

REGULATION OF INSULIN-LIKE GROWTH FACTOR-I
AND INSULIN-LIKE GROWTH FACTOR BINDING
PROTEIN EXPRESSION IN THE RAT

BY

JIANGMING LUO

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Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements for the
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TABLE OF CONTENTS

ACKNOWLEDGMENTS.....	i
ABSTRACT.....	ii
PUBLICATIONS.....	vi
LIST OF ABBREVIATIONS.....	x
LIST OF TABLES.....	xiv
LIST OF FIGURES.....	xvi

CHAPTER 1: REVIEW OF THE LITERATURE

1.1	Insulin-like growth factor-I (IGF-I).....	1
1.1.1	Historical perspective.....	1
1.1.2	Protein structure.....	4
1.1.3	Gene and mRNA structure.....	10
1.1.4	Regulation of gene expression.....	13
1.1.5	Biological actions.....	23
1.2	Insulin-like growth factor binding proteins (IGFBPs)	
1.2.1	IGFBP-1.....	28
1.2.2	IGFBP-2.....	48
1.2.3	IGFBP-3.....	54
1.2.4	IGFBP-4.....	67
1.2.5	IGFBP-5.....	70
1.2.6	IGFBP-6.....	71
1.2.7	Relationship among the six cloned	

IGFBPs.....	73
1.3 Research objectives.....	74

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials.....	77
2.2 Animals.....	77
2.3 Molecular Probes.....	78
2.4 Labelling of probes.....	79
2.5 Large scale plasmid DNA preparation.....	82
2.6 RNA extraction and RNA blot hybridization....	84
2.7 Nuclear run-off assay.....	87
2.8 IGF-I radioimmunoassay.....	88
2.9 Immunoblotting.....	89
2.12 Ligand blotting.....	90
2.11 Determination of serum glucose, glycosylated haemoglobin.....	90
2.12 Statistics.....	91

CHAPTER 3: TRANSCRIPTIONAL REGULATION OF RAT IGFBP-1 EXPRESSION BY GROWTH HORMONE

3.1 Introduction.....	92
3.2 Experimental design.....	93
3.3 Results.....	93
3.4 Discussion.....	95

**CHAPTER 4: REGULATION OF EXPRESSION OF IGF-I AND IGFBPs
BY DEXAMETHASONE**

4.1	Introduction.....	99
4.2	Experimental design.....	102
4.3	Results	
4.3.1	Dexamethasone inhibits growth hormone-induction of IGF-I gene expression in hypophysectomized rats and reduces IGF-I mRNA levels in the pituitary intact rats.....	104
4.3.2	Dexamethasone increases hepatic IGFBP-1 mRNA abundance and serum IGFBP-1 concentrations in the rats.....	114
4.3.3	Dexamethasone enhances hepatic IGFBP-3 mRNA abundance and serum IGFBP-3 concentrations in the rats.....	123
4.4	Discussion.....	127

CHAPTER 5: EXPRESSION OF IGF-I AND IGFBPs IN DIABETIC RATS

5.1	Introduction.....	138
5.2	Experimental design.....	141
5.3	Results	
5.3.1	Expression of IGF-I gene in the streptozotocin (STZ)-induced	

	diabetic rats.....	143
5.3.2	Expression of IGFBP-1 gene in the STZ-induced diabetic rats.....	148
5.3.3	Effect of insulin on IGF-I and IGFBP-1 mRNA abundance in STZ- induced diabetic rats.....	153
5.3.4	Expression of IGFBP-1, -2, -3, and -4 in the spontaneously diabetic BB/W rats and the effects of intensive insulin treatment on these IGFBPs.....	153
5.4	Discussion.....	163
CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS.....		175
REFERENCES.....		178

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ABSTRACT

Insulin-like growth factor-I (IGF-I) plays an important role in promoting growth. IGF-I is bound to insulin-like growth factor binding proteins (IGFBPs) in the circulation and tissue fluids, and the actions of IGF-I are believed to be modulated by IGFBPs. Both IGF-I and IGFBPs are regulated by hormones and nutrition. Poor growth and decreased expression of IGF-I in growth hormone (GH) deficiency are well documented. Glucocorticoid excess and diabetes are associated with retarded growth but the mechanisms involved are poorly understood. In this thesis, I have examined the expression of genes encoding IGF-I and IGFBPs in three animal models: hypophysectomized (hypox) rat, glucocorticoid treated rat and diabetic rat. In all of the three animal models, impaired growth is a common phenomenon but is attributable to the disturbance of different hormones in each model. I tested the hypothesis that the perturbation of both IGF-I and IGFBP gene expression may contribute to the stunted growth in these animals. Serum IGF-I and IGFBPs were measured by radioimmunoassay, immunoblotting or ligand blotting methods. IGF-I, IGFBP-1, -2, -3 and -4 mRNA abundance was determined by Northern analysis or slot blot analysis, and the transcriptional rate of IGFBP-1 was determined by nuclear run-off assay.

In hypox rats, hepatic IGFBP-1 mRNA level and transcription rate were elevated 4 to 5-fold compared to sham-operated pituitary-

intact rats. After a single injection of GH (100 μ g/100 g body weight), the hepatic IGFBP-1 transcription rate in hypox rats was reduced within 30 min to the levels seen in the sham-operated control rats. Similarly, hepatic IGFBP-1 mRNA abundance was reduced after acute GH injection.

In hypox rats, acute administration of a glucocorticoid, dexamethasone (DXM), inhibited GH induction of serum IGF-I and IGF-I mRNA abundance in a dose-dependent manner in multiple tissues including liver and tibia. Injection of DXM at higher doses acutely reduced steady state IGF-I mRNA abundance in multiple tissues in pituitary-intact rats. In addition, dose-dependent increases in hepatic IGFBP-1 and -3 mRNA abundance, and serum IGFBP-3 levels were seen in pituitary-intact rats after the administration of DXM. When DXM was administered daily for 6 days to pituitary-intact rats, body weight gain and hepatic and tibial IGF-I mRNA abundance were significantly reduced, whereas hepatic IGFBP-1, -3 mRNA and serum IGFBP-1 and -3 levels were significantly increased.

In streptozotocin (STZ)-induced diabetic rats, IGF-I mRNA abundance was reduced in various tissues including liver, kidney, lung, diaphragm, quadriceps, heart, and adipose tissue. As early as 7 days after STZ administration there was a modest reduction (10-30%) in IGF-I mRNA abundance in the tissues examined. A further reduction in IGF-I mRNA abundance was seen in many tissues,

32 and 91 days after STZ administration. In contrast to the decrease in IGF-I mRNA, the IGFBP-1 mRNA levels were significantly increased in the liver and kidney of diabetic rats. Treatment of diabetic rats with insulin for 1 week resulted in a significant decrease in renal IGFBP-1 mRNA abundance. In spontaneously diabetic rats, significant increases in the amount of hepatic IGFBP-1 and -2 mRNA were seen 1 month and 3 months after the onset of diabetes. Intensive insulin treatment for 3 weeks normalized the amount of hepatic IGFBP-1 mRNA in diabetic rats and resulted in a decrease in hepatic IGFBP-2 mRNA. In contrast to the increase in IGFBP-1 and -2 mRNA, a significant decrease in hepatic IGFBP-3 mRNA and serum IGFBP-3 was seen in diabetic rats. Intensive insulin treatment for 3 weeks did not restore the IGFBP-3 mRNA level in diabetic rats.

The findings are consistent with the hypothesis that inhibition of IGF-I gene expression together with reduced IGF-I action due to the perturbation of expression of IGFBP genes may contribute to the growth retardation seen in GH deficiency, glucocorticoid excess and diabetes. The information obtained from the studies described in this thesis is useful for further developing additional experiments aimed at elucidating the molecular mechanisms involved in regulation of IGF-I and IGFBPs in GH deficiency, glucocorticoid excess and diabetes. The understanding of these mechanisms may lead to new therapeutic approaches, with the hope of improved growth in children with these

disorders.

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1. Jiangming Luo and L. J. Murphy. Cycloheximide blocks growth hormone but not estragon induction of insulin-like growth factor-I mRNA accumulation. Combined Meeting of American Society for Cell Biology and American Society for Biochemistry and Molecular Biology, San Francisco, February, 1989.
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3. Jiangming Luo and L.J. Murphy. Dexamethasone inhibits growth hormone induction of insulin-like growth factor-1 mRNA. 71th Annual Meeting of the Endocrine Society, Seattle, June, 1989.
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Washington, April, 1989.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
bp	base pairs
bw	body weight
°C	degrees centigrade
Ci	curie
CPM	counts per minute
CTP	cytosine triphosphate
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothretiol
DXM	dexamethasone
E ₂	17β-estradiol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EtBr	ethidium bromide
FSH	follicle-stimulating hormone
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GH	growth hormone
GTP	guanosine triphosphate
HCl	hydrochloric acid
hr	hour
hGH	human growth hormone

hypox	hypophysectomized
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
i.p.	intraperitoneally
i.v.	intravenously
kb	kilobase
KDa	kilodalton
KCl	potassium chloride
L	litre
LH	luteinizing hormone
M	molar
mg	milligram
MgCl ₂	magnesium chloride
min	minutes
ml	millilitre
mM	millimolar
MOPS	3N-morpholino-propanesulfonic acid
mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
NP-40	Nonidet P-40
OD	optical density
O/N	overnight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PDGF	platelet-derived growth factor
poly(A)	containing polyadenylic acid tail
PRL	prolactin
PTH	parathyroid hormone
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
RPM	revolutions per minute
SDS	sodium dodecyl sulfate
Sec	second
SSC	standard saline citrate
STZ	streptozotocin
T	thymidine
TBE	tris borate EDTA buffer
TE	tris-EDTA
TGF	transforming growth factor
tRNA	transfer RNA
μCi	microcurie
μg	microgram
μl	microlitre
μM	micromolar
UTR	untranslated region
UTP	uridine triphosphate
UV	ultraviolet
vol	volume
v/v	volume per volume

wt	weight
w/v	weight per volume
%	percent

LIST OF TABLES

Table	Page No.
Table 1-1.	Homology between IGF-I, IGF-II and proinsulin in various domains.....5
Table 1-2.	Biological actions of IGF-I.....29
Table 1-3.	Effects of some hormones on IGFBP-1.....43
Table 1-4.	Regulation of IGFBP-2.....51
Table 1-5.	Regulation of IGFBP-3.....59
Table 1-6.	Some characteristics of six cloned IGFBPs.....75
Table 2-1.	cDNA probes.....80
Table 2-1a.	The sequences of the rat IGFBP-2, -3 and -4 oligonucleotide probes.....81
Table 5-1.	Effects of STZ-induced diabetes on body weight, body weight gain and serum glucose.....144
Table 5-1a.	The relative values of IGF-I mRNA abundance in various tissues of STZ-induced diabetic rats and age-matched non-diabetic control rats.....146
Table 5-2.	Effects of spontaneous diabetes on body weight, serum glucose and glycosylated haemoglobin in control rats and rats diabetic for 1 and 3 months.....156
Table 5-3.	Densitometric quantitation of serum IGFBPs in control rats and rats

diabetic for 1 and 3 months.....162

LIST OF FIGURES

Figure	Page No.
Fig. 1-1. Comparison of human, cow, pig, rat, mouse, chicken, and xenopus IGF-I peptides.....	8
Fig. 1-2. Structure and expression of the rat IGF-I gene.....	12
Fig. 3-1. The effects of GH on hepatic IGFBP-1 expression in hypox rats.....	94
Fig. 4-1. Protocol of treatment in the study of effects of DXM on IGF-I gene expression.....	103
Fig. 4-2. Northern blot analysis of poly (A) ⁺ RNA from hypox rats treated with GH and DXM.....	105
Fig. 4-3. The effect of DXM on GH-induced increase in IGF-I mRNA abundance in various tissues of the hypox rat.....	106
Fig. 4-4. The effects of DXM and GH on serum IGF-I concentrations in the hypox rat.....	108
Fig. 4-5. The Northern blot analysis of poly (A) ⁺ RNA from pituitary-intact rats treated with DXM....	110
Fig. 4-6. The effect of DXM on steady state IGF-I mRNA abundance in various tissues of pituitary- intact rats.....	111
Fig. 4-7. The effects of DXM on serum IGF-I concentrations in the pituitary intact rats....	112
Fig. 4-8. The effect of chronic administration of DXM	

