 41B, and the resulting sequence is shown in Fig 26. Furthermore, Unterman et al (259) reported the sequence of some 941bp of the rIGFBP-1 gene 5'FL region which agrees with the data reported in this thesis.

3.2.2.III. Consensus Sequences

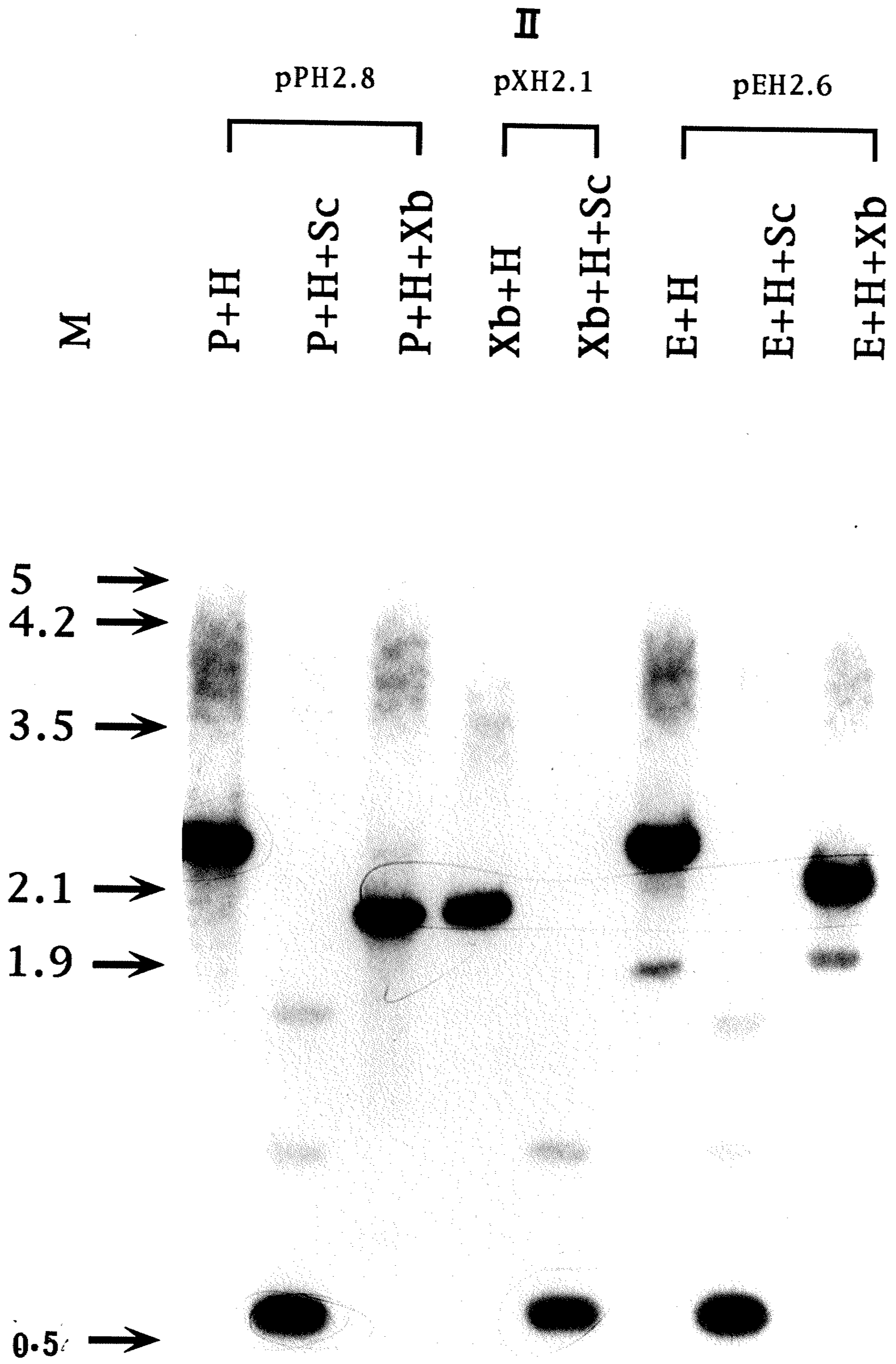
Computer aided analysis was performed on 1627 bp of rIGFBP-1 5' FL region for consensus sequences of insulin response elements (IREs), glucocorticoid response elements (GRE), AP-1, AP-2, and OCT binding sites as well as TATA and CAAT boxes (Table 4).

Figs 37-I and -II. Restriction Endonuclease Analysis of 5' Flanking Genomic Subclones of D-3

Plasmids containing subcloned fragments PH-2.8, XH-2.1, and EH-2.6 were subjected to restriction enzyme digestion as indicated at the top of each lane. The EtBr stain (I) and the Southern blot probed with PS400 (II) are shown. Restriction enzymes are abbreviated as before (Fig 18). Mol wt markers (M) are depicted in kb on the left margin. Electrophoretic and Southern hybridization conditions are as in MATERIALS AND METHODS.



M
P+H
P+H+Sc
P+H+Xb
Xb+H
Xb+H+Sc
E+H
E+H+Sc
E+H+Xb
2.1
1.7
1.5
0.8
0.7
0.5



II
pPH2.8
pXH2.1
pEH2.6
M
P+H
P+H+Sc
P+H+Xb
Xb+H
Xb+H+Sc
E+H
E+H+Sc
E+H+Xb
5
4.2
3.5
2.1
1.9
0·5

Fig 38. Restriction Enzyme Mapping of the Endogenous Rat IGFBP-1 Gene

Rat liver genomic DNA (50μg) was digested with appropriate restriction enzyme/s and analyzed as in MATERIALS AND METHODS. Southern blots were probed either with PS400 (I) or with CS15 (II). Restriction enzymes used, and abbreviated as before (Fig 18), are represented at the beginning of each lane.

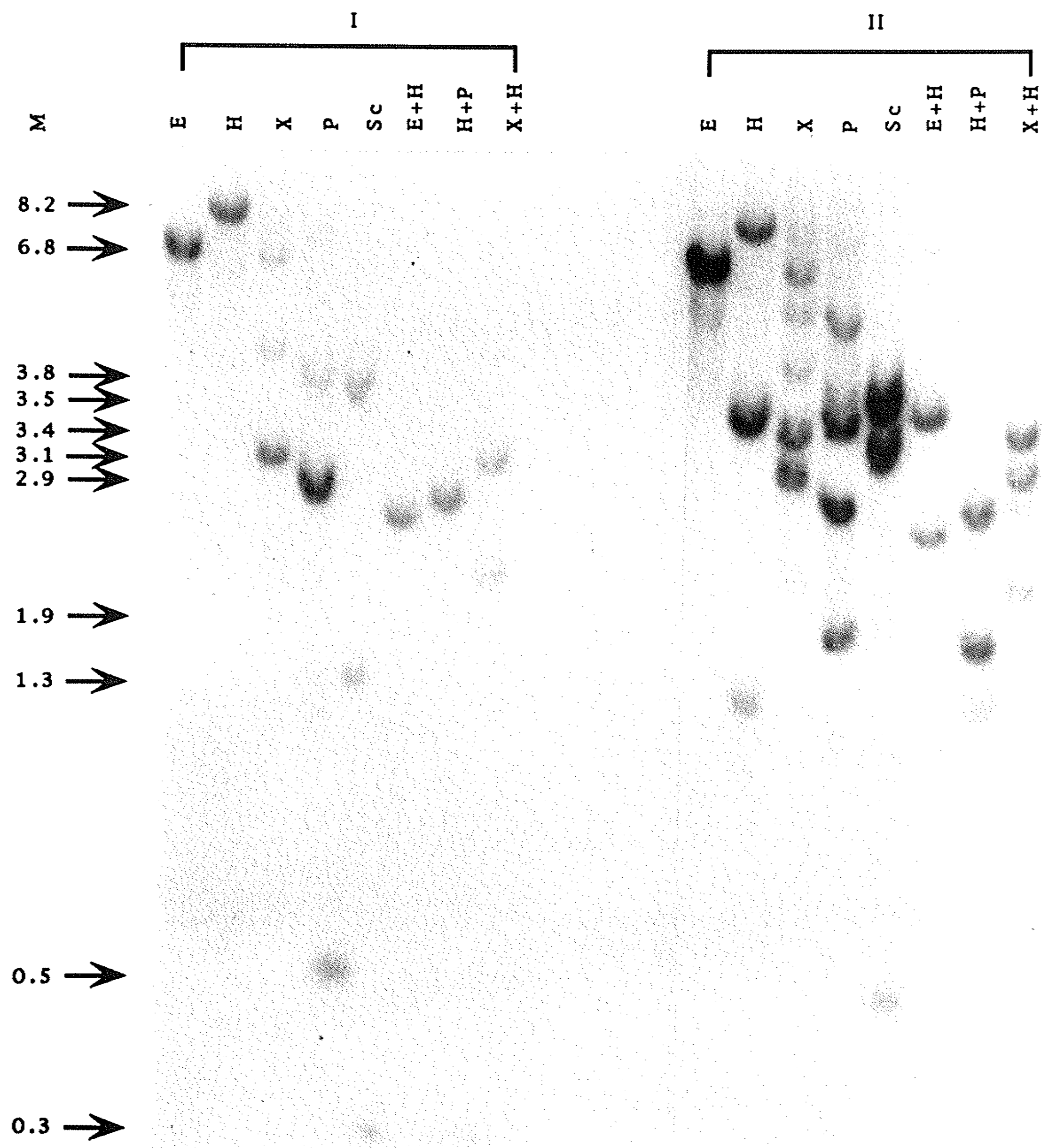
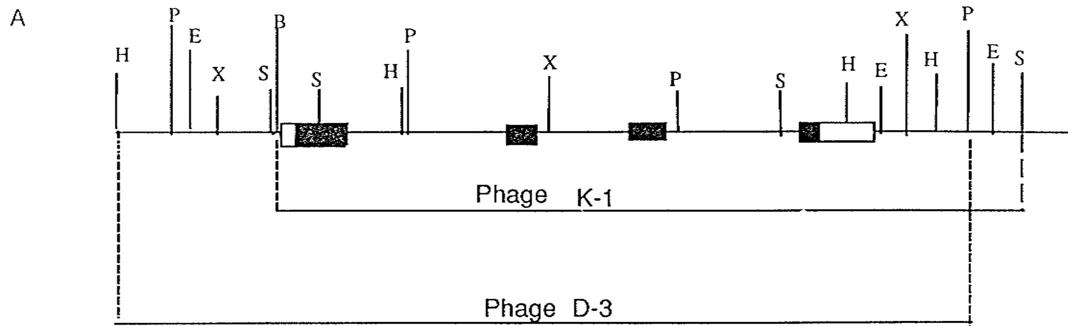
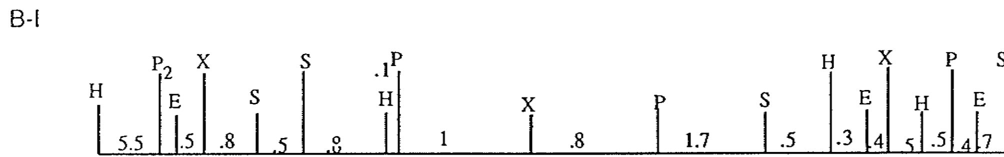