on body wt gain, serum IGF-I concentration, hepatic and proximal tibial IGF-I mRNA abundance in pituitary-intact rats.....	113
Fig. 4-9. The dose-dependent induction of serum total IGF binding activities by DXM.....	116
Fig. 4-10. The time course of induction of hepatic IGFBP-1 mRNA following DXM administration.....	117
Fig. 4-11. The effect of DXM on hepatic IGFBP-1 mRNA abundance.....	118
Fig. 4-12. The effect of DXM on IGFBP-1 transcription.....	120
Fig. 4-13. The effects of chronic administration of DXM on hepatic IGFBP-1 mRNA abundance.....	121
Fig. 4-14. The effect of chronic DXM administration on serum IGFBP-1 concentration.....	122
Fig. 4-15. The time course of induction of serum IGFBPs following DXM administration.....	125
Fig. 4-16. The time course of induction of hepatic IGFBP-3 mRNA following DXM administration.....	126
Fig. 4-17. The dose-dependent induction of IGFBP-3 expression by DXM.....	128
Fig. 4-18. The effects of chronic administration of DXM on IGFBP-3 expression.....	129
Fig. 5-1. The effect of STZ-induced diabetes on IGF-I mRNA abundance in various tissues of the rat...	145
Fig. 5-2. Representative autoradiograms of Northern blots demonstrating IGF-I mRNA abundance in	

tissues from diabetic and non-diabetic rats....	147
Fig. 5-3. Correlation between serum glucose and hepatic IGF-I mRNA abundance.....	149
Fig. 5-4. The effect of STZ-induced diabetes on IGFBP-1 mRNA abundance in the liver and kidney.....	150
Fig. 5-5. Northern blot analysis of hepatic and renal RNA from diabetic rats at various times after STZ administration.....	151
Fig. 5-6. Correlation between serum glucose and hepatic IGFBP-1 mRNA abundance.....	152
Fig. 5-7. The relative tissue abundance of IGF-I and IGFBP-1 mRNAs in diabetic rats following treatment with insulin.....	154
Fig. 5-8. The effects of spontaneous diabetes and insulin treatment on hepatic IGFBP-1 mRNA abundance....	157
Fig. 5-9. The effects of spontaneous diabetes and insulin treatment on hepatic IGFBP-2 mRNA abundance....	159
Fig. 5-10. The effects of spontaneous diabetes and insulin treatment on hepatic IGFBP-3 mRNA abundance....	160
Fig. 5-11. Ligand blot of serum from diabetic and non-diabetic rats.....	161
Fig. 5-12. The effects of spontaneous diabetes and insulin treatment on hepatic IGFBP-4 mRNA abundance....	164

Chapter 1. Review of the Literature

1.1 Insulin-like growth factor-I

1.1.1 Historical perspective

The insulin-like growth factors (IGFs) were discovered by three different lines of research. In 1957, Salmon and Daughaday (1957) reported an important observation. They found that the GH-inducible factor in serum was able to stimulate the incorporation of [^{35}S] sulphate into incubated cartilage. The serum of hypox rat lacked this sulphation factor or "sulphation activity". Administration of GH to hypophysectomized (hypox) rats restored the serum sulphation activity despite GH itself having no direct effect on incorporation of [^{35}S] sulphate into cartilage. The normal rat serum also contained such sulphation activity. The serum containing this GH-dependent sulphation factor also stimulated the incorporation of thymidine into DNA (Daughaday & Reeder, 1966) and proline into the hydroxyproline of collagen (Daughaday & Mariz, 1962). The name "sulphation activity" was subsequently changed to "somatomedin" to designate these GH-dependent growth factors in serum (Daughaday *et al.*, 1972).

By using different bioassays for identification of somatomedin and using different purification procedures, three different

somatomedins were purified. The purified neutral polypeptide, which stimulated the sulphate incorporation into chick cartilage, was designated somatomedin A (Uthne, 1973; Sievertsson *et al.*, 1975). Using the same purification procedure, a second polypeptide, which was GH-dependent and with a more acidic isoelectric point, was purified. It was designated somatomedin B (Uthne, 1973; Sievertsson *et al.*, 1975). Another group of researchers identified a factor, which was responsible for stimulation of sulphate uptake and thymidine incorporation by rat cartilage, and called it somatomedin C. This polypeptide is GH-dependent and has a basic isoelectric point (Van Wyk *et al.*, 1974).

Independently, Froesch *et al.* (1963) observed that serum insulin-like effects are much greater than would be expected on the basis of the insulin content of serum. Furthermore, most of the insulin effects of serum were not neutralized by the use of anti-insulin serum. This insulin-like activity was termed "nonsuppressible insulin-like activity" or "NSILA". During the purification procedure of NSILA, two kinds of NSILA were found. NSILA-S was present in the supernatant and NSILA-P was present in the precipitate after an acid-ethanol extraction. NSILA-P was shown to be less stable than NSILA-S and difficult to purify further. In 1976, two related polypeptides having insulin-like effects and cell-growth promoting effects *in vitro* were purified from NSILA-S by Rinderknecht and Humbel (1976a). They called these two polypeptides NSILA-I and NSILA-II, which are different in

molecular weight and amino acid composition. After amino acid sequences of the N-terminal of NSILA-I and II were determined, a striking structural similarity was found between NSILA and the insulin B chain. They renamed NSILA-I and II IGF-I and IGF-II (Rinderknecht & Humbel, 1976b).

The third line of investigation was conducted by Pierson & Temin (Pierson & Temin 1972). In 1972, they extracted a growth promoting activity from serum, which they named multiplication-stimulating activity (MSA). This activity was found to be able to stimulate DNA synthesis and cell division in chicken embryo fibroblasts, and had non-suppressible insulin-like activity. In 1973, Dulak and Temin (1973) found that Buffalo rat liver cells (BRL-3A) secrete MSA into the culture medium and these conditioned media supported growth in chick embryo fibroblasts. Later MSA was shown to have functional similarities to insulin and somatomedin (Smith & Temin, 1974). In 1980, Moses *et al.* (1980) reported that MSA was present at higher level in fetal rat sera compared to maternal sera, and gradually declined after birth, suggesting that MSA may function as a growth factor in the fetal rat.

It became apparent that most of the activities that have been described by various names (somatomedin A, somatomedin C, IGF-I, IGF-II, MSA) represented two different peptides: IGF-I and IGF-II. Analysis of amino acid sequence of somatomedin C purified by Van Wyk revealed that it is identical to IGF-I purified by Rinderknecht

(Klapper *et al.*, 1983). Somatomedin A appeared to be a deaminated form of IGF-I (Enberg *et al.*, 1984). MSA was demonstrated to be the rat homologue of human IGF-II, differing in only five amino acid residues (Marquardt *et al.*, 1981). It was renamed rat IGF-II. The primary structure of somatomedin B was determined and showed that somatomedin B is a protease inhibitor unrelated to somatomedin (Fryklund *et al.*, 1978). In 1987, the terms IGF-I and IGF-II were recommended to avoid confusion in nomenclature (Daughaday *et al.*, 1987).

1.1.2 Protein structure

IGF-I is a single chain, basic polypeptide of 70 amino acids with three intrachain disulphide bridges. The calculated molecular weight is 7,649. IGF-II is a single chain, slightly acidic polypeptide cross-linked by three intrachain disulphide bonds consisting of 67 amino acids, with a calculated molecular weight of 7,471 (Humbel 1984). These two peptides have about 70% identity in their amino acid sequences, and the homology in B, C, A, and D domains are 76%, 38%, 77% and 80%, respectively. Also they are related closely to proinsulin in structure sharing at least 60% sequence homology (Rinderknecht & Humbel 1978). The homology between human IGF-I, IGF-II and proinsulin in each domain is shown in Table 1-1. Similar to proinsulin, the IGFs contain an amino-terminal B domain and an A domain that are separated by a short connecting C domain. In the A and B domains, the homology between

Table 1-1. Homology between IGF-I, IGF-II and proinsulin in various domains. N - no homology.

	DOMAINS			
	B	C	A	D
IGF-I/IGF-II	76%	38%	77%	80%
IGF-I/Proinsulin	48%	N	52%	-
IGF-II/Proinsulin	40%	N	57%	-

IGFs and insulin is approximately 45%. The C domains of IGF-I and IGF-II are 12 and 8 amino acids respectively, being shorter and not homologous with the C domain of proinsulin. In contrast to proinsulin, there is no evidence that the IGFs undergo cleavage to release a C-peptide. Also unlike proinsulin, the IGFs contain an additional D-domain which extends from the C-terminal. The D-domain is 8 and 6 amino acids in length for IGF-I and IGF-II, respectively. Amino acid sequences derived from the nucleotide sequences indicate that both IGF-I and IGF-II protein precursors contain E-domains at the carboxyl termini. In the rat, IGF-II E-domain is 89 amino acids. Two different IGF-I E-domains which arose by alternate RNA splicing were identified in the rat. IGF-IA propeptide contains 35 amino acids in E-domain while IGF-IB propeptide contains 41 amino acids in E-domain. They share the same amino acid sequence of 16 amino acids in N-terminus of E-domain but are different in other amino acids in C-terminus. The computer-aided modelling analysis of the IGFs suggests that the IGFs and proinsulin have similar three-dimensional structures (Blundell & Humbel, 1980; Blundell *et al.*, 1983). Another member of insulin-like growth factor family is relaxin which is a polypeptide hormone from the corpus luteum and is responsible for dilation of the symphysis pubis prior to parturition. Relaxin has A and B chains linked by disulphide bridges. The similarity in the tertiary structure of insulin and relaxin was suggested by model building, although they have low (20%) sequence homology (Blundell & Humbel, 1980).

Mature IGF-I is highly conserved in different species (Fig. 1-1). Bovine, porcine and human IGF-I are identical, whereas rat and mouse IGF-I differ from human by three and four amino acid residues, respectively. Only one amino acid residue difference is seen between rat and mouse IGF-I. Chicken and xenopus IGF-I differ from human IGF-I by 8 and 11 amino acid residues. IGF-II is also highly conserved, but shows somewhat greater variability with the coding sequence of the mature peptide. Bovine, rat and mouse IGF-II differ from human IGF-II at positions B32, C35, and C36. In addition, rat and mouse IGF-II substitute glycine for serine at position 22, and mouse IGF-II also substitutes arginine and serine for glycine at position 3 and 5, respectively (Rechler & Nissley 1989). Remarkable conservation of sequence and structure of IGFs in several species indicates that there have been strong constraints inhibiting evolutionary divergence of these proteins (Daughaday & Rotwein, 1989).

Variant forms of both IGF-I and IGF-II have been identified. A truncated form of IGF-I which lacks the first three residues at N-terminus has been isolated (Carlsson-Skwirut *et al.*, 1986; Sara *et al.*, 1986) and this amino-terminus truncation presumably results from post-translational modification of proIGF-I or of mature IGF-I. Variants of IGF-II have also been identified. These variants contain either a tetrapeptide substitution at ser-29 or a tripeptide substitution at ser-33 and a 21-amino acid residue extension peptide at carboxyl-terminus (Zumstein *et al.*, 1985). A

B Domain

Human	G	P	E	T	L	C	G	A	E	L	V	D	A	L	Q	F	V	C	G	D	R	G	F	Y	F	N	K	P	T
Cow	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pig	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-
Mouse	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-
Chicken	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-
Xenopus	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-

C Domain

Human	G	Y	G	S	S	S	R	R	A	P	Q	T
Cow	-	-	-	-	-	-	-	-	-	-	-	-
Pig	-	-	-	-	-	-	-	-	-	-	-	-
Rat	-	-	-	-	-	I	-	-	-	-	-	-
Mouse	-	-	-	-	-	I	-	-	-	-	-	-
Chicken	-	-	-	-	-	-	-	-	L	H	H	K
Xenopus	-	-	-	-	N	N	-	-	S	H	H	R

A Domain

Human	G	I	V	D	E	C	C	F	R	S	C	D	L	R	R	L	E	M	Y	C	A
Cow	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pig	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mouse	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chicken	-	-	-	-	-	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-
Xenopus	-	-	-	-	-	-	-	-	Q	-	-	-	F	-	-	-	-	-	-	-	-

D Domain

Human	P	L	K	P	A	K	S	A
Cow	-	-	-	-	-	-	-	-
Pig	-	-	-	-	-	-	-	-
Rat	-	-	-	-	T	-	-	-
Mouse	-	-	-	-	T	-	A	-
Chicken	-	I	-	-	P	-	-	-
Xenopus	-	A	-	-	-	-	-	-

Fig. 1-1. Comparison of human, cow, pig, rat, mouse, chicken, and xenopus IGF-I peptides.

variant form of IGF-II lacking an N-terminal alanine has been identified and it comprises approximately 25% of the IGF-II in human plasma (Rinderknecht & Humbel, 1978). Since my project is related to IGF-I and IGFBPs, in the following sections I will concentrate on IGF-I and IGFBPs rather than IGF-II.