I
II
M
E
H
X
P
Sc
E+H
H+P
X+H
E
H
X
P
Sc
E+H
H+P
X+H
8.2
6.8
3.8
3.5
3.4
3.1
2.9
1.9
1.3
0.5
0.3

Fig 39. Alignment of Restriction Maps of Cloned Rat IGFBP-1 Gene with the Endogenous Rat Gene

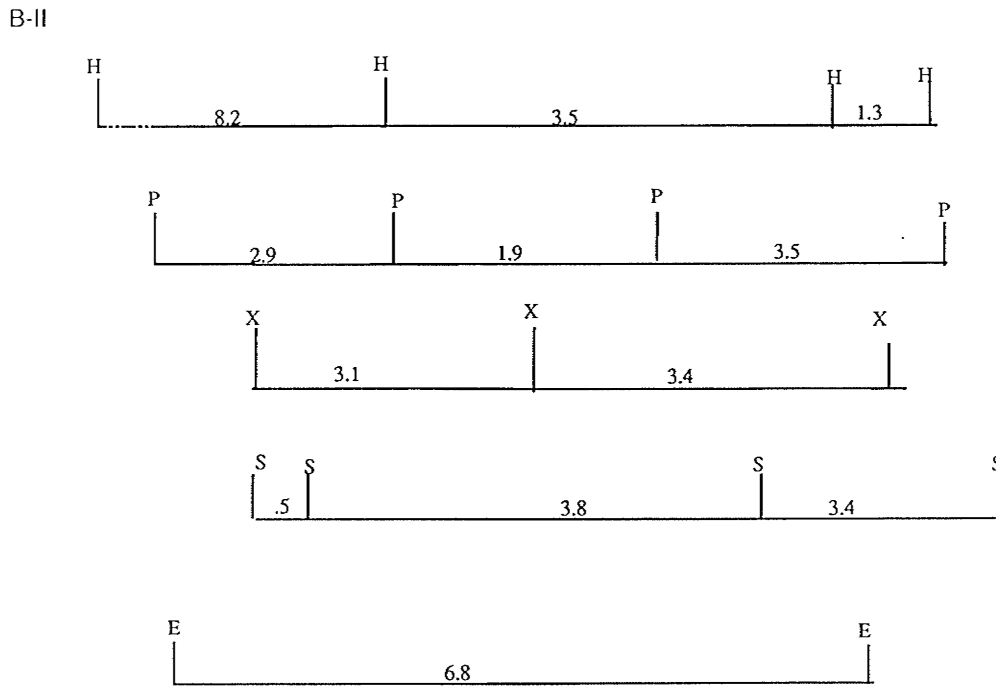
A: Structure of the cloned rat IGFBP-1 gene from recombinant phages K-1 and D-3. Exons are represented as boxes and the solid part is the translated region. Restriction endonuclease sites as abbreviated before (Fig 18) and the part of the gene present in each phage, is depicted. B-I: Restriction enzyme map of the endogenous rat gene. B-II: Restriction fragments produced by individual restriction enzymes.



A
H
P
E
X
S
B
S
H
P
X
P
S
H
E
X
H
P
E
S
Phage K-1
Phage D-3



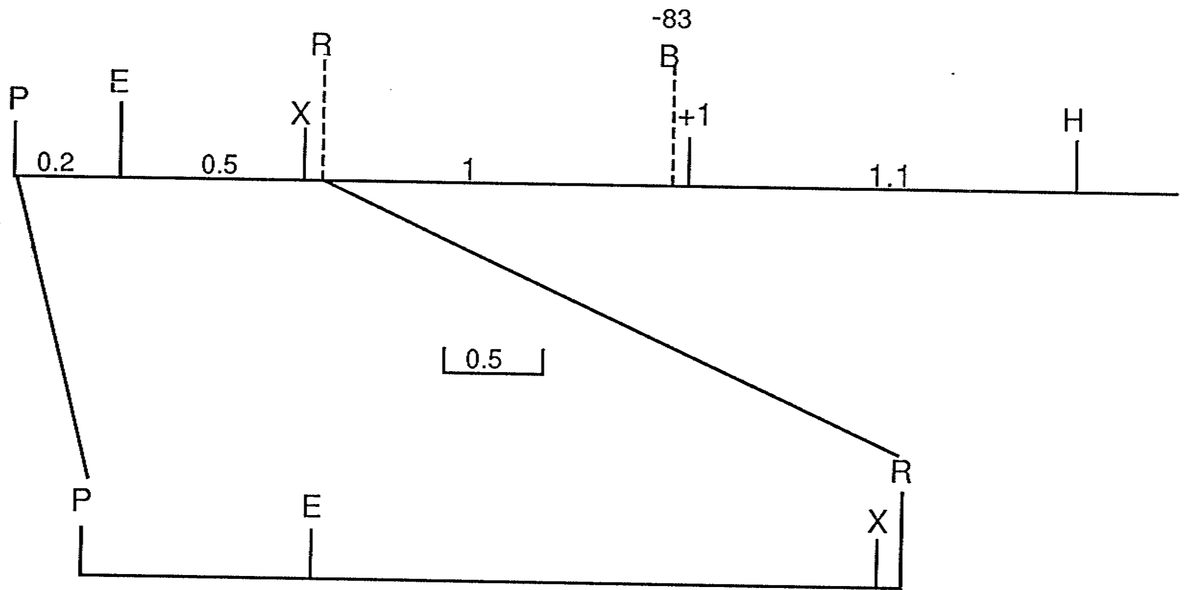
B-I
H
P2
E
X
S
S
.1P
H
X
P
S
H
E
X
H
P
E
S
5.5
.5
.8
.5
.8
1
.8
1.7
.5
.3
.4
.5
.5
.4
.7

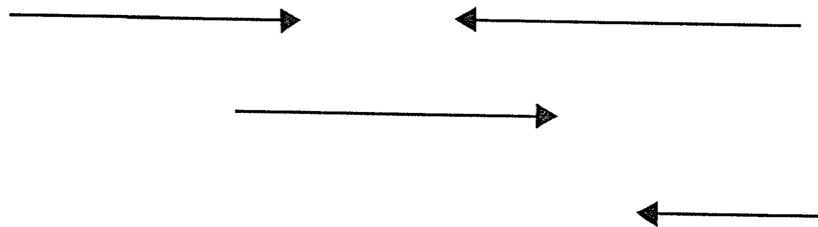


B-II
H
H
H
H
8.2
3.5
1.3
P
P
P
P
2.9
1.9
3.5
X
X
X
3.1
3.4
S
S
S
S
.5
3.8
3.4
E
E
6.8

Fig 40. Sequencing Strategy for the 5'Flanking Region from Pst1 (P) to Rsa1 (R) Restriction Sites

The 5'flanking region of rIGFBP-1 gene spanning from the Pst1 site at the 5' end to the HindIII (H) site in the intron 1 is shown. Restriction sites, EcoR1(E), Xba1(X), Rsa1(R), BamH1(B) are also represented. The estimated length between restriction sites are represented in kilobases. The B site (-83) located 83 nt 5' to the transcription start site (+1) is depicted. Below, the enlarged P/R fragment is shown; direction and length of the sequencing is represented by the direction and the length of the arrows respectively.

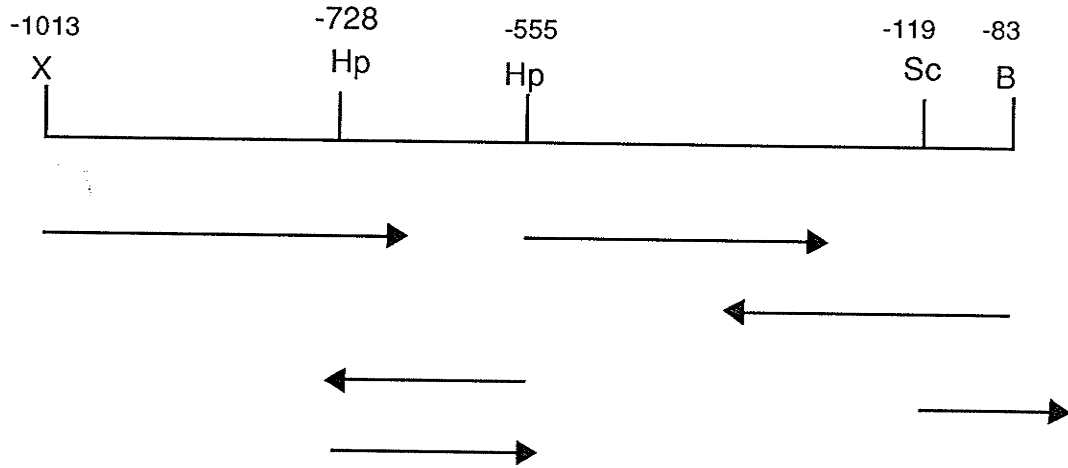




0.1kb

Fig 41A. Sequencing Strategy of the 5'Flanking Region from -1013 to -83 of the rIGFBP-1 Gene

The restriction site Hpa II (Hp) and Sac I (Sc) and others abbreviated as in Fig 40, are depicted. The number above each site represents the nt 5' to the transcription start site. Length of arrows depict the sequence length; direction of sequencing is represented by the direction of the arrows.



0.5

The *cis*-DNA elements that are responsible for the basal promoter activity such as TATA and CCAAT boxes were located in the rIGFBP-1 5' FL; the former (TATA box) was located in the -29 to -24 region while the latter (CAAT box) spanned from -62 to -58 of the rIGFBP-1 5' FL region (Fig 26).

Two octamer binding sites (abbreviated as OCT or OTF) were located beginning at -111 and the other at -1295 respectively in the 5'FL region of the gene (Fig 26). The consensus octanuleotide ATGCAAAT matched at 6 out of 8 positions with that of the first OTF site, while in the second OTF, this match (between consensus the OTF and the element) increased to 7nt out of 8 (Table 4). This element is conserved both in ubiquitously expressed genes such as histone H2B and tissue-specific expressed genes exemplified by the immunoglobulins. The former binds nuclear factor OTF-1 while the latter binds OTF-2 (243,244).

The CACCC consensus sequence (distal sequence) starts at position -136 relative to the transcription start site (Fig 26). These distal sequence elements are highly conserved among adult mammalian globin genes that are β-like (245).

There are three insulin response elements (IRE) belonging to two classes. The two elements begining at -294 and -369 respectively, belong to the type present in the glucagon gene. The sequence of the core consensus element is 5' G/C GCCTG 3' and was identical at 5 positions out of six in the first IRE (-294/-289) while in the second IRE (-369 to -364) all the six positions were conserved (Table 4). The core consensus of the 2nd type of IRE is the amylase/proximal PEPCK-2nd element type (246) and was situated at -111/-102 (Fig 26). This IRE was identical at 7 nt out of 10nt to the conserved

Fig 41B. Sequence Strategy for the Partial Rat IGFBP-1 Genomic Sequence

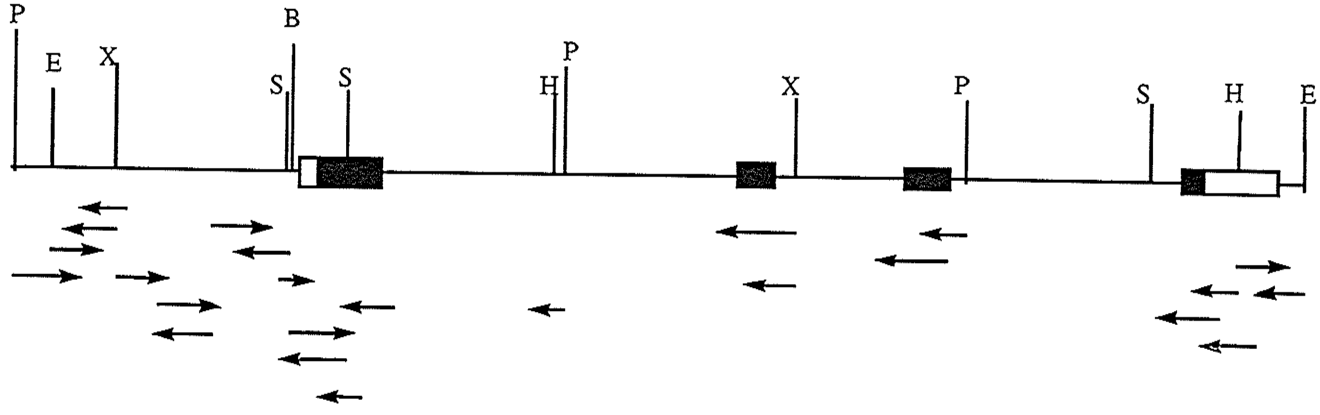

P
E
X
S
B
S
H
P
X
P
S
H
E

TABLE 4. Consensus Sequences of the Rat IGFBP-1 Gene

Amylase/proxymal PECK	
Consensus **IRE**	**T A G T C A A A C A**
rIGFBP-1 (-111 to -102)	a **A G** c a **A A A C A**
Glucagon type Consensus **IRE**	**G/C G C C T G**
rIGFBP-1(-294 to -289)	a **G C C T G**
(-369 to -364)	**G G C C T G**
Consensus **GRE**	**(T/G) G T A C A nnn T G T T C T**
rIGFBP-1(-353 to -339)	**G G T** c t **A ATC T G T T C T**
(-1078 to -1064)	**T G T** c **C A GGG T G T T C T**
Consensus **AP-1**	**T G A G/C T C/A A**
rIGFBP-1 (-1101 to -1095)	**T G** g **G T C A**
Consensus **AP-2**	**C C C A/C N G/C G/C G/C**
rIGFBP-1(-377 to -369)	**C C C** t **T**t **G G G**
(-532 to -525)	t **C C** t **G G G G**
(-730 to -723)	**C** t **C C G G G** a
(-791 to -784)	**C** a **C C T G G G**
Consensus **OCT binding site**	**A T G C A A A T**
rIGFBP-1(-111 to -107)	**A** a **G C A A A** a
(-1295 to -1288)	**A** c **G C A A A T**

consensus sequence (Table 4). In this respect the rat gene differed from the human counterpart because the IRE present in the 5' flanking of the human gene was similar to that of phosphoenolpyruvate carboxykinase (PEPCK) distal element type (125). The core consensus sequence is different from that of glucogon gene (246). However, similar regulation results in that both types of IREs (glucagon and PEPCK) are negative elements. The gene expression is down-regulated in response to insulin.

There are two glucocorticoid response elements (GREs) present in the 5'FL region of rIGFBP-1 (Table 4). The first is located at -353/-339 and the second at -1078/-1064 (Fig 26). According to Imai et al (247), the 15nt core consensus is (T/G)GTACANNNTGTTCT. The first GRE of the rIGFBP-1 is identical to this consensus at 13 out 15nt positions (13/15), while in the more upstream element (-1078/-1064) the identity increased to 14/15 (Table 4).

There are four AP2 sites in the 1627 bp 5' FL of the rIGFBP-1 gene. The element locations going from 3' to 5' are: i) -377/-369, ii) -532/-525, iii) -730/-723, and (iv) -791 to -784. These AP2 sites are implicated in TPA, cAMP and forskolin induced gene expression; the core consensus is 5' CCC A/C N G/C G/C G/C 3' (248). All but the fourth AP2 sites are identical at 6/8 positions whereas at the fourth site, this was increased to 7/8 (Table 4).