Analysis of mutant IGF molecules generated by *in vitro* mutagenesis and chemical synthesis has determined the role of various parts of IGF structure. The B domain of IGF-I is required for IGFBP binding. De Vroede *et al.* (1985) reported that insulin-IGF hybrid molecules containing the B domain of IGF-I were recognized by binding proteins. A truncated form of IGF-I molecules lacking the N-terminal tripeptide of the B domain has a markedly reduced affinity for binding proteins (Szabo *et al.*, 1988). Four-point mutations (Gln3, Ala4, Tyr15, Leu16) in B domain of IGF-I reduced the affinity for binding proteins 600-fold (Bayne *et al.*, 1988). Some amino acids in B, C, and A domain of IGF-I are important for maintaining high-affinity binding to the IGF-I receptor. The mutants of IGF-I altered at Tyr24 decreased the affinity for the IGF-I receptor (Cascieri *et al.*, 1988a; Bayne *et al.*, 1990). Bayne *et al.* (1989; 1990) found that the replacement of the C domain of IGF-I with four glycine bridge or single point mutation at Tyr31 in C domain decreased the affinity for IGF-I receptor. Tyr60 replaced with Leu in A domain resulted in 20-fold loss in affinity for IGF-I receptor (Bayne *et al.*, 1990). Several regions of A domain of IGF-I are involved in recognition to the

IGF-II receptor. The mutant of IGF-I changed in Thr49, Ser50, Ile51 has at least 20-fold lower affinity for the IGF-II receptor, but has no effect on affinity for IGF-I receptor (Cascieri *et al.*, 1989). Mutation at Tyr60 resulted in the loss of affinity with IGF-II receptor (Bayne *et al.*, 1990).

1.1.3 Gene and mRNA structure

The human, ovine, rat, and chicken IGF-I genes have been characterized (Rotwein *et al.*, 1986; Shimatsu & Rotwein, 1987a; Dickson *et al.*, 1991; Kajimoto & Rotwein, 1991). The rat IGF-I gene is a single copy gene and is very large, extending over at least 70 kb (Shimatsu & Rotwein, 1987a). It consists of at least six exons (Shimatsu & Rotwein, 1987a; Bucci *et al.*, 1989). Parts of exon 3 and exon 4 encode the 70 amino acid mature peptide. Exon 1 and 2 encode the 5' untranslated region, and each contain transcription initiation sites, with either exon 1 or exon 2 being spliced to exon 3. Exon 5 and 6 encode the E domain and exon 6 contains the particularly long 3'-untranslated region. The processing of the primary IGF-I gene transcript in the rat appears to be complicated. Alternative mRNA splicing occurs at both 5' and 3' ends of the gene, and there are at least 5 functional polyadenylation sites at the 3' end of exon 6. cDNA clones with three distinct 5' untranslated regions in the rat have been identified, and designated class A, B, and C (Roberts *et al.*, 1987a). The class B sequence diverges from the class A sequence 72

bp upstream from the ATG at Met-22 of the pre-pro-IGF-I amino acid sequence, while the class C sequence diverges from the class A and B sequences 15 bp upstream of this codon. The class C 5' untranslated region has been identified in the exon 1 of rat IGF-I gene (Shimatsu & Rotwein 1987a), while class B 5' untranslated region has been located in the exon 2 in the rat IGF-I gene (Bucci *et al.*, 1989). However, the class A 5' untranslated region has not yet been positioned on the rat IGF-I genomic map, and the sequence unique to class A cDNA was thought recently to be artifactual (Adamo *et al.*, 1991). In addition, differential splicing of the primary IGF-I gene transcript results in either IGF-Ia (exon 1, 3, 4 and 6) or IGF-Ib (exon 1, 3, 4, 5 and 6) which contains a 52-base insertion after residue 16 of the E domain. The structure and expression of the rat IGF-I gene are shown diagrammatically in Fig. 1-2.

In Northern blot analysis, 3 major groups of IGF-I transcripts of 0.8-1.2, 1.7-2.0 and 7.0-7.5 kb in rat have been detected (Lund *et al.*, 1986; Murphy *et al.*, 1987a). The intermediate forms of 2.6 and 3.9 kb also have been observed. The major size heterogeneity of IGF-I RNAs occurs at the 3' end (Lund *et al.*, 1989). The 7.0-7.5 kb IGF-I mRNAs have an unusually long 3' untranslated region of greater than 6 Kb (Lund *et al.*, 1989). The class B and C IGF-I mRNAs and IGF-IA, IGF-IB mRNAs in rat are present in all sizes of transcripts (Shimatsu & Rotwein 1987b; Lowe *et al.*, 1987; 1988). The 7.0-7.5 kb IGF-I mRNAs have been shown to have shorter half-

Rat IGF-I gene, mRNA and protein structure

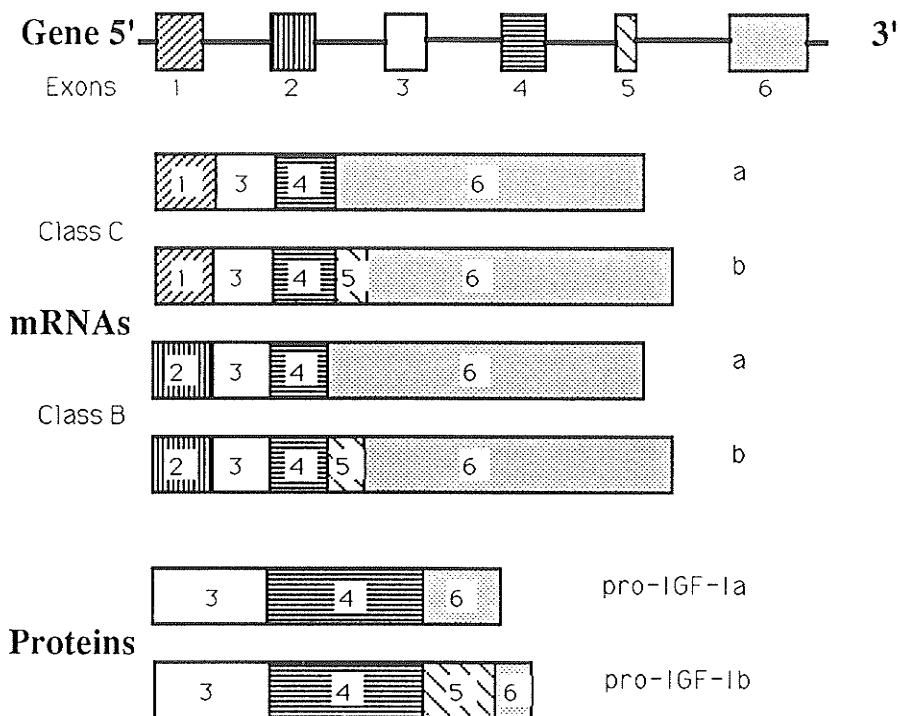


Fig. 1-2. Structure and expression of the rat IGF-I gene.

lives than the smaller IGF-I mRNAs both *in vitro* and *in vivo* studies (Hepler *et al.*, 1990). These 7.0-7.5 kb IGF-I mRNAs were essentially undetectable in polysomal RNA, while only the shorter species of IGF-I mRNA were found in polysomes, suggesting that some aspect of the long 3' untranslated region may prevent translation (Foyt *et al.*, 1991).

1.1.4 Regulation of gene expression

Developmental expression

IGF-I plays an important role in growth and development, especially in the postnatal period. The changes in IGF-I gene expression during development have been observed by many investigators. It was demonstrated that IGF-I mRNA is present in 11-day whole rat embryos, and IGF-I mRNA increased by 9-fold between day 11 and day 13 of gestation. By 14-19 days of gestation, IGF-I mRNA was detected in several fetal rat tissues, including intestine/stomach, liver, lung and brain (Lund *et al.*, 1986, Hoyt *et al.*, 1988). IGF-I mRNA is lower in fetal and neonatal rat liver than in adult liver (Lund *et al.*, 1986; Roberts *et al.*, 1986; Hoyt *et al.*, 1988). This is consistent with the low level of IGF-I in rat fetal and neonatal serum (Daughaday *et al.*, 1982). The gene expression of IGF-I in fetal liver is less than that in certain extra-hepatic tissues. Liver IGF-I mRNA levels increase dramatically between birth and adulthood (Lund *et al.*, 1986;

Rotwein *et al.*, 1987; Beck *et al.*, 1988; Adamo *et al.*, 1989), and the liver becomes a quantitatively predominant source of IGF-I. This results in the large postnatal increase in serum IGF-I. The differential expression of IGF-I in extra-hepatic tissues occurs during development. IGF-I mRNA levels in stomach, intestine and testis decline, while those in heart and kidney increase (Lund *et al.*, 1986; Hoyt *et al.*, 1988; Adamo *et al.*, 1989).

Serum IGF-I concentrations have been extensively studied in man, particularly in childhood because of the regulatory role of IGF-I in growth (Furlanetto *et al.*, 1977, 1987; Hall *et al.*, 1979, 1980; Bala *et al.*, 1981; Zapf *et al.*, 1981; Sibergeld *et al.*, 1986). At birth, the concentration of IGF-I in cord blood is about half of that in normal adult serum (Bala *et al.*, 1981). Serum IGF-I concentrations rise progressively during childhood and reach the level of normal adults just before puberty (Bala *et al.*, 1981; Sibergeld *et al.*, 1986). During puberty, 2 to 3-fold rise in serum IGF-I concentrations is seen and is believed to be related to the increase in sex steroids which occur at this time (Bala *et al.*, 1981; Rosenfield *et al.*, 1985; Sibergeeld *et al.*, 1986). A gradual decline of the high pubertal IGF-I concentrations occurs and after age 25 year serum IGF-I concentrations remain relatively constant at adult levels until the age of 60. Thereafter, there is a progressive decline in serum IGF-I levels due to a decrease in GH secretion with age (Rudman *et al.*, 1981; Sara *et al.*, 1982; Clemmons & Van-Wyk, 1984; Florini *et al.*, 1985; Hammerman, 1987).

Growth hormone

The plasma IGF-I levels are dependent on the growth hormone status of animals, being low in GH-deficient animals and elevated in animals with GH-secreting pituitary tumours (Clemmons & Van wyk, 1984; Scott *et al.*, 1985). Administration of GH to hypopituitary animals results in the increase in plasma IGF-I levels (Furlanetto *et al.*, 1977; Froesch *et al.*, 1982). Plasma IGF-I and tissue IGF-I then stimulate longitudinal growth, and as such mediate the growth promoting effect of GH. Although IGF-I is synthesized by multiple tissues (Murphy *et al.*, 1987a, 1987b; Robert *et al.*, 1987b), the liver appears to be quantitatively the most important source of plasma IGF-I (Schwander *et al.*, 1983; D'Ercole *et al.*, 1984; Scott *et al.*, 1985). Circulating GH interacts with hepatic GH receptor and results in the stimulation of synthesis and secretion of IGF-I. The effect of GH on IGF-I synthesis in liver involves changes in IGF-I mRNA. The amount of IGF-I mRNA is markedly decreased in liver from hypox rat, and all of the IGF-I mRNA species were affected. Administration of GH to hypox rat restores hepatic IGF-I mRNA levels (Roberts *et al.*, 1986; Hynes *et al.*, 1987; Murphy *et al.*, 1987a, 1987b). All three sizes of IGF-I mRNA are increased (Roberts *et al.*, 1986; Murphy *et al.*, 1987a). A time course study of hepatic IGF-I mRNA accumulation after GH administration showed that the increase of IGF-I mRNA levels was seen at 1-3 h after GH and IGF-I mRNA levels reached to the maximal at 6-12 h, which

paralleled the changes in serum immunoreactive IGF-I (Murphy *et al.*, 1987a). The increase in hepatic IGF-I mRNA levels after the GH administration was demonstrated to be at least partially due to the increase in transcription rate of IGF-I gene (Mathews *et al.*, 1986; Johnson *et al.*, 1989). Furthermore, the direct effect of GH on IGF-I mRNA abundance has been demonstrated on hepatocytes (Norstedt & Moller 1987; Johnson *et al.*, 1989). Different species of IGF-I mRNA differing in their 5' UTR and in 3' regions are differentially regulated by GH. In liver, class B transcripts were increased 6.4-fold by GH treatment, while class C transcripts were elevated only 2.2-fold (Lowe *et al.*, 1987). GH treatment increased IGF-IB mRNA levels about 8-fold, but only increased IGF-IA mRNA levels 2.4-fold (Lowe *et al.*, 1988).