The AP1 site with a consensus sequence of 5' TGA G/C T C/A A 3', has been suggested to participate in TPA and cAMP mediated gene expression (248). This site binds homodimers of JUN or heterodimers of JUN and FOS or JUN and ATF family proteins (248). Such a site (AP1) was located at -1101/-1095 of rat IGFBP-1/5'FL region

where the identity or the match was 6/7 positions (Table 4).

3.2.2.IV. Growth Hormone Response Element

The rIGFBP-1 is transcriptionally down-regulated by GH. In GH reduced conditions such as hypophysectomy, the gene transcription is higher than in normal rats and when treated with GH, within 30 min, transcription is significantly reduced as we have reported before (99). Also the stable mRNA abundance in the liver decreased in a similar fashion as seen in Sec 2.2.

This observation led to the hypothesis of a negative growth hormone response element (HRE) in the 5' FL region of the rIGFBP-1 gene. The objective of the following experiments was to test the hypothesis that the transcriptional down-regulation of hepatic IGFBP-1 by GH is mediated by DNA-nuclear factor interactions in the 5'FL of the gene and, if so, to delineate the DNA element involved. The experimental strategy employed to delineate such elements, was mobility-shift assays (see MATERIALS AND METHODS for details). Fragments containing the 5' FL region from -83 to -728 were subcloned and used as probes; the lengths ranged from 91 bp to 196 bp (Fig 42). Each of these DNA probes was labeled with ^{32}P either at the 5' end or at the 3' end (refer MATERIALS AND METHODS for details).

Three groups of rats were employed in the study: i) sham operated ii) hypox , and iii) hypox rats treated chronically for 8 days with human growth hormone. This protocol was identical to the treatment given in a previous study where it was observed that the

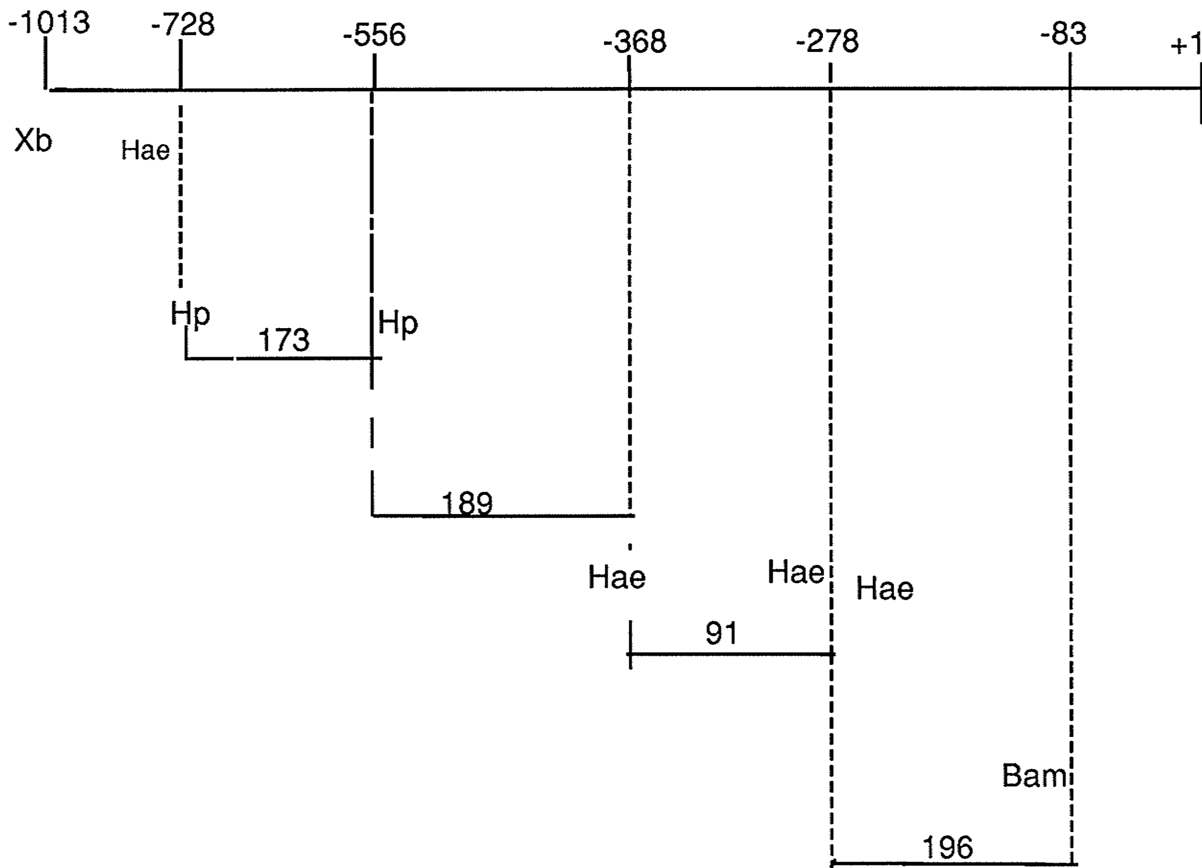
hepatic mRNA abundance of IGFBP-1 was inversely regulated by hGH (see section 2.2). After the treatment rats were sacrificed and hepatic nuclear extracts were prepared as detailed in the MATERIALS AND METHODS section.

Mobility-shift assays were performed with the above pooled nuclear extracts (from four individual animals per pooled sample). The first three DNA probes, Hp 173 (173 nt -728/-556 Hpa11 fragment), Hp/Hae 189 (189 nt -556/-368 HpaII/HaeIII fragment), and Ha 91 (91 nt -368/-278 HaeIII fragment) demonstrated protein-DNA interactions as a retarded band or bands with hypox rat nuclear extracts when compared to sham controls. But GH treatment did not effect the interactions qualitatively or quantitatively (Figs 43-I and -II). Whereas interactions with the most proximal probe Hae/Bam 196 (196nt -278/-83 Hae111/BamH1 fragment), and hypox nuclear extracts were different to that of the sham control, and upon GH treatment of hypox rats, the interactions resembled the sham control profile (Fig 44). When the incubations were carried-out in the presence of 50 molar excess unlabeled probe, the mobility-shifted bands disappeared suggesting the specificity of these interactions (Fig 44).

As this (mobility-shift experiments) represents a first attempt to identify DNA/protein interactions in the 5'-end of the IGFBP-1 gene, the functional significance of these interactions is not known at this point of time.

Fig 42. 5 'Flanking DNA Probes Generated for Mobility Shift Assays

The transcription start site depicted as +1. Length of each probe is given in nt as well as starting and the end points in the 5'FL region depicted. Restriction enzyme sites HpaII (Hp), HaeIII (Hae), and BamH1 (Bam), that were used to generate the probes are represented.



-1013
-728
-556
-368
-278
-83
+1
Xb
Hae
Hp
173
Hp
189
Hae
Hae
Hae
91
Bam
196

Fig 43-I and -II. Mobility Shift Assays with Probes - 728/-556(Hp-173), -556/-368(Hp/Hae-189) and -368/-278(Hae/Hae-91)

Probes were generated as shown in Fig 42 and the experiments performed as described in MATERIALS AND METHODS. Briefly, groups of rats (4-5 rats/group), first sham operated (S), second a hypox (H) and third hypox rats treated chronically with hGH for 8 days (G) were sacrificed and nuclear extracts prepared from individual rats and then pooled. The nuclear extracts were equilibrated at RT with the relevant ^{32}P-end labeled probe (shown over the bracket) and analyzed on 5% native polyacrylamide gels, dried and autoradiographed. Mobility shifts with probes -556/-368, -368/-278 (I) and -728/-556 (II) are shown. P;probe only-without any nuclear extracts, E; 50 molar excess unlabeled DNA probe.