The GH regulation of IGF-I mRNA in extra-hepatic tissues has also been reported. Increased IGF-I mRNA level after GH treatment was observed in a variety of tissues including heart, lung, kidney, spleen, pancreas, muscle, uterus, testis, fat, bone, and pituitary (Mathews *et al.*, 1986; Doglio *et al.*, 1987; Hynes *et al.*, 1987; Murphy *et al.*, 1987a; Roberts *et al.*, 1987; Turner *et al.*, 1988; Fagin *et al.*, 1988, 1989a; Isgaard *et al.*, 1988a, 1989; Closset *et al.*, 1989; McCarthy *et al.*, 1989; Gaskins *et al.*, 1990). Furthermore, IGF-I mRNAs are regulated by GH in a tissue-specific manner. Murphy *et al.* (1987a) reported that the increase in IGF-I mRNAs by GH was most marked in skeletal and cardiac muscles (9 to 10-fold) and least marked in the brain. In their report, GH

induced 4-fold increase in IGF-I mRNAs in the liver. The different effects of various modes of GH administration on IGF-I mRNA levels have been described. In hypox rats, pulsatile GH administration (which mimics the pattern of GH secretion in male rats) is more effective than continuous GH infusion in increasing IGF-I mRNA levels in skeletal muscle and rib growth plate, but not in liver (Isgaard *et al.*, 1988b). In a human study, it was shown that small but frequent i.v. boluses and continuous infusion of GH are equally effective in increasing serum IGF-I in GH-deficient patients, whereas the same amount of GH given as two large boluses results in a significantly smaller increase in serum IGF-I (Jorgensen *et al.*, 1990).

Estrogen

In the uterus, estrogen is a more potent stimulus to IGF-I mRNA expression compared to GH. Murphy *et al.* (1987d; 1988c) reported that 17 β -estradiol (E₂) increased uterine IGF-I mRNA by 14 to 22-fold in ovariectomized or ovariectomized hypophysectomized rat (ovx-hypox), while GH increased uterine IGF-I mRNA only by 4-fold in ovx-hypox rat. Bonus injection with E₂ alone had no significant effect on hepatic IGF-I gene expression and serum IGF-I levels (Murphy & Friesen, 1988c). It has been reported that E₂ treatment increased the steady state levels of IGF-I mRNA 2- to 2.5-fold in bone cells which might explain the anabolic effects of E₂ on bone (Ernst *et al.*, 1989). The autocrine or paracrine actions

of IGF-I in bone and other tissues after E₂ treatment may play an important role since estrogen replacement decreased or did not change the circulating IGF-I levels in the rat and in postmenopausal women (Dawson-hughes *et al.*, 1986; Bellantoni *et al.*, 1991). Chronic estrogen treatment resulted in a decline in serum IGF-I levels and reduces the magnitude of GH stimulation of hepatic IGF-I mRNA in the rat (Murphy & Friesen, 1988c).

Insulin

Circulating IGF-I levels are low in diabetic rats in association with growth retardation (Maes *et al.*, 1983; 1986). Decreased IGF-I mRNA levels in liver, lung, kidney and uterus have been found in STZ-induced diabetic rats (Goldstein *et al.*, 1988; Murphy, 1988c; Fagin *et al.*, 1989b; Boni-Schnetzler *et al.*, 1989). Reduced IGF-I mRNA levels in multiple tissues suggest that the growth retardation seen in diabetic rats may be associated with diminished tissue availability of IGF-I from both endocrine and autocrine/paracrine sources. Goldstein *et al.* (1988) compared the effects of different doses of STZ and found that with higher dosages of STZ (144 and 288 mg/mg), hepatic IGF-I mRNA levels fell 63% and 88% below control levels. Furthermore, reduction of hepatic IGF-I mRNA was better correlated with ketonemia than hyperglycemia (Goldstein *et al.*, 1988). Interestingly, there is a recent report that kidney IGF-I mRNA levels increased 50-100% in postpubertal diabetic rats compared with postpubertal controls 12

h after STZ injection, and fell to approximately 50% of control levels between day 2 and 7 (Bach *et al.*, 1991). It has been shown that exogenous GH was ineffective in inducing tissue IGF-I mRNA levels in the presence of diabetes (Fagin *et al.*, 1989b). Also, estrogen-stimulated uterine IGF-I expression was diminished in diabetic rats (Murphy, 1988c). Insulin treatment of STZ-induced diabetic rats restored basal, GH-stimulated and E₂-stimulated IGF-I mRNA levels (Murphy, 1988c; Boni-Schnetzler *et al.*, 1989, Fagin *et al.*, 1989b), suggesting a permissive role for insulin. Acute injection (10 U) or chronic infusion (2.4 U/day) with insulin increased hepatic IGF-I mRNA levels in normal rats (Salamon *et al.*, 1989). The direct stimulating effect of insulin on IGF-I mRNA content was demonstrated in cultured hepatocytes (Johnson *et al.*, 1989). Effect of diabetes on pituitary IGF-I may be different. It was reported that pituitary IGF-I content increased by 69% in STZ-induced diabetic rats with no change in IGF-I mRNA. Insulin treatment completely reversed the rise of pituitary IGF-I peptide content. Elevated IGF-I levels in the pituitary may suppress GH synthesis and secretion seen in STZ-induced diabetic rats (Olchovsky *et al.*, 1991).

Other hormones

The glucocorticoid, DXM decreased steady state IGF-I mRNA levels in rat neuronal and glial cells in culture (Adamo *et al.*, 1988). It was shown that cortisol decreased IGF-I mRNA levels by

60% in osteoblast cultures with 6 hours of treatment, and the concentrations of immunoreactive IGF-I by 50% after 24 hours. Cortisol also opposed parathyroid hormone (PTH)-induction of IGF-I mRNA and peptide levels. The inhibitory effect of cortisol on IGF-I mRNA was seen in fibroblast/proosteoblast culture as well. Cortisol inhibited the GH-induction of IGF-I mRNA levels in these cells (McCarthy *et al.*, 1990). These effects may contribute to the inhibitory influence of glucocorticoid on bone formation.

Acute administration of ovine prolactin (PRL) resulted in an increase in hepatic IGF-I mRNA and a modest increase in serum IGF-I (Murphy *et al.*, 1988d). Ovine PRL was about 50% as potent as ovine GH. In contrast to this result, it was reported that rat PRL did not increase hepatic IGF-I mRNA (Isgaard *et al.*, 1988b). Kleinberg *et al.* (1990) reported that hPRL increased rat mammary IGF-I mRNA content but was less potent than hGH.

Acute injection of thyroid hormone to hypox rats slightly increased hepatic IGF-I mRNA levels, while chronic administration of thyroid hormone had no effect on hepatic IGF-I mRNA levels. Thyroid hormone treatment prior to GH treatment did not influence the effect of GH, while administration of thyroid hormone after a chronic GH treatment decreased IGF-I mRNA levels. Injection of thyroid hormone and GH at the same time resulted in hepatic IGF-I mRNA levels greater than those seen after GH alone (Wolf *et al.*, 1989). Similar effect of potentiation of the GH-induced IGF-I

expression by thyroid hormone in primary rat hepatocytes was reported by Tollet *et al.* (1990). Triiodothyronine (T3) stimulated IGF-I mRNA contents in the rat pituitary cell line GH3, although it may be mediated by stimulating endogenous GH secretion (Fagin *et al.*, 1989a).

PTH increased IGF-I mRNA abundance and IGF-I levels in cultured osteoblast, and as such regulated bone growth (McCarthy *et al.*, 1989; 1990). Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) stimulate testicular IGF-I mRNA levels in rats without stimulating an increase in serum IGF-I concentrations (Closset *et al.*, 1989).

Nutritional states

Nutritional status play an important role in IGF-I gene expression. Emier and Schalch (1987) reported that hepatic IGF-I mRNA levels fall to 39% of control in rats after 30 h of fasting. Refeeding fasted rats resulted in a marked rise in major hepatic IGF-I mRNA transcript (8Kb) by 6 h, and an 18-fold increase at 30 h. Similar results were reported by Lowe *et al.* (1989). Lowe *et al.* (1989) have also shown that the response of IGF-I mRNA levels to fasting was quantitatively different in different tissues. The lower levels of IGF-I mRNA in starvation are consistent with the effect of fasting on circulating IGF-I levels (Clemmons *et al.*, 1981; Emier & Schalch, 1987). The molecular mechanisms for

regulation of hepatic IGF-I mRNA by fasting were reported to be at transcriptional as well as post-transcriptional levels (Straus & Takemoto, 1990). Decreased hepatic GH receptor mRNA levels in fasting rats were described (Bornfeld *et al.*, 1989; Straus & Takemoto, 1990) which may contribute to the hepatic GH resistance seen in fasting rats. In the rat, maternal fasting caused a decrease in fetal hepatic IGF-I mRNA levels and the fetal growth retardation (Straus *et al.*, 1991a).

Dietary protein also effects the IGF-I gene expression. Neonatal or young rats fed with protein-restricted diets had decreased IGF-I mRNA levels in liver and skeletal muscle (Bornfeldt *et al.*, 1989; Moats-staats *et al.*, 1989; VandeHaar *et al.*, 1990), and a decreased serum IGF-I concentrations (Prewitt *et al.*, 1982; VandeHaar *et al.*, 1990).

Tissue regeneration

Jennische *et al.* (1987) reported that ischemic damage to a skeletal muscle in rats resulted in an increase in immunoreactive IGF-I in skeletal muscle. Increased IGF-I mRNA levels in the damaged muscle was demonstrated by Edwall *et al.* (1989). Following unilateral nephrectomy, hypertrophy of the remaining kidney occurs. Increases in both IGF-I content and IGF-I mRNA levels in remaining kidney were observed (Stiles *et al.*, 1985; Fagin & Melmed, 1987). However, another investigator reported no increases in kidney IGF-I

mRNA levels following unilateral nephrectomy (Lajara *et al.*, 1989). After partial hepatectomy, no change in IGF-I mRNA levels in regenerating liver was observed (Norstedt *et al.*, 1988).

1.1.5 Biological actions

In vivo

1. Insulin-like growth action

Some earlier experiments by Froesch *et al.* (1966) and Oelz *et al.* (1970) demonstrated that non-suppressible insulin-like activity containing IGF-I and II lowered the blood sugar and stimulated glycogen and lipid synthesis in rats when injected intravenously. Similar results were obtained by intravenous bolus injections of pure IGF-I in rat: the blood sugar was decreased with a nadir between 15-30 min, the disappearance of U-[¹⁴C] glucose from serum was enhanced, U[¹⁴C] glucose incorporation into diaphragm glycogen was increased (Zapf *et al.*, 1986). In a human study, both insulin and IGF-I induced hypoglycaemic responses, but on a molar basis IGF-I was only 6% as potent as insulin in this regard (Guler *et al.*, 1987). The hypoglycaemic response after intravenous IGF-I injection was demonstrable only with large amounts of IGF-I that override the binding capacity of the binding proteins (Zapf *et al.*, 1986; Guler *et al.*, 1987).

2. Growth-promoting action

The stimulating effect of IGF-I on animal growth was demonstrated at an earlier time with IGF-I preparations purified from human serum. The IGF-I preparation was able to increase body length and weight in pituitary-deficient Snell dwarf mice (Buul-Offers *et al.*, 1979). Similar stimulating effects of IGF-I preparation on body growth were reported in hypox rats (Schoenle *et al.*, 1982). Russell and Spencer (1985) found that tibial epiphyseal width increased after the injection of IGF-I directly into the growth plates of hypopituitary rats. The availability of large quantities of recombinant IGF-I facilitated such studies and confirmed the early findings. Subcutaneous infusions of recombinant IGF-I stimulate the growth of hypox rat (Skottner *et al.*, 1987; Guler *et al.*, 1988), neonatal rat (Phillips *et al.*, 1988), growing postweaning normal rat (Hizuka *et al.*, 1986), as well as Snell dwarf mice (Buul-Offers *et al.*, 1986). The growth-promoting effect of IGF-I was also demonstrated in transgenic mice carrying IGF-I gene (Mathews *et al.*, 1988; Behringer *et al.*, 1990). Transgenic mice which carry both IGF-I transgene and diphtheric toxin gene ablating GH gene expression, express IGF-I in the absence of GH. These mice grew larger than their GH-deficient transgenic littermates and exhibited weight and linear growth indistinguishable from that of their nontransgenic siblings (Behringer *et al.*, 1990). Although IGF-I can mimic the growth-promoting effects of GH, specific distinct patterns for the growth-

promoting actions of IGF-I and GH may exist. For example, GH induced a significant weight gain of the liver, kidney, heart, thymus and spleen in Snell Dwarf mice, whereas IGF-I only produced a significant weight gain in kidney and spleen (Buul-Offers *et al.*, 1988). Analysis of the independent effect of GH and IGF-I in transgenic mice reveals that GH is necessary for the attainment of normal liver size, while IGF-I can stimulate the weight gain of the brain (Behringer *et al.*, 1990).

3. Other actions

In addition to the insulin-like metabolic action and growth-promoting actions of IGF-I, some other biological response to IGF-I *in vivo* were also reported. For instance, infusion of IGF-I promptly increased glomerular filtration rate and effective renal plasma flow (Ritz *et al.*, 1991). Infusion of IGF-I into the mammary arterial supply enhanced milk secretion and mammary blood flow in intact, conscious goats (Prosser *et al.*, 1990). IGF-I at physiological concentration is a potent inhibitor of both glucose- and arginine-induced insulin secretion (Leahy, 1990).

In vitro

1. Insulin-like action

The insulin-like effects of the IGF-I *in vitro* can be seen in

all typical target tissues of insulin. In the adipose tissue, IGF-I stimulated glucose transport, glucose oxidation, lipogenesis from glucose, and inhibited lipolysis (Zapf *et al.*, 1978). The IGF-I appears to act via the insulin receptor in adipose tissue (Zapf *et al.*, 1981b). However, insulin-like action of IGF-I in muscle appears to be mediated by the IGF-I receptor since IGF-I is much more potent with regard to insulin like activity in muscle [Meuli *et al.*, 1975; Poggi *et al.*, 1979).

2. Cell proliferation

The stimulating effects of IGF-I on DNA synthesis and cell proliferation have been demonstrated repeatedly in primary cell culture, established cell lines, or organ explants (Zapf *et al.*, 1978; Froesch *et al.*, 1979; 1985; Takada *et al.*, 1990). For instance, IGF-I stimulated DNA synthesis 3-4-fold in human fibroblasts and chick embryo fibroblasts. IGF-I increased the cell number of chick embryo fibroblasts by 150% in 4 days compared with an increase of 25% in control cultures (Rechler *et al.*, 1978). However, IGF-I alone could only account for less than 10% of mitogenic effect of whole serum (Clemmons & Van Wyk., 1984). It has been suggested that platelet-derived growth factor (PDGF) initiate the cell cycle and allow these competent cells to respond to IGF-I. The IGF-I acts as a progression factor to stimulate these competent cells through the DNA synthesis phase of the cell cycle (Stiles *et al.*, 1979). The mitogenic effects of IGF-I are

believed to act via the IGF-I receptor (Froesch *et al.*, 1985). It has been shown that IGF-I can stimulate the expression of c-fos in rat skeletal muscle cells and murine osteoblasts (Ong *et al.*, 1987; Merriman *et al.*, 1990) and stimulate c-myc and c-fos in rat thyroid cells (Isozaki & Kohn, 1987).

3. Cell differentiation

IGF-I plays a role in stimulating cellular differentiation. It was demonstrated that IGF-I can promote the differentiation of myoblasts, osteoblasts, adipocytes and oligodendrocyte (Schmid, *et al.*, 1983; 1984; Florini *et al.*, 1986; McMorris, *et al.*, 1986; Smith *et al.*, 1988). For example, Schmid *et al.* (1983) showed that the low concentration of IGF-I can stimulate the differentiation of myoblasts and the formation of myotubes in primary cultures of chick embryonic cells. In the presence of IGF-I, more myotubes were seen. Acetylcholinesterase activity, which serves as a marker enzyme of muscle cells, rose in the presence of IGF-I, indicating that the cells were muscle cells. It was shown recently that the phorbol 12-myristate 13-acetate (PMA) induced differentiation of adrenergic neuroblastoma cell is strongly potentiated by IGF-I, as determined by morphology and markers for neuronal differentiation (Pahlman *et al.*, 1991). IGF-I also is a differentiating factor for testicular and ovarian function (Adashi, *et al.*, 1984; Veldhuis *et al.*, 1986; Chatelain *et al.*, 1987). IGF-I has been identified as an erythroid cell growth and colony formation factor both *in vitro*

and vivo (Kurtz *et al.*, 1982; 1988). The biological actions of IGF-I are summarized in Table 1-2.

1.2 Insulin-like growth factor binding proteins (IGFBPs)

It has been recognized that both IGF-I and IGF-II in serum and tissue fluids are tightly bound to specific IGF binding proteins. IGFBPs are believed to be able to modulate IGF actions. At least six distinct IGFBPs exist in tissue fluids and plasma from man and rat. These six IGFBPs will be reviewed in the following sections.

1.2.1 IGFBP-1

Human IGFBP-1 was isolated from mid-term human amniotic fluid and characterized by Povia *et al.* (1984a). They also reported the amino acid composition and sequence of the first ten amino acids. Subsequently, about 40% of the amino acid sequence was determined by Brewer *et al.* (1988). An IGF-binding protein was isolated from the conditioned media of the human hepatocarcinoma cell line HepG2 by Povia *et al.* (1985) and was shown to have identical amino acid composition and amino-terminal protein sequence. The protein placenta protein 12, PP-12, which was the product of secretory endometrium and decidua (Rutanen *et al.*, 1985; 1986) and purified from human placenta, was reported to be able to bind IGF-I and have the same N-terminal amino acid sequence and composition to the IGFBP-1 from amniotic fluid (Koistinen *et al.*, 1986). Another protein called pregnancy-associated α 1-globulin purified from human

Table 1-2. Biological actions of IGF-I

In vivo:

insulin-like action: hypoglycaemia; clearance of U-[¹⁴C] glucose from serum; U-[¹⁴C] glucose incorporation into glycogen.

growth-promoting action: increase of body length and weight; increase of epiphyseal width; increase of weight gain in certain organs, e.g. kidney, spleen, brain.

other actions: increase of glomerular filtration rate and effective renal plasma flow; enhanced milk secretion and mammary blood flow; inhibition of glucose- and arginine-induced insulin secretion.

In vitro:

insulin-like action: stimulation of glucose transport; increased synthesis of lipid, glycogen and protein; inhibition of lipolysis.

cell proliferation: stimulation of DNA synthesis and cell replication in many primary cell culture, cell lines or organ explants; stimulation of expression of the oncogene c-fos and c-myc.

cell differentiation: stimulation of the differentiation of myoblasts, osteoblasts, adipocytes, oligodendrocytes; differentiating factor for ovarian and testicular function; induction of erythropoiesis and granulopoiesis.

other action: enhanced catecholamine secretion from chromaffin cells.

endometrium was also shown to be identical to IGFBP-1 (Bell & Keyte, 1988). It became apparent that the human amniotic fluid binding protein, HepG2 binding protein, PP-12 and the pregnancy-associated α 1-globulin are identical, and designated IGFBP-1. IGFBP-1 has an apparent molecular mass of 32-34 KDa (reduced) or 28 KDa (nonreduced) on SDS-PAGE.

Molecular biology

Human IGFBP-1 cDNA was cloned by several groups from cDNA libraries of human placenta (Brinkman *et al.*, 1988a), decidua (Brewer *et al.*, 1988; Julkanen *et al.*, 1988), liver (Brinkman *et al.*, 1988a) and HepG2 hepatocarcinoma cells (Lee *et al.*, 1988). The sequence of each clone predicts a mature protein of 234 amino acids and a signal peptide of 25 amino acids. The calculated molecular mass of mature IGFBP-1 protein is 25 KDa. The deduced amino acid sequence is consistent with that determined by protein sequencing (Brewer *et al.*, 1988). Human IGFBP-1 gene is a single copy gene (Brewer *et al.*, 1988; Brinkman *et al.*, 1988b; Julkannen *et al.*, 1988) and is located on chromosome 7 (Brinkman *et al.*, 1988b; Alitalo *et al.*, 1989). It is organized in four exons and spans 5.9 Kb (Brinkman *et al.*, 1988b). Suwanichkul *et al.* (1990) have characterized the promoter of hIGFBP-1 gene. They identified the CCAAT box region which begins 72 bp upstream from cap side as the major cis element involved in basal IGFBP-1 promoter activity in HepG2 cells. The liver factor B1 is the major trans-acting factor

which stimulate basal IGFBP-1 promoter activity in HepG2 cells by interacting with CCAAT box region. The predicted IGFBP-1 mRNA size is 1.5 Kb which is consistent with the size seen on Northern blot.

Rat IGFBP-1 cDNA was isolated by Murphy *et al.* (1990) from rat decidua and liver cDNA libraries. The predicted protein is 272 amino acids in length and contains a 25-residue hydrophobic region at the N-terminal end which presumably represents the signal peptide. Rat IGFBP-1 and human counterpart share 66% amino acid identity with greatest similarity in N-terminal and C-terminal region of mature protein. Rat IGFBP-1 cDNA has been expressed in Cos-1 cell, and produced a 29 KDa ^{125} I-IGF-I binding protein identified by ligand blotting. This rat IGFBP-1 cDNA hybridized with a 1.6 kilobase transcript in RNA blot. Rat IGFBP-1 gene and 5' flanking sequence were recently cloned in Murphy's laboratory. It contains 4 exons similar to human IGFBP-1 gene (Seneviratne *et al.*, personal communication).

Both hIGFBP-1 and rIGFBP-1 contain 18 cysteine residues in an identical position. There are no classical N-glycosylation sites in both hIGFBP-1 and rIGFBP-1. However, there are five and two potential O-glycosylation sites in hIGFBP-1 and rIGFBP-1, respectively (Brinkman *et al.*, 1988a; Murphy *et al.*, 1990). An Arg-Gly-Asp (RGD) sequence which has been identified as a consensus sequence for interacting with cell surface integrin receptors, is present in the COOH sequence of hIGFBP-1 and rIGFBP-1. Human IGFBP-

1 had higher affinity for IGF-I than for IGF-II (D'Ercole *et al.*, 1985; Baxter & Cowell, 1987a). The affinity of human IGFBP-1 for IGF-I is lower compared to the that of IGFBP-3 for IGF-I (Martin & Baxter, 1986). It was reported that there is a single binding site for IGF per IGFBP-1 molecule (Baxter *et al.*, 1987c).

The tissue distribution of IGFBP-1 mRNA in adult and fetal rats has been reported. In adult rat, the order of abundance of rIGFBP-I mRNA was decidual tissue > liver >> kidney >> uterus > brain. No rIGFBP-1 mRNA signal was detected in diaphragm, heart and lung in Northern blot analysis (Murphy *et al.*, 1990). IGFBP-1 mRNA is more abundant in liver than in other tissues in the 21-day gestation rat. Other tissues in which IGFBP-1 mRNA can be clearly detected include stomach, lung, heart, and kidney. High levels of IGFBP-1 mRNA persists at 1 day of age, but are greatly reduced in livers from 21-day postnatal and adult rats (Ooi *et al.*, 1990).

Post-translational modifications

It has been reported that different preparations of IGFBP-1 that appeared to have identical amino acid sequences and molecular sizes showed contrasting biologic activity. It has been proposed that an unidentified post-translational modification may account for these differences (Busby *et al.*, 1988a). Frost and Tseng (1991) have shown that IGFBP-1 is present in human endometrial stromal cell conditioned medium in nonphosphorylated and phosphorylated forms.

Phosphovariants of IGFBP-1 are also found in amniotic fluid, suggesting that IGFBP-1 is phosphorylated *in vivo*. Phosphorylation of IGFBP-1 occurs exclusively on serine. Another group (Jones *et al.*, 1991) have shown that human hepatoma (HepG2) cells secrete phosphorylated IGFBP-1, and this form of IGFBP-1 has 6-fold higher affinity for IGF-I than it does after dephosphorylation. It also was demonstrated that human amniotic fluid and human fetal serum contain a large proportion of nonphosphorylated IGFBP-1, whereas HepG2 cell and human decidual cell secrete predominantly the phosphorylated IGFBP-1, suggesting that IGFBP-1 is secreted as a phosphoprotein and is subsequently dephosphorylated *in vivo*. The lower affinity, dephosphorylated IGFBP-1 may potentiate the effects of IGF-I on stimulated DNA synthesis, whereas phosphorylated IGFBP-1 may not because of its high affinity for IGF-I.

Regulation

Development

Fetal serum IGFBP-1 levels are 13-fold higher than those in term cord serum that are 5-fold higher than those in adult serum (Drop *et al.*, 1984). Serum IGFBP-1 levels decline from birth to puberty (Drop *et al.*, 1984; Hall *et al.*, 1988). The mean level is 4 times higher during the first year of life than in the age group 14-16 year. In adults aged 20-66 years, no significant change in serum IGFBP-1 is found (Hall *et al.*, 1988).