I

-556 to -368 -368 to -278

P S H G P S H G

-728 to -556

P S H G E

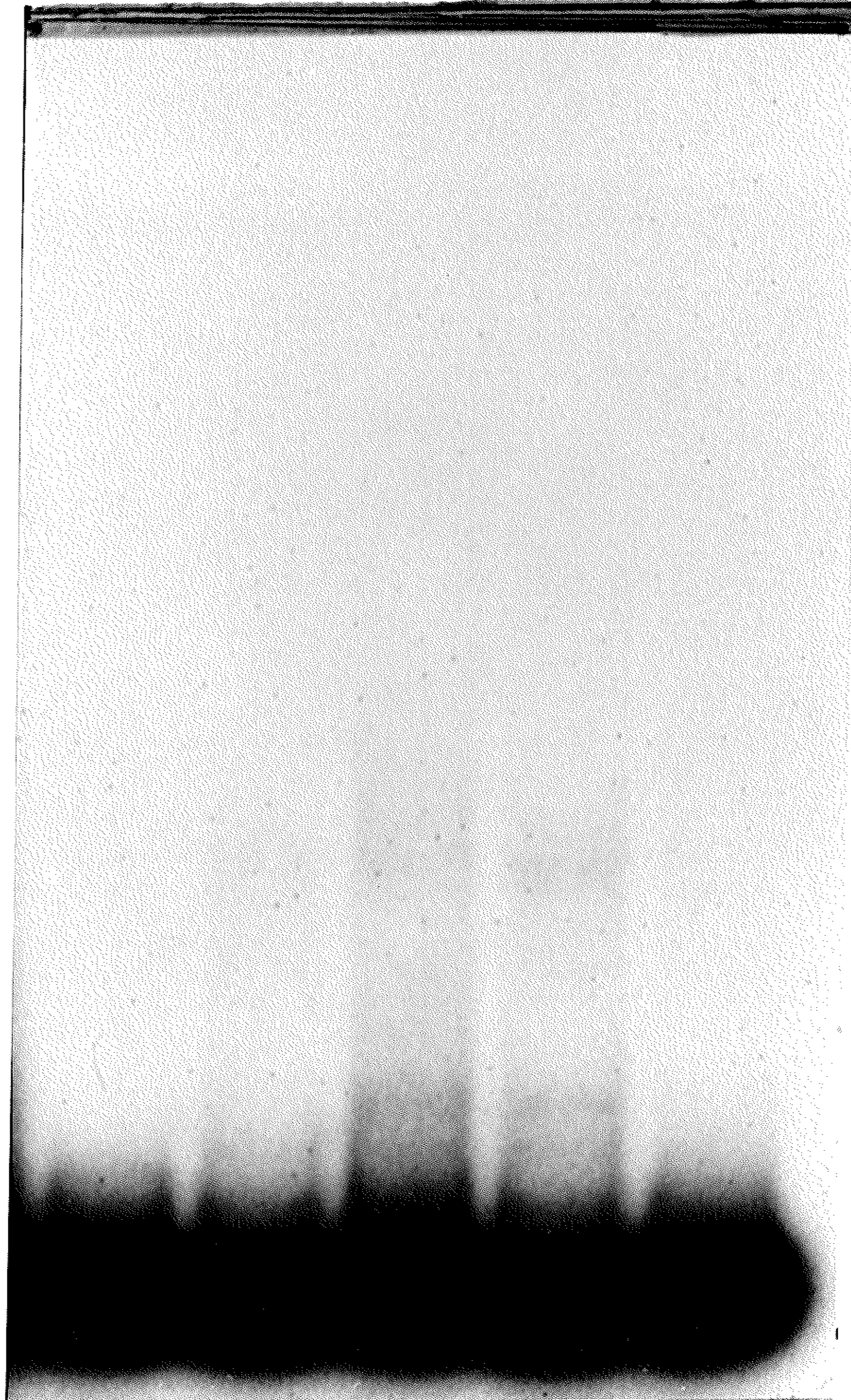
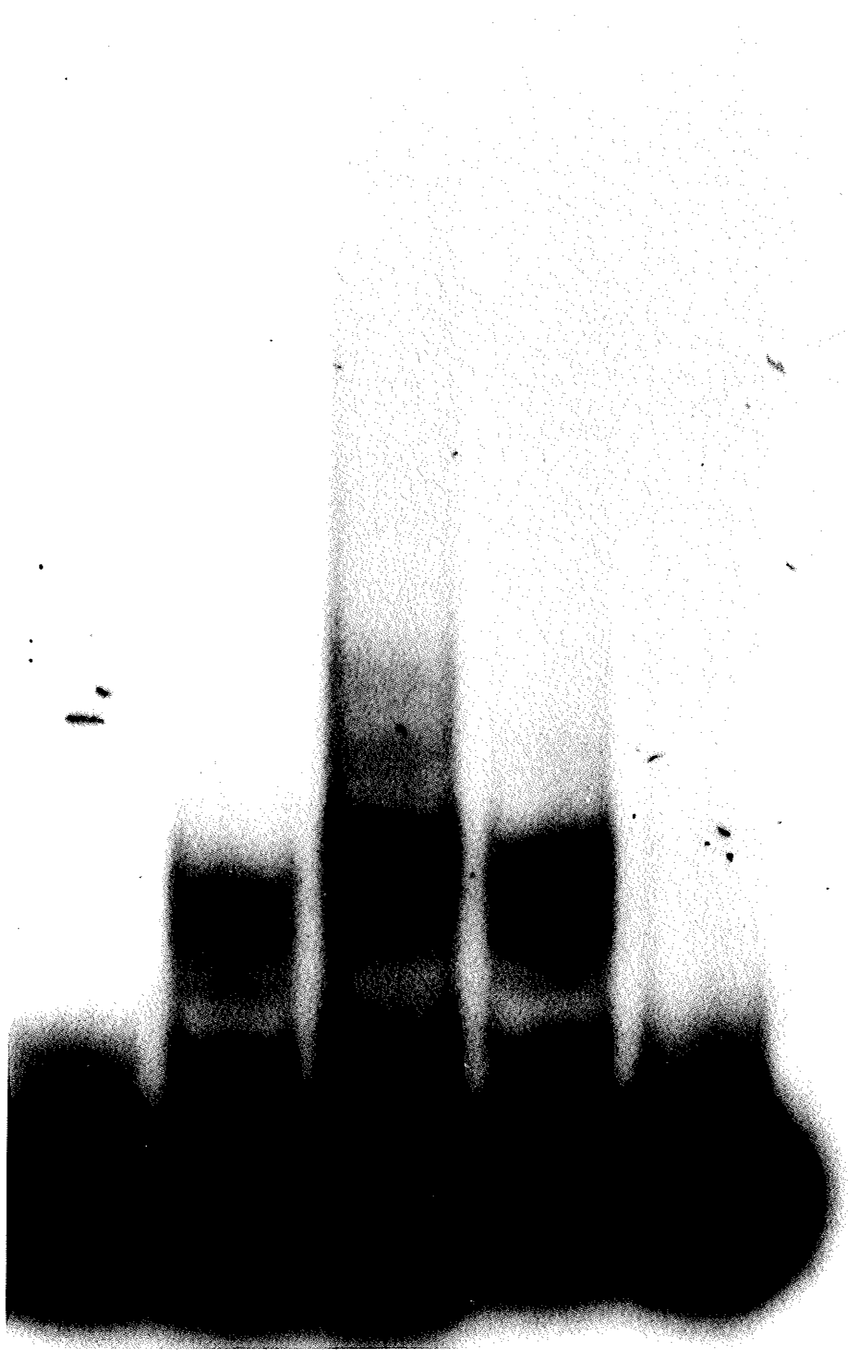


Fig 44. Mobility Shift Assays

Same as the previous figure (Fig 43) except the probe used is -278/-83 (196nt-HaeIII/BamH1); 50molar excess of unlabeled probe (E) was included in the reaction mixture of the last lane in addition to the contents in H lane. This is to ascertain the specificity of the retarded bands.

-278 to -83

P S H G E



mutants was observed. When Cys-226 was changed to Tyr, loss of IGF binding capacity was observed (68).

The predicted amino acid sequence of the rat IGFBP-1 was longer by 13 residues than its human counterpart (98); no classical N-linked glycosylation signals were found in the rat IGFBP-1(Asn-X-Ser or Asn-Ser-Thr). There are two potential regions in the polypeptide that can be O-glycosylated; residues 96-119 and 164-176 (69). Frost and Tseng in 1991 (78) showed that human IGFBP-1 secreted from cells decidualised in vitro, secreted IGFBPs that were both phosphorylated as well as non-phosphorylated. Such forms are also present in vivo and occur (phosphorylation) exclusively on serine. Another group (257) demonstrated that a human hepatoma cell line Hep G2 secreted both phosphorylated and non-phosphorylated forms and the former had 6-fold higher affinity compared to the latter (71). This may be one of the mechanisms whereby IGFBP affinity to IGFs is regulated.

Other IGFBPs 2 through 6 are glycosylated to varying degrees (71). The Arg-Gly-Asp (RGD) tripeptide sequence is found in many matrix proteins including elastin, fibronectin, and often serves as a recognition sequence for the cell surface intergrin receptor (89, 166). Both of human and rat IGFBP-1 and -2 contain RGD sequences in their COOH -terminal ends.

Regulation of IGFBP-1 in the Rat

It is clear that IGFBP-1 regulation as indicated from mRNA abundance studies,

is multifactorial. From this and other studies it is becoming increasingly apparent that the regulation of IGFBP-1 is carried out by hormonal factors including insulin, GH, IGFs, estrogen, and nutritional status- such as starving and protein restriction. Since these factors are intertwined, deciphering any one causative factor is difficult (91,92). For example our finding that 24 h fasting increased the IGFBP-1 expression by 10-fold (mRNA) and the dramatic decrease by 1hr of refeeding (inverse regulation) may be occuring via GH, IGFs, insulin, or may be a direct effect.