Diurnal variation and fasting

The serum IGFBP-1 levels display a pronounced diurnal rhythm in normal subjects (Baxter & Cowell, 1987a; Degerblad *et al.*, 1989). Baxter and Cowell (1987a) reported that serum IGFBP-1 levels started to rise at about 2400 h, reaching a peak at 0600-0700 h and falling rapidly after 0800 h. The mean peak-to-basal difference was about 13-fold. Neither peak nor basal IGFBP-1 levels were significantly associated with mean or peak nocturnal GH secretion. A light meal taken at 0130 h interrupted the increase in IGFBP-1 levels, whereas prolonged fasting sustained the increase in IGFBP-1 levels, indicating that diurnal variation is not a true circadian rhythm. Instead, it is related to feeding time (Cotterill *et al.*, 1988).

Several studies have demonstrated that human IGFBP-1 levels in serum were increased 4- to 12-fold during fasting (Busby *et al.*, 1988b; Cotterill *et al.*, 1988). The serum IGFBP-1 level in the fasting state was decreased after a meal (Suikkari *et al.*, 1988). Similar results were reported in rats. Fasting for 24 h resulted in an increase in serum IGFBP-1 levels in rat, and about a 10-fold increase in hepatic IGFBP-1 mRNA abundance (Murphy *et al.*, 1991). Refeeding caused a rapid decrease in hepatic IGFBP-1 mRNA abundance to an undetectable level for at least 24 h. Injection of GH, but not insulin resulted in a decline in elevated hepatic IGFBP-1 mRNA levels in fasted rats, suggesting that enhanced expression of

IGFBP-1 in fasted rat may be a consequence of GH deficiency rather than insulin deficiency (Murphy *et al.*, 1991).

Insulin

Several earlier clinical studies suggested that insulin plays a role in the regulation of the serum IGFBP-1 concentration: serum IGFBP-1 levels are elevated in diabetic patients, decreased in acute hyperinsulinemia as well as insulinoma patients (Brismar *et al.*, 1988; Suikkari *et al.*, 1988). *In vivo* studies, exogenous insulin caused a 3- to 4-fold increase rather than a decrease in serum IGFBP-1 levels due to the insulin-induced hypoglycaemia (Cotterill *et al.*, 1988; Yeoh & Baxter, 1988). The stimulating effects of lower glucose in medium and lower intracellular glucose on production of IGFBP-1 were also demonstrated *in vitro* (Lewitt & Baxter, 1989; 1990). In glucose clamping studies, exogenous insulin caused a dose-dependent decrease in the serum IGFBP-1 levels during hyper- and normoglycemic conditions (Suikkari *et al.*, 1988; 1989). Also the insulin effects were independent of the glucose levels. The direct effects of insulin on IGFBP-1 production have been demonstrated in an *in vitro* system. Studies with explants of human fetal liver (Lewitt & Baxter, 1989), human hepatoma cells (Conover & Lee, 1990a; Singh *et al.*, 1990), human decidual cells (Thraikill *et al.*, 1990) and rat H4IIE hepatoma cells (Unterman *et al.*, 1991) showed that insulin can directly reduce the production of IGFBP-1. Powell *et al.* (1991) proved that

the effect of insulin on IGFBP-1 occurred at the level of IGFBP-1 gene transcription. Treatment with 100 nM insulin markedly lowered the abundance of IGFBP-1 mRNA to 40% of control levels after 2 h, and to 9% of control levels after 4 h in human HepG2 hepatoma cells. Transcription rate of IGFBP-1 gene from these insulin treated cells was approximately one third of the control. The region spanning from 103 to 529 base pairs 5' to the IGFBP-1 mRNA cap site in IGFBP-1 gene was necessary to demonstrate the inhibitory effect of insulin in a CAT assay. Unterman *et al.* (1991) demonstrated the inhibitory effect of insulin on IGFBP-1 gene transcription rate and/or processing of IGFBP-1 mRNA in rat H4IIE hepatoma cells. In their experiment, insulin lowered the IGFBP-1 mRNA levels but did not effect the half-life of IGFBP-1 mRNA. The inhibitory effect of insulin on IGFBP-1 synthesis in human HepG2 cell was shown to be mediated through specific interaction with the insulin receptor (Conover *et al.*, 1990). It was reported that insulin caused an increased movement of IGFBP-1 from the vascular space to tissues of the rat heart (Bar *et al.*, 1990).

In patients with insulin-dependent diabetes, serum IGFBP-1 levels are elevated (Brismar *et al.*, 1988; Suikkari *et al.*, 1988). Suikkari *et al.* (1988) reported that the mean serum IGFBP-1 levels were 4-fold higher in type-1 diabetic patients compared with normal subjects. Elevated serum IGFBP-1 levels were also found in streptozotocin-induced diabetic rats by ligand blotting and

immunoblotting (Unterman *et al.*, 1989; 1990; Graubert *et al.*, 1991). Graubert *et al.* (1991) examined the dose-response effects of STZ on serum IGFBP-1 levels in rats. When rats received 72, 144, and 288 mg/kg STZ, serum IGFBP-1 levels increased 3-, 8-, and 17-fold, respectively. At the time when the research work of this thesis was conducted, there were no data on IGFBP-1 gene expression in diabetic animals. Subsequently, there were some reports in IGFBP-1 gene expression in diabetic rats. Ooi *et al.* (1990) demonstrated that hepatic IGFBP-1 mRNA was increased more than 100-fold in STZ-induced diabetic rats, suggesting that hepatic production of IGFBP-1 was increased and contributed to elevated circulating levels of IGFBP-1 in these animals. However, there is not other data at present to indicate that the elevated IGFBP-1 mRNA levels in STZ-induced diabetic rats is due to the changes in transcription rate of IGFBP-1 gene or in stability of IGFBP-1 mRNA. Treatment with insulin normalized serum IGFBP-1 levels in both insulin-dependent diabetic patients and STZ-induced diabetic rats. Treatment with insulin for 4 days lowered the serum IGFBP-1 levels in STZ-induced diabetic rats. Two days after insulin was discontinued, serum IGFBP-1 levels rose again (Unterman *et al.*, 1990). The elevated serum IGFBP-1 levels can be reversed by insulin in STZ-induced diabetic rats, indicating that the increased levels of IGFBP-1 is not a nonspecific toxic effect of STZ. Graubert *et al.* (1991) found that serum IGFBP-1 levels in diabetic rats receiving low-dose insulin declined significantly less than in diabetic rats receiving high-dose insulin. It was reported that

treatment with insulin for 4 days completely restored the hepatic IGFBP-1 mRNA levels in diabetic rats (Ooi *et al.*, 1990).

Serum from diabetic patients and rats contains elevated levels of somatomedin inhibitors (Phillips *et al.*, 1979; Phillips *et al.*, 1985; Avasthy *et al.*, 1986; Taylor *et al.*, 1987; 1990). These inhibitors are able to inhibit IGF-I action in cartilage bioassays. Two groups described these inhibitors with molecular masses in the ranges of 1, 20-30, 30-50, and >150 KDa (Phillips *et al.*, 1979; Taylor *et al.*, 1987). The relationship between these inhibitory factors and IGFBPs is not clear. Taylor *et al.* (1990) proposed that IGFBP-1 is one of the IGF-I inhibitors observed in diabetic patient's serum. They showed that both serum IGFBP-1 concentrations and inhibitory bioactivity reached a peak at 0800 h and displayed similar changes over the 24-h period. Both serum IGFBP-1 and IGF-I inhibitor levels showed a similarity in the insulin dependence. Graubert *et al.* (1991) found that the profile of rising IGFBP-1 levels in diabetic rats (IGFBP-1 rise before levels of IGFBP-1 fall) strongly resembles the rise in IGF-I inhibitor activity, suggesting that IGFBP-1 may be an IGF-I inhibitor. It is likely that IGF-I inhibitors in diabetic animals not only contain IGFBP-1, but also other molecules. For example, it was reported that one protein in IGF-I inhibitor preparation in diabetic rats has a unique N-terminal amino acid sequence different from that reported for IGF-binding proteins (Gagliardi *et al.*, 1990).

Growth hormone

Several clinical studies documented that serum IGFBP-1 is elevated in GH-deficiency patients and reduced in patients with acromegaly (Drop *et al.*, 1984; Pova *et al.*, 1984b; Busby *et al.*, 1988b; Hall *et al.*, 1988). Hall *et al.* (1988) reported that serum IGFBP-1 concentrations in GH deficient patients were 2-fold elevated, whereas the serum IGFBP-1 levels in patients with acromegaly were reduced by 50%. Holly *et al.* (1991) analyzed the inter-relations between GH, insulin, IGF-I and IGFBP-1 in patients with acromegaly. They found that serum fasting insulin levels remained significantly elevated in acromegaly patients, and also the insulin levels were unrelated to mean GH levels but correlated with those of serum IGF-I. Furthermore, the low levels of serum IGFBP-1 were unrelated to mean GH but were inversely related to fasting insulin levels. They proposed that low IGFBP-1 in acromegaly patients is the consequence of high insulin levels which result from hypersecreting GH-induced elevated IGF-I levels in these patients. In another study in which GH was given on alternate days, the serum levels of both IGF-I and insulin were significantly higher in GH-treated than in placebo-treated women during the 11-day follow-up period (Tapanainen *et al.*, 1991). These results support the idea that long term elevated GH increases serum IGF-I, and insulin which results in low levels of IGFBP-1. The effect of acute GH on production of IGFBP-I may not be secondary to the elevated levels of insulin. In the same study by

Tapanainen *et al.* (1991), serum IGFBP-1 levels decreased significantly during the first 4 h after GH injection, whereas serum IGF-I and insulin did not change during this 4 h period. One evidence that supports the direct GH-mediated mechanism of IGFBP-1 regulation came from the study in which octreotide was used to treat acromegalic patients (Ezzat *et al.*, 1991). Octreotide is a somatostatin analogue which can effectively control hypersomatotropism in acromegaly. Injection of 100 μ g of octreotide maximally attenuated GH to 18% of control levels, and IGF-I to 46% of control levels after 4 h. Serum IGFBP-1 levels were increased 1.5-fold after 2 h and 3-fold after 4 h. Serum insulin levels did not change during this time, suggesting that the effect of GH on IGFBP-1 may not be mediated by insulin. One study from our laboratory (Seneviratne *et al.*, 1990) demonstrated that IGFBP-1 expression is inversely regulated by GH in the rats. Hepatic IGFBP-1 mRNA levels were increased 6.5- to 17.9-fold in hypox rat compared to that in pituitary-intact rats. The abundance of 30 kDa IGF-I binding protein was increased in both hepatic extracts and serum from hypox rats. Furthermore, both acute and chronic GH treatment reduced hepatic IGFBP-1 mRNA levels. As described earlier in this chapter, a study from Murphy *et al.* (1990) demonstrated that GH, not insulin, lowered the elevated hepatic IGFBP-1 mRNA levels in fasted rats. It seems unlikely that effect of GH on IGFBP-1 mRNA levels is mediated by insulin. Unterman *et al.* (1991) used rat H4IIE hepatoma cells to examine the effect of GH on IGFBP-1 mRNA levels *in vitro*. Three h or 24 h

treatment with 1 μ M rat GH had no effect on IGFBP-1 mRNA abundance in these cells. One explanation for these negative results is that the rat H4IIE hepatoma cells may have lost GH receptors and therefore do not respond to GH. Whether GH has direct effect on IGFBP-1 gene expression will await for more investigations.

Glucocorticoid

It has been reported that the nocturnal rise in serum IGFBP-1 levels were suppressed in patients with Cushing's disease. A nocturnal rhythm of serum IGFBP-1 occurred in the patient after treatment with pituitary adenomectomy or stereotactic pituitary irradiation. In the patients with glucocorticoid deficiency, the morning serum levels of IGFBP-1 did not differ from levels in normal control (Degerblad *et al.*, 1989). *In vitro*, dexamethasone suppressed IGFBP-1 production in human fetal liver explants (Lewitt *et al.*, 1989), but had no effect on IGFBP-1 production in HepG2 hepatoma cells (Conover *et al.*, 1989). There has not been any data concerning the regulation of IGFBP-1 mRNA by glucocorticoid at the time of commencing this thesis. Orłowski *et al.* (1990a) reported that dexamethasone stimulated the IGFBP-1 gene expression in H4IIE rat hepatoma cells. In their study, incubation with 10^{-6} M DXM for 48 h increased the amount of IGFBP-1 in conditional medium by approximately 10-fold, and increased the IGFBP-1 mRNA abundance by 7-fold. DXM at concentrations equivalent to physiological concentrations was able to increase the IGFBP-1 concentration in

conditioned medium. The increased IGFBP-1 mRNA abundance was observed as early as 3 h after the addition of DXM. DXM caused an increase in half-life of IGFBP-1 mRNA (from 1.7 h to 2.5 h). The authors claimed that this small difference in half-life of mRNA can not account for the 10-fold increase in steady state levels of IGFBP-1 mRNA, suggesting that DXM stimulate the IGFBP-1 gene transcription rate. The similar stimulating effect of DXM on IGFBP-1 mRNA and protein levels in H4IIE cells was confirmed by another study (Unterman *et al.*, 1991). The discrepancy in the effect of glucocorticoid on IGFBP-1 production *in vitro* systems may probably be due to the difference in the target tissues or cells. The increased expression of IGFBP-1 by glucocorticoid was speculated to contribute to growth retardation seen in glucocorticoid excess. It was noted that glucocorticoid excess is associated with an increase in circulating somatomedin inhibitors (Unterman & Phillips, 1985). In some respects, these glucocorticoid-induced somatomedin inhibitors are similar to IGFBP-1: they rise in diabetes and fasting. However, the exact relationship between these inhibitors and IGFBP-1 remains to be determined.