When an animal is deprived nutrition, the maintenance of the basal metabolism for survival takes priority over growth. Nutritional deprivation results in many effects including decreased levels of insulin, IGF-1, glucose in circulation both in humans and rodents while the GH levels decrease only in rodents and not in humans (95). Therefore the factor increasing hepatic IGFBP-1 expression could be any one of the above factors or a combination of them. Recently, Strauss and Takemoto (79) demonstrated that growth hormone receptor (GHR) content also decreased in starved rat liver membranes. This (GHR down regulation) may play a role in food-deprived increases in IGFBP-1 gene expression.

There is also evidence to suggest the importance of IGFBPs in glucose counter regulation. Insulin, the primary regulator of blood glucose, is present in circulation in very low amounts compared to that of IGFs (0.2 ng insulin vs 100-600 ng IGFs in the rat). If all the IGFs are present in a "free" state, it will result in severe hypoglycemia. This is because IGFs can act like insulin in stimulating the glucose transporter in many tissues and cells (97). Therefore, by complexing to IGFs and sequestering them under food

deprived conditions, IGFBPs can decrease "free" IGFs. This will lower insulin-like activity and thus contribute to glucose homeostasis. Because of this role-glucose counter regulation, IGFBPs may have clinical implications in treating pathological states such as diabetes, tumor derived hypoglycemia, etc.

There is a growing body of evidence to suggest that the IGFBPs modulate the actions of IGFs (reviews 1,71,83 and references therein). IGFs affect both growth and differentiation via endocrine, paracrine and/or autocrine mechanisms. Under growth retarding conditions, such as fasting basic metabolism must be maintained at the expense of growth and it has been shown that IGF-1 and -II levels decrease in the circulation (112-113). Also the availability of IGFs already in circulation is decreased by an increase in the level of high affinity low mol weight binding proteins such as IGFBP-1 which contribute to the sequestering of IGFs, hence, optimizing blockade of the growth mediated by the IGFs.

There is evidence suggesting the presence of two types of IGF binding complexes in the circulation : (i) A higher molecular weight form of 150-200 kD that contains IGFBP-3 and is the predominant form in the adult circulation (102) and (ii) concentration correlates positively with the growth status. The evidence comes from pathological states such as hypopituitarism where GH levels are decreased when compared to the normal situation and correlatively the higher molecular weight complex concentration decreases while that of IGFBP-1 containing smaller molecular weight complex increases (114). Conversely, in acromegaly which is a hyperpituitary condition, the reverse is true. This notion is further reinforced because in physiological growth spurts of puberty, again such

up-regulation of the larger molecular weight complex and the concurrent down-regulation of the smaller molecular weight complex are observed (115).

The larger complex consists of glycosylated IGFBP-3, an 85 kD acid labile sub-unit (ALP), and IGF-1 or -II (83). Whereas the smaller complex (40 kD), is composed of IGFBP-1, -2, or -6 and contains most of the unsaturated IGF binding sites in the serum (71).

In the adult human and the rodent 95% of the IGF in circulation is bound to IGFBP-3 and this binding protein has serum concentration of 5 ug/ml; IGFBP-2, 150 ng/ml and IGFBP-1 is the lowest with 50 ng/ml. It is known that the IGFBPs with or without binding to IGFs, cross the vasculature; insulin has been shown to stimulate the transport of IGFBP-1 but not IGFBP-2, across intact capillaries which may be one of the mechanisms whereby insulin lowers the IGFBP-1 concentration in circulation (88).

Insulin is also decreased in the blood following an overnight fast. This observation formed the rationale to examine insulin as a potential regulator of IGFBP-1 expression. As seen in the RESULTS Section 2.3, insulin was unable to affect the elevated IGFBP-1 mRNA levels after a 24h fast. In strvation many co-factors required for the insulin action may be absent , rendering the action of insulin bio-inactive.

It has been shown before that under caloric restricted conditions, that IGFs become bio-inactive (188). This was the case with GH regulation of IGFBP-2 (96); under caloric restrictions, GH regulation of IGFBP-2 was inhibited and postulated to be due to the bioinactivation of GH in the state of caloric restriction. But under various other conditions, investigators have observed the inverse regulation of IGFBP-1 by insulin

including in type I diabetes where lower insulin levels than normal are present, and the increased levels of hepatic IGFBP-1 present (in type I diabetics) compared to that of normal while insulin administration lowered the levels. In human hepatic slices, insulin again down-regulated the dexamethasone induced expression of IGFBP-1 (123). Similar results were observed in both human (122) and rat hepatoma cell lines (151,194). Recently Powell's group delineated a negative insulin response element in the human IGFBP-1 gene 5' FL (-529/-103) region (125). Our collaborators down in Atlanta, Ga. have delineated even further one of the rat IGFBP-1 insulin response silencer elements to -119/-83 region of the 5' FL of the gene relative to the transcription start site. This is to be discussed further later in the DISCUSSION.

Growth hormone regulation

The working hypothesis of this thesis work was the inverse regulation of IGFBP-1 by GH; mRNA levels increased when rats were hypophysectomized and upon reconstitution of GH via ip injections, decrement of the IGFBP-1 message was observed (RESULTS Sec 2.2). The nuclear-run off assays performed by J L Luo when in our laboratory, proved that the major contributor to increased mRNA abundance was the elevated transcription (99). Therefore two questions were raised: 1. What is the mechanism by which GH regulates IGFBP-1 gene expression? and 2. Is the rat a good animal model to get a handle on the human situation? In order to answer these questions, cloning of the gene (including the 5'FL region) was undertaken.

The salient features of the structure of the rat gene (IGFBP-1) are remarkably similar to that of the human (Table 3). Such evolutionary conservation generally suggests the functional importance of the gene. This evolutionary conservation also extends to other members of the family that has been cloned so far; human IGFBP-1 (170), rat IGFBP-1 (reported here), human and rat IGFBP-2 (126,164 respectively), human IGFBP-3 (107), and IGFBPs 4-6, where only the cDNAs have been cloned (127-129).

The exon/intron structure of IGFBP genes cloned so far from different mammalian species including the rat cloned here points out to the significant conservation of the genomic structure among these genes.

The IGFBP-1 gene of the rat cloned and sequenced for the first time and reported here proves further the conservation of IGFBP gene structure across species. This probably suggests a common founding primordial gene for the members of the IGFBP family of genes.

Exons

Both human and rodent IGFBP -1, and -2 genes consist of four exons while the human IGFBP-3 has an extra 5th exon. Exon 1 is homologous in all three genes (IGFBP-1,-2, and -3) and out of the 18 Cys residues that are conserved in the binding proteins, 12 are encoded by exon 1. Exon 2 is the most variable suggesting its importance in dictating functional specificity to the binding proteins.

Similar to exon 1, 3rd exons of human and rodent IGFBPs are remarkably similar in size and encoding protein motifs. Rat and human IGFBP-1 have 129bp exons while

that of IGFBP-2 is 141bp in both rat and human. IGFBP-3 has the smallest third exon with 120bp.

Size of the exon 4 varies from the smallest at 141bp of human IGFBP-3 to the largest, 706bp of the human IGFBP-1. Others fall between with 174bp of rat IGFBP-2 and 496bp for the human IGFBP-2 while that of the rat IGFBP-1 which is cloned here is slightly smaller than its human counterpart (706- human vs 646-bp) as shown in Table 3.

In the rat gene, 131nt are translated thus giving-rise to a 515nt 3' UT region which was comparable in length to that of the human gene where the 4th exon is 129nt and the 3'UT region is 579nt long (TABLE 3). In IGFBP-2, 163nt were translated resulting in a 311-3'UT region. Human IGFBP-3, the translatable region of the exon 4 is 141nt long and the rest of the exon encodes a 15nt 3'UT region.

Exon 5 of the human IGFBP-3 which is 1482nt is all 3'UT hence the total IGFBP-3 3' UT region is 1497nt in length. The function of this exon is unknown. It might be important in conferring stability to hnRNA of IGFBP-3.

Introns

All of intron/exon splice junctions conform to the classical GT/AG rule. Furthermore, the deviations from the consensus splice sequences for the vertebrates, are also conserved between the rat and human IGFBP-1 genes (TABLE 3). These sorts of conservation also extends to other IGFBP genes.

The intron-1 in the human IGFBP-3 is reported to be 3.285kb (107) while that of

human IGFBP-2 is 27kb or more (126); intron-1 of IGFBP-1 in the human and the rat was similar in lengths (1.2kb rat vs 1.6kb human). Also the type and phase of intron-1 is conserved across binding proteins 1, 2 and 3 .

In the case of intron-2 again the phase which is 0 and conserved in the IGFBP genes including rat IGFBP-1 reported here. Intron lengths are also similar in rat IGFBP-1 and -2 (IGFBP-1 0.8kb vs 0.7kb for IGFBP-2) whereas in the human, intron-I is 1.24kb and 1.1kb in the IGFBP-2 (164,170,126) and the IGFBP-3 had the smallest intron-1 with 0.544kb (107).