Other hormones

The effects of other hormones on IGFBP-1 are summarized in the Table 1-3. When boys were given androgens serum IGFBP-1 levels were suppressed, whereas in girls receiving estrogens serum IGFBP-1

Table 1-3. Effects of some hormones on IGFBP-1

Hormone	Effect
Estrogen	increased serum IGFBP-1 levels in girls (Drop et al., 1984)
Androgens	suppressed serum IGFBP-1 levels in boys (Drop et al., 1984)
Progesterone	stimulated IGFBP-1 production in human endometrial stromal cells (Bell et al., 1991) and in HepG2 hepatoma cells (Conover et al., 1989)
Relaxin	stimulated IGFBP-1 production in human decidual cell culture (Thraillkill et al., 1990) and in endometrial stromal cells (Bell et al., 1991)
IGF-I	inhibited production of IGFBP-1 in human decidual cell culture (Thraillkill et al., 1990) enhanced IGFBP-1 production in human fetal fibroblasts (Hill et al., 1989) no effect on IGFBP-1 production in rat H4IIE hepatoma cells (Orlowski et al., 1990)

levels were increased (Drop *et al.*, 1984). It has been reported that high dose estrogen antagonizes the action of GH and inhibits dietary intake. This may explain why estrogen increased IGFBP-1 levels. In human decidual cell culture, IGF-I inhibited production of IGFBP-1, whereas relaxin stimulated IGFBP-1 production. Furthermore IGF-I could block the relaxin stimulated IGFBP-1 production (Thrailkill *et al.*, 1990). Relaxin was also found to induce IGFBP-1 production in human endometrial stromal cells (Bell *et al.*, 1991). In human fetal fibroblasts, addition of exogenous IGF-I enhanced IGFBP-1 production (Hill *et al.*, 1989), but in rat H4IIE hepatoma cells, IGF-I had no effect on IGFBP-1 production (Orlowski *et al.*, 1990a). The different effects of IGF-I on IGFBP-1 production may depend on species or cell types. It has been noted that progesterone stimulated the IGFBP-1 production in human endometrial stromal cells (Bell *et al.*, 1991) and in HepG2 hepatoma cells (Conover *et al.*, 1989).

Pregnancy

The serum IGFBP-1 levels are increased during normal pregnancy (Rutanen *et al.*, 1982; Yeoh & Baxter, 1988; Giudice, *et al.*, 1990). Rutanen *et al.* (1982) used radioimmunoassay to examine the serum IGFBP-1 concentrations in pregnant women. They found that the serum IGFBP-1 levels in pregnant women were higher than those in nonpregnant women from the seventh week of gestation. The IGFBP-1 levels gradually increased until 22 to 23 weeks, then slightly

declined at 32 to 33 weeks, and rose again toward term. IGFBP-1 levels at 22 to 23 weeks of gestation were approximately 5-fold higher compared with those in nonpregnant women. Giudice *et al.* (1990) reported that serum IGFBP-1 levels detected by ligand blotting and immunoprecipitation steadily increased throughout gestation. The origin of IGFBP-1 synthesis in pregnancy was thought to be decidua. IGFBP-1 was believed to play an inhibitory role in placental and fetal growth, since maternal serum IGFBP-1 levels were inversely related to infant birth weight (Howell *et al.*, 1985), and were further elevated in preeclampsia (Iino *et al.*, 1986).

Biological activities of IGFBP-1

The biological role of IGFBP-1 is not completely defined at present. The inhibitory effects of IGFBP-1 on IGF-I action *in vitro* have been demonstrated in endometrium (Rutanen *et al.*, 1988), cultured choriocarcinoma cells (Ritvos *et al.*, 1988), rat FRTL5 thyroid follicular cells (Frauman *et al.*, 1989) and chick embryo pelvic cartilage (Burch *et al.*, 1990). Burch *et al.* (1990) showed that both basal and IGF-I-stimulated cartilage weight gain, [³H]thymidine incorporation into DNA, and ³⁵SO₄ incorporation into proteoglycan were inhibited by IGFBP-1. Although most reports described the inhibitory activities of IGFBP-1 in IGF-I actions, stimulating activities of IGFBP-1 have also been observed. Elgin *et al.* (1987) reported that a preparation of IGFBP-1 markedly

potentiated replication and DNA synthesis on human, mouse, chicken embryo fibroblasts and porcine aortic smooth muscle cells in response to IGF-I. The stimulating effect of IGFBP-1 is not just dependent on the interaction between IGFBP-1, IGF-I and IGF-I receptor. A factor in platelet-poor plasma and in cerebrospinal fluid was reported to be required for the potentiating effect of IGFBP-1 (Clemmons & Gardner, 1990). The discrepancy of biological activities of IGFBP-1 is not fully understood. It may be due to the differences in preparation of IGFBP-1, experimental conditions, target cells or different forms of IGFBP-1. The possibility of different form of IGFBP-1 was suggested by the studies of Busby et al. (1988a). They purified two forms of IGFBP-1 after the ion exchange chromatography: form B showed the stimulating effect same as that reported by Elgin et al. (1987); form C inhibited both basal and IGF-I-stimulated DNA synthesis. Form B was shown to bind to the cell surface in association with [¹²⁵I] IGF-I, whereas form C did not bind. The inhibitory action of form C was thought to compete with receptors for IGF binding and therefore inhibit binding of IGF-I to cell receptors. In contrast, the stimulating effect of form B on IGF-I action is not easily explained. It was suggested that form B may facilitate the cell growth responses to IGF-I by altering IGF-I degradation or by increasing binding of IGF-I to the IGF-I receptor (Elgin et al., 1987). The inhibitory effect of IGFBP-1 on IGF-I action in the past studies could be due to the preparation of IGFBP-1 which might contain excess amounts of inhibitory form of IGFBP-1. The exact difference in the nature of

these two forms of IGFBP-1 is not clear. These two forms of IGFBP-1 could not be distinguished by molecular mass, isoelectric point, amino acid composition or N-terminal amino acid sequence, and do not contain carbohydrate (Busby et al., 1988a). A difference of these two forms of IGFBP-1 is the surface charge properties, since they can be distinguished from each other by ion-exchange chromatography. It suggested that some kind of protein modifications may account for the difference in biological activities of these two forms of IGFBP-1. It was reported recently that IGFBP-1 is present in nonphosphorylated and phosphorylated forms (Forst & Tseng, 1991; Tone et al., 1991). These modifications may occur in form B or form C IGFBP-1, although there is no report which demonstrated that the difference between form B and form C of IGFBP-1 is phosphorylation. It has been reported that phosphorylated IGFBP-1 has 6-fold higher affinity for IGF-I than dephosphorylated IGFBP-1 does. Since form B and form C of IGFBP-1 have similar affinity for IGF-I (Busby et al., 1988a), other kind of protein modification present in form B or form C IGFBP-1 is possible. A role of IGFBP-1 in modulating blood glucose levels *in vivo* was reported recently (Lewitt et al., 1991). Co-infusion of equimolar concentrations of human IGFBP-1 and IGF-I inhibited the IGF-I induced fall in plasma glucose in the rats. Injection of IGFBP-1 alone increased plasma glucose levels. These results suggest that IGFBP-1 levels might modulate hypoglycaemic activity of unbound IGFs in the circulation.

1.2.2 IGFBP-2

Rat IGFBP-2 was purified from conditioned medium of BRL-3A rat liver cells, and the N-terminal amino acid sequence of this protein was determined (Lyons & Smith, 1986; Mottola *et al.*, 1986). It has an apparent molecular mass of 31.5-33 kDa (reduced), and 34-36 kDa (nonreduced) on SDS-PAGE. Rat IGFBP-2 is the predominant IGFBP in fetal rat serum and is present at greatly reduced levels in adult rat serum (Lyons & Smith, 1986; Romanus *et al.*, 1986; Yang *et al.*, 1989). It has been shown that IGFBP-2 is the predominant form of IGF-binding proteins in the adult rat cerebrospinal fluid (Tseng *et al.*, 1989). IGFBP-2 binds IGF-I and IGF-II with similar high affinity (Brown *et al.*, 1989).

Molecular biology

Rat IGFBP-2 cDNA was cloned from BRL-3A cDNA library (Brown *et al.*, 1989) and rat adult liver cDNA library (Margot *et al.*, 1989) by using oligonucleotide probes. The deduced binding protein precursor consists of a 34-amino acid prepeptide and a 270-amino acid mature protein. The predicted molecular weight is 29,500. Similar to rIGFBP-1, rIGFBP-2 contains 18 cysteine, and an Arg-Gly-Asp (RGD) tripeptide motif. It does not contain N-glycosylation sites. A single rIGFBP-2 RNA species of 1.6 or 2.0 Kb is seen in Northern blot. IGFBP-2 mRNA is expressed in BRL-3A cells and in multiple fetal rat tissues including liver, kidney, stomach,

intestine, lung and brain (Brown *et al.*, 1989; Margot *et al.*, 1989; Ooi *et al.*, 1990). Rat IGFBP-2 mRNA, like IGFBP-1 mRNA, was highly expressed in fetal and neonatal rat liver. In the term gestation rats, both IGFBP-2 and IGFBP-1 mRNAs in liver are more abundant than in other tissues. The levels of IGFBP-2 and IGFBP-1 mRNAs in livers are greatly reduced in 22-day post natal and adult rats (Ooi *et al.*, 1990). IGFBP-2 mRNA abundance in many other tissues in adult rats showed a similar great reduction compared to that in fetal tissues. IGFBP-2 mRNA is persistently expressed in adult rat brain although the abundance is reduced. In contrast, IGFBP-1 mRNA was expressed at very low levels in adult brain, and in fetal brain. The relatively high expression of IGFBP-2 mRNA in brain may explain why IGFBP-2 is present in cerebrospinal fluid in high concentrations (Ocran *et al.*, 1990).

The cloning of rat IGFBP-2 gene has been reported (Brown & Rechler, 1990). The rat IGFBP-2 gene spans at least 8 kb and consists of 4 exons. It was demonstrated that a 1260 nt fragment containing 1144 nt of 5' flanking region has promoter activity. Rat IGFBP-2 gene lacks a TATA box immediately up-stream from the transcription initiation site. However, several potential transcription factor binding sites or motifs were observed in this 5' flanking region. The functional role of these sites remains to be determined. Both human IGFBP-2 gene and cDNA have been cloned. Human IGFBP-2 gene is located at chromosome 2 and consists of four exons (Binkert *et al.*, 1992). Human IGFBP-2 cDNAs were cloned from

a human fetal liver cDNA library (Binkert *et al.*, 1989) and from HepG2 hepatoma cell cDNA library (Zapf *et al.*, 1990). The predicted human IGFBP-2 precursor contains a prepeptide region of 39 amino acids and the mature binding protein with 289 amino acids (31 kDa). Human IGFBP-2 shares extensive amino acid homology (>85%) with the rat IGFBP-2 (Binkert *et al.*, 1989). Both human adult and fetal liver contain the 1.6-1.8 kb IGFBP-2 transcript (Zapf *et al.*, 1990). Human IGFBP-2 appears to be major form of lower molecular weight binding proteins in adult serum (Zapf *et al.*, 1990).