Intron-3 of rat IGFBPs 1 (TABLE 3), and 2 (164) are 1.4kb and 1.9kb respectively whereas the human IGFBP-3 intron number 3 is 1.595kb in length (107). Intronic phase in all the above was 0. Only the IGFBP-3 had the additional 4th intron with a 0.94kb in length and phase 0 in type (107).

Because of the above features of the IGFBP gene family, some investigators have proposed a divergent model of evolution for these genes. That is the IGFBPs started as a single primordial gene and due to evolutionary pressures evolved to a current family with six or more members (126).

5' Flanking Sequences and Tissue Specific Expression

As seen before (RESULTS Sec 3.2.2.III), the rat IGFBP-1 gene 5' flanking region possessed many potential *cis*-acting DNA elements. These (response elements) are purely empirical and need to be functionally demonstrated in order be of any significance.

The negative insulin response elements (IRE/IRS) have been reported for the hIGFBP-1 gene and their functional existence has been demonstrated (125). But insulin was unable to depress the 24h starvation induced increase in the IGFBP-1 expression. This is probably suggestive of the critical role that nutrition plays in the insulin response. This is not the first time this phenomenon has been observed. When the gene expression of IGFs were examined, similar nutritional status dependence was observed. There is a large body of evidence suggesting the positive correlation between GH levels and IGF expression. Upon fasting bioactive and immuno-reactive IGFs declined and the administration of exogenous GH to these fasting animals did not restore the somatomedin (IGF) levels suggesting some sort of GH resistance (188) akin to the insulin resistance that was observed for the rIGFBP-1 expression. But in other experimental conditions, insulin has been observed to down-regulate both human and rat IGFBP-1 gene expression. Using the rat cDNA that was cloned here (CS15) and provided, Unterman's group demonstrated that in a rat hepatoma cell line, H4-II-E, insulin down regulated the dexamethasone-induced expression of IGFBP-1 (130). This was also the case with rat and human liver organ cultures (123). In the human liver cell line Hep G2, the same insulin counter regulation was demonstrated (122).

There is also in vitro evidence coming from pathological states such as diabetes for the inverse insulin regulation of IGFBP-1 expression. Both in rats and humans in insulin dependent diabetes (IDDM/type I diabetes), the expression of the IGFBP-1 gene increased and upon insulin treatment, this trend was reversed suggesting inverse in vivo insulin regulation (119-121).

Against this background we along with our collaborators in Atlanta, Ga. USA, set-out to define the IRE in the 1627nt of the 5' FL region of the gene I cloned-rIGFBP-1. To this end I made a CAT construct where the 1627nt 5'FL region was put in front of a CAT gene and this was subsequently dispatched to them (Fig 45). Deletion analysis of this construct led to the definition of an IRE spanning from -83 to -119 which is a 37bp IRE in this gene and these data were presented at the 1993 Annual Endocrine Society meeting held in Las Vegas USA (104).

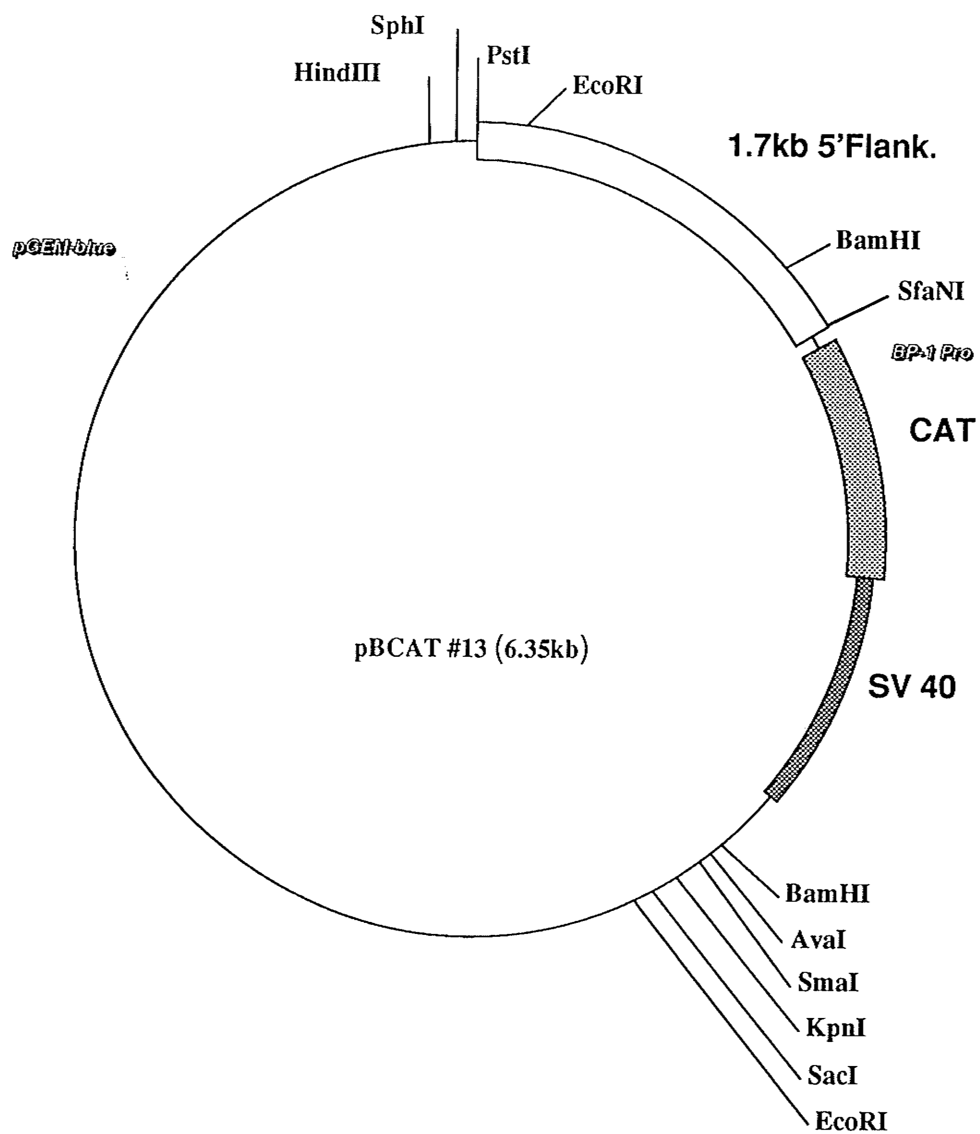
When this 37bp region was examined with the aid of a computer, it suggested the presence of a proximal PEPCK type IRE, as seen in Table 4 which was different to that found in the human gene. This is the first time that an IRE of any mammalian IGFBP has been so completely mapped. In the human IGFBP-1, the IRE was mapped only to a -529/-103 region (125) and was an IRE of distal PEPCK type (in the human) as opposed to the proximal PEPCK type observed in the rat gene cloned here.

Concurrently two other groups have reported the characterization of elements of the rat IGFBP-1 5'FL region; Unterman's group reported that a 1100bp 5' flanking region that mediated the insulin dependent down-regulation of the gene expression (131) whereas Suh and Ooi were more specific in that they managed to delineate the IRE to -328/+1 region of the gene (132). Both the above groups transfected the CAT constructs to the H-4-II-E rat hepatoma cell line. In contrast, our collaborators in Atlanta, Ga. did so in primary rat hepatocytes (104). One can argue that the latter is a more physiologically relevant model.

My working hypothesis based on the regulation of hypox animal studies was that

Fig 45. Chloramphenicol acetyltransferase (CAT) construct containing a rIGFBP-1 5'flanking region

The 5' flanking region of the rat IGFBP-1 gene spanning from -1627 (Pst1) to +167 (SfaN1) represented by an open box. CAT gene is depicted as a box with solid dots. The box with open dots marked as SV40 contains the SV40 early region poly (A)+ and transcription termination signals. Rest of the construct, clockwise from BamH1 to Pst1 is the pGEM-blue plasmid vector DNA.


SphI
HindIII
PstI
EcoRI
1.7kb 5'Flank.
pGEM-blue
BamHI
SfaNI
BP-1 Pro
CAT
pBCAT #13 (6.35kb)
SV 40
BamHI
AvaI
SmaI
KpnI
SacI
EcoRI

GH has a negative effect on IGFBP-1 gene expression. Initially with the aid of a computer I scanned the whole of the 5' flanking region that was cloned for possible "growth hormone response" elements. But such manipulations are of limited value since only very few such elements have been characterized so far. One of them ("growth hormone response" element) has been the in the rat serine protease inhibitor 2.1 gene (133) which is a 45bp element. Comparing our gene with it did not yield significant similarity.

The growth hormone target genes identified so far are: IGF-I (93), epidermal growth factor receptor gene (140), alpha $_{2u}$ globulin (139), steroid 5-α reductase and liver cytochrome 450 enzyme encoding genes (138). Since very few "growth hormone response" elements have been characterized the value of these empirical comparisons are really limited. Even the IGF-1 promoter which is a good place to start , has not been characterized in details for such elements. Thus the amount of work that remains to be done in this area is enormous.

When the 5' flanking region of the human IGFBP-1 gene from -1205 to +1 reported by Powell et al (125) when maximally aligned with the corresponding region of the rat gene, highlighted many interesting features. A 102bp region spanning from -234 to -133 of the human gene exhibited 65% sequence identity with a 104bp 5'FL region (-219/-116) of the rat IGFBP-1 gene. It is of interest that many important *cis*-elements have been mapped to the vicinity of this region including the basal promoter region of the human IGFBP-1 that contains the DNA element which interacts with the liver specific transcription factor LB-F1 and the IRE (125). Also our collaborators in Atlanta

demonstrated the presence of the basal promoter in the -83/+1 region of the rat IGFBP-1 (Dr David Robertson, personal communications). Furthermore, Powell's group (125) located the IRE to this region (-103/-529) which encompasses this conserved region and the IRE in the rat IGFBP-1 gene also falls adjacent to this region (-119/-83). From the mobility shift assays the 5' flanking region that exhibited a hint of growth hormone response also localizes to this vicinity (-278/-83) as shown before (Sec 3.2.2.IV). Thus the remarkable phylogenetical conservation of this region may indicate the possible importance in the regulation of IGFBP-1 gene expression.

Further analysis of this region using chloroamphenicol acetyl transferase (CAT) assays and DNA footprinting techniques would be of interest. The postulated "growth hormone response" element would not only be a first but also contribute significantly to the understanding of the molecular mechanisms in terms of DNA-protein interaction governing IGFBP-1 expression at the transcriptional level.

I have summarized the findings of this thesis with what is already known and unknown in the form of a model shown in Fig 46. Growth enhancing conditions including increased circulating-GH, -insulin, and fed state, result in the shifting of the equlibrium towards the 150-200kD "larger complex" which in turn results in the elevation of the circulating levels compared to that of the "smaller complex". This "larger complex", with its IGFBP-3, has lower affinity for IGFs than the "smaller complex" which has IGFBP-1 as one of the binding proteins (Fig 46). Therefore higher "free" IGF could now interact with its receptor and increase the gene expression of its target genes which include genes that are responsible for growth and differentiation as revised in the INTRODUCTION.

Increased levels of GH in the circulation, through the interaction of its receptor that would trigger off a cascade of biochemical signals culminating in the interaction of nuclear factor/s with the regulatory sequences in the postulated "growth hormone response" element that may reside in the -83/-278 region of the IGFBP-1 5'FL region. According to the results discussed here this interaction will turn-down gene expression.

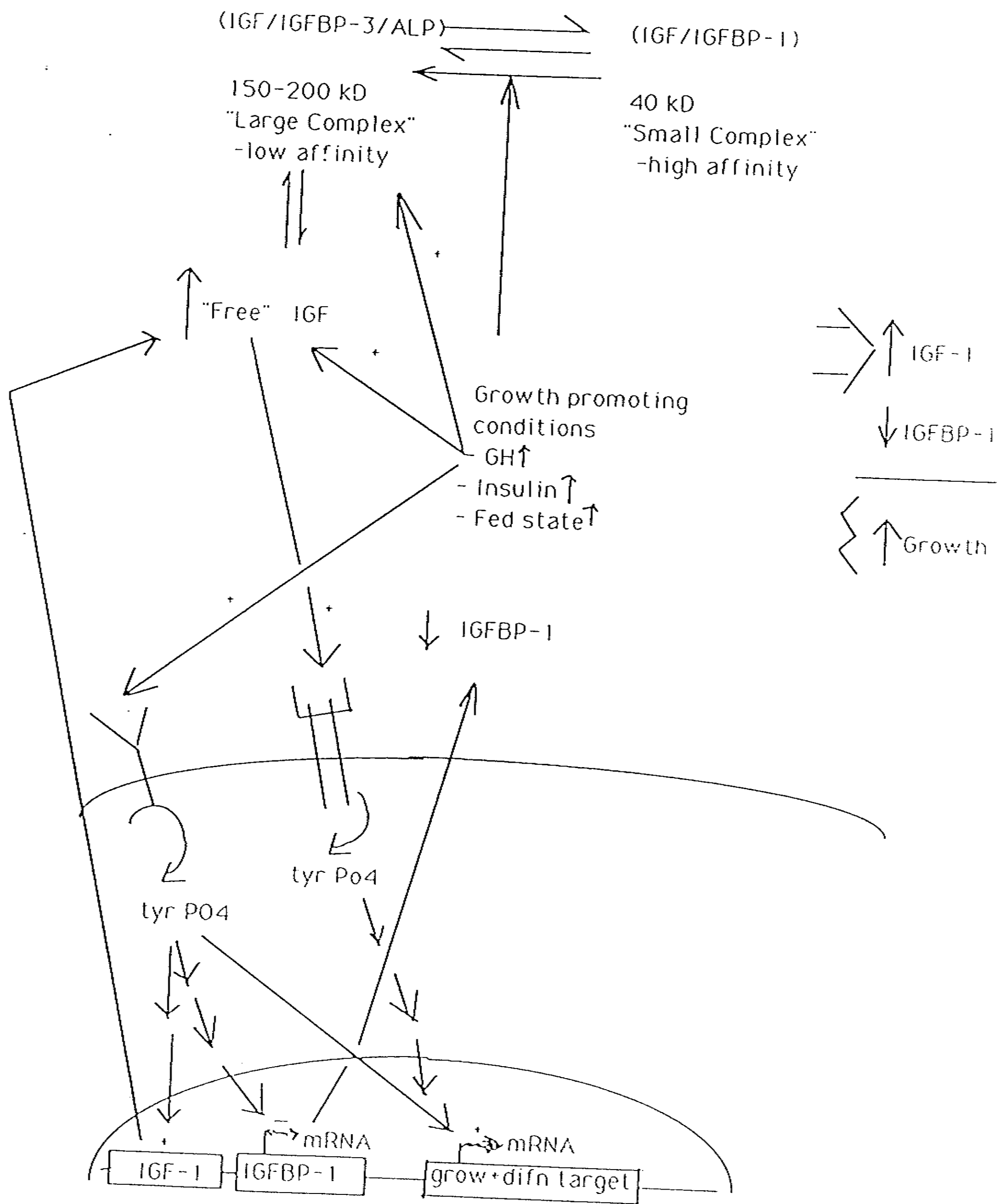
Therefore, the final out come of all the above events would be decreased IGFBP-1 levels, elevated levels of growth and differentiation affecting target genes turned on by IGFs, due to the increased circulating levels of "free" IGFs.

In growth retarding conditions such as type I diabetes, nutritionally deprived states, and decreased GH conditions (hypophysectomy), the reverse is true, which would result in the increased IGFBP-1, decreased transcription of growth and differentiation causing genes, resulting from the lowering of the "free" circulating IGF levels.

From the above model the contribution that has been made by this Thesis can be appreciated in deciphering the complex regulation that goes on in the GH/IGF/IGFBP-axis and the regulatory role played by the IGFBP-1 in it.

Fig46

GH/IGF/IGF-Binding Protein Axis
Growth Modulation Model



Conclusions and Future directions

Regulation of the IGFBP-1 gene expression is multifactorial. Factors including nutritional status, GH regulate its gene expression. But these regulatory factors are interconnected, hence to decipher the primary effects on gene expression, reduced systems have to be employed. The cloning the rat IGFBP-1 gene including 1627 bp of the 5' flanking region will enable such experiments to be performed. Regulatory sequences for each of these factors can be delineated by using regulatory sequence-reporter constructs and transfecting appropriate cell culture systems.

DNA/protein interaction that are responsible in mediating hormonal response of IGFBP-1 gene expression, can be elucidated via mobility-shift and DNase protection assays. Furthermore, such DNA regulatory sequences can be used in affinity chromatography to isolate the corresponding nuclear-protein/s.

Cloning of the rat IGFBP-1 also makes it possible to investigate the role of IGFBP-1 in vivo by two strategies, (i) producing mice that carry the trans-gene for rat IGFBP-1 and (ii) producing homologous recombinant mice in whom the endogenous IGFBP-1 gene expression is disrupted. Both the above strategies are in progress.

Finally, I hope this thesis has made a contribution in understanding the complex regulation of IGFBP-1 gene expression by demonstrating GH and nutritional regulation of the IGFBP-1 gene, cloning of a full length IGFBP-1 cDNA, as well as cloning and elucidating the structure of the IGFBP-1 gene. It is my vehement hope that the tools now at hand for IGFBP-1 will enable further gains in understanding the role of IGFBP-1.

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