Regulation

Regulation of IGFBP-2 is summarized in Table 1-4. Regulation of IGFBP-2 is similar to IGFBP-1 in many respects. The expression of IGFBP-2 appears to be elevated in some situations related to growth retardation. Fasting for 48 h in the rats results in a 10- to 20-fold induction of IGFBP-2 mRNA levels in the liver (Ooi *et al.*, 1990; Orłowski *et al.*, 1990b) and approximately 7-fold increase of IGFBP-2 in the serum (Orłowski *et al.*, 1990b). It has been demonstrated that fasting causes a pronounced reduction in liver GH receptors and GH binding. One explanation for elevated IGFBP-2 mRNA in fasted rats is that the impaired signalling from GH due to reduction in GH receptor in fasted rats may have similar effect on IGFBP-2 gene expression as in hypox rats in which production of GH is diminished, although the direct demonstration

Table 1-4. Regulation of IGFBP-2

Factor	Effect
Fasting	Increase in serum IGFBP-2 levels and hepatic IGFBP-2 mRNA
Refeeding	Decrease in elevated hepatic IGFBP-2 mRNA
Protein-limited diet	Increase in hepatic IGFBP-2 mRNA levels
Energy-restricted diet	Increase in hepatic IGFBP-2 mRNA levels
Hypophysectomy	Increase in serum IGFBP-2 levels and hepatic IGFBP-2 mRNA abundance
Growth hormone	No effect
Streptozotocin-induced diabetes	Increase in hepatic IGFBP-2 mRNA levels
Insulin	Decrease in elevated hepatic IGFBP-2 mRNA levels
Dexamethasone	Decrease in hepatic IGFBP-2 mRNA levels and IGFBP-2 production in rat osteoblast-like cells
IGF-I	Inhibit IGFBP-2 mRNA levels in rat hepatocyte, but stimulate IGFBP-2 production in rat osteoblast-like cells
Retinoic acid	Stimulate IGFBP-2 mRNA levels in rat hepatocytes

has not been done. Three days after refeeding, elevated hepatic IGFBP-2 mRNA levels completely return to normal (Ooi *et al.*, 1990). The abundance of hepatic IGFBP-2 mRNA was increased in the rats on the protein-limited diets (same total caloric intake compared to the control), and on the energy-restricted diets (Straus & Takemoto, 1990; 1991a). Hypophysectomy caused a marked increase in hepatic IGFBP-2 mRNA levels (20-fold) and in serum IGFBP-2 concentrations (Ooi *et al.*, 1990; Orlowski *et al.*, 1990b). In contrast to the effect on IGFBP-1 mRNA levels, the GH treatment alone (4-8 days) failed to suppress the elevated hepatic IGFBP-2 mRNA levels (Margot *et al.*, 1989; Ooi *et al.*, 1990). Injection of GH with combination of T4, cortisone acetate and testosterone decreased hepatic IGFBP-2 mRNA to about 50% of the levels in hypox rats (Ooi *et al.*, 1990), suggesting that other hormones acting independently or in conjunction with GH can suppress IGFBP-2 mRNA production. It was also demonstrated that GH has no affect on IGFBP-2 mRNA levels in rat hepatocytes (Boni-Schnetzler *et al.*, 1990). In streptozotocin-induced diabetic rats, hepatic IGFBP-1 mRNA levels were increased (Boni-Schnetzler *et al.*, 1989; Ooi *et al.*, 1990) and were reduced to control levels by treatment with insulin for 7 days (Boni-Schnetzler *et al.*, 1989). The direct inhibitory effect of insulin on IGFBP-2 expression was further demonstrated in rat hepatocytes (Boni-Schnetzler *et al.*, 1990). Retinoic acid stimulates IGFBP-2 expression in rat hepatocytes and insulin suppresses retinoic acid-induced IGFBP-2 expression (Schmid *et al.*, 1992). Treatment with DXM to 4-day-old rats for 4 days

resulted in a 50% decrease in hepatic IGFBP-2 mRNA abundance (Orlowski et al., 1990b). This effect is in contrast to the stimulating effect of DXM on IGFBP-1 expression. DXM was also shown to reduce the level of IGFBP-2 in rat osteoblast-like cell cultures (Chen et al., 1991). The effect of IGF-I on IGFBP-2 expression may depend on types of tissues or cells. IGF-I in 10 nM concentration slightly inhibited IGFBP-2 mRNA levels in rat hepatocyte (Bonisch-Schnetzler et al., 1990), whereas IGF-I stimulated IGFBP-2 production by 3.5-fold in rat osteoblast-like cells in the same concentration (Chen et al., 1991). It is not known whether IGF-I has an affect on IGFBP-2 translation rate. In humans, serum IGFBP-2 decreases steadily throughout gestation and this reduction may be related to a pregnancy-related protease (Giudice et al., 1990). In term of regulation, although IGFBP-1 and IGFBP-2 are similar in many respects, there are some obvious differences (e.g. DXM and GH treatment, and in pregnancy). These differences may reflect a distinct biological role of these two binding proteins.

Biological activities

All documented results showed that IGFBP-2 has inhibitory effect on IGFs actions. Knauer and Smith (1980) showed that rat IGFBP-2 inhibited IGF-II-stimulated glucose transport and DNA synthesis in chicken embryo fibroblasts by inhibiting IGF-II binding to the cell receptor. Rat IGFBP-2 was also reported to inhibit IGF-II binding to IGF-II receptor in rat hepatoma H35 cells

(Mottola *et al.*, 1986). Han *et al.* (1988) found that IGFBP-2 reduced IGF-I-stimulated ^3H -thymidine incorporation by astroglial cells in a dose-dependent manner. IGFBP-2 inhibited ^{125}I -IGF-I binding to astroglial cells, suggesting that the IGFBP-2 inhibits the mitogenic actions of IGF-1 by preventing IGF-I binding to its receptor. These results suggest that IGFBP-2 may have an important regulatory role in the development of the nervous system. It was reported that IGFBP-2 inhibited FSH-stimulated DNA synthesis by the granulosa cells (Bicsak *et al.*, 1990b). Since IGF-I had not effect on FSH-stimulated DNA synthesis, the authors proposed an IGF-independent mechanism for the effect of IGFBP-2 in granulosa cell proliferation. There is a possibility that the IGF-I receptors on granulosa cell were already saturated by endogenous IGF-I produced by stimulation of FSH. Therefore, any added exogenous IGF-I would not have any further effect. This possibility was not discussed and ruled out by the authors. Recently, Feyen *et al.* (1991) showed that a recombinant human IGFBP-2 inhibited both basal and IGF-I-stimulated DNA synthesis and collagen synthesis in fetal rat calvaria, suggesting that IGFBP-2 may have an regulatory role in bone metabolism whereby the activity of locally produced IGF-I may be controlled.

1.2.3 IGFBP-3

IGFBP-3 was known as BP-53 before the introduction of standardized nomenclature. It is the most abundant IGF-binding

protein in adult human and rat serum, and is present predominantly in a ternary complex of approximately 140 kDa (Baxter & Martin, 1989a). The ternary complex consists of an acid-stable glycoprotein of 40-50 kDa (IGFBP-3, or β -subunit), an acid-labile glycoprotein of 85 kDa (α -subunit), and either IGF-I or IGF-II (γ -subunit). A model of this ternary complex was proposed by Furlanetto (1980) and was confirmed by Baxter (1988). On SDS-PAGE, human IGFBP-3 (β -subunit) appears as a glycoprotein doublet bands of 53 kDa nonreduced (43 kDa reduced) and 47 kDa nonreduced (40 kDa reduced). Since the first 15 amino acids of these doublets are the same, it was suggested that these two bands are glycosylation variants of the same protein (Baxter *et al.*, 1986c; Baxter & Martin 1987b). IGFBP-3 contains a high affinity binding site ($K_a=2-3 \times 10^{10} \text{ M}^{-1}$) for both IGFs with slightly higher affinity for IGF-II than for IGF-I (Martin & Baxter 1986) and a lower affinity binding site ($K_a=4-6 \times 10^8 \text{ M}^{-1}$) for the α -subunit (Baxter *et al.*, 1989a). Acid labile subunit (α -subunit) appears as a doublet of apparent molecular mass 86 and 84 kDa under either nonreducing or reducing conditions on SDS-PAGE. The doublet comprises of glycosylation variants since the proteins migrate as a single band after deglycosylation with N-glycanase (Baxter *et al.*, 1989a). Studies on the binding of the three subunits indicate that the interaction of α -subunit with β -subunit binding site requires the presence of an IGF (Baxter *et al.*, 1989a; Baxter & Martin, 1989b). β -subunit does not bind to α -subunit to form a dimer. Only when the β -subunit binds to γ -subunit, the affinity of the β -subunit for the

α -subunit increases and the ternary complex can form. Although the presence of γ -subunit is required for α -subunit binding to β -subunit, γ -subunit binding site occupancy is not the only determinant of α -subunit binding. It was shown recently that binding affinity of the α -subunit for β - γ complexes is strongly dependent on the structure of the IGF in the complex. Deletion of D-domain or replacement of Tyr24 of the B-domain in IGF-I molecule decreased the binding affinity of β - γ complex for α -subunit (Baxter *et al.*, 1992). IGFs and IGFBP-3 are found in serum in approximately equimolar concentrations (Baxter & Martin, 1986b), whereas the α -subunit is present in a 2-3-fold molar excess (Baxter, 1990). Much of the circulating α -subunit is present in the uncomplexed form. This may be important in maintaining most of IGFBP-3 in the ternary form.

When rat serum IGFBPs are analyzed by Western ligand blotting, six distinct bands are detected. Three large molecular mass bands (49, 45, and 42 KDa) are believed to be related to IGFBP-3. Their differences in apparent size are due to variable degrees of glycosylation, since N-glycanase digestion converts the 42-49 KDa triplet into a single band with an apparent molecular mass of 37 KDa, and amino acid sequences of 42 and 45 KDa proteins give the same sequence of the first 31 amino acids (Zapf *et al.*, 1988; 1989). It is interesting that a 31-32 KDa binding protein in serum was found to have same 31 N-terminal amino acid sequences as IGFBP-3 in both human and rat. These 31-32 KDa proteins are thought to

be a degradation products of IGFBP-3, which lack the COOH terminus (Zapf *et al.*, 1988; 1990).

Molecular biology

Both human and rat IGFBP-3 cDNAs have been cloned (Wood *et al.*, 1988; Shimasaki *et al.*, 1989). The predicted sequence from human IGFBP-3 cDNA contains a 27 amino acid putative signal peptide followed by a mature protein of 264 amino acids. The predicted molecular mass is 28.7 KDa. There are three potential N-linked glycosylation sites and two O-linked glycosylation sites. The discrepancy between predicted molecular weight and molecular mass in SDS-PAGE can be explained by glycosylation of IGFBP-3. The predicted sequence of rat IGFBP-3 from cloned cDNA reveals a mature protein of 265 amino acid which is one amino acid longer than mature human IGFBP-3 (Shimasaki *et al.*, 1989). Rat IGFBP-3 contains four Asn-linked glycosylation sites. There is approximately 82% amino acid homology between human IGFBP-3 and rat IGFBP-3, and most of the amino acid substitutions occur in the mid-region of the molecules. Both human and rat IGFBP-3 contain 18 cysteine residues clustered near the N- and C-terminus which are conserved in other IGF-binding proteins. Unlike IGFBP-1 and -2, IGFBP-3 lacks the RGD sequence in. A single 2.5-2.6 kb band of IGFBP-3 transcript can be detected in Northern blot of human and rat tissues (Wood *et al.*, 1988; shimasaki *et al.*, 1989). In rat, expression of IGFBP-3 occurs in multiple tissues, including liver,

kidney, stomach, heart, adrenal, ovary, testis, spleen, lung, small and large intestine. IGFBP-3 mRNA is more abundant in liver than in other tissues examined. Human IGFBP-3 gene has been cloned (Cubbage *et al.*, 1990). It is a single gene and spans 8.9 kb. There are five exons in human IGFBP-3 gene and four of them encode protein. Human IGFBP-3 gene contains a typical TATA box and consensus GC upstream promoter element. The promoter activity of IGFBP-3 gene has been demonstrated.

Regulation

Regulation of IGFBP-3 is quite different with that of IGFBP-1, -2, and is summarized in Table 1-5.

Age

Similar to IGF-I, serum IGFBP-3 shows strong age-dependence. Human serum IGFBP-3 levels are low at birth, rise during the first weeks of life, and continue to increase in young children until the end of puberty. A gradual decline in serum IGFBP-3 levels are seen from adulthood to old age (Baxter & Martin, 1986b; Blum *et al.*, 1990a). IGFBP-3 mRNA is present in the kidney and liver of 1-day-old rats and rises significantly in both organs by week 1. Thereafter, levels remain relatively constant, particularly in the liver (Albiston & Herington, 1992).

Table 1-5. Regulation of IGFBP-3

Factor	Effect
GH	Increase serum IGFBP-3 and 150-200 KDa complex in hypox rat; no effect on production of IGFBP-3 in human fibroblasts <i>in vitro</i>
IGF-I	Increase serum IGFBP-3 and production of IGFBP-3 both <i>in vivo</i> and <i>in vitro</i>
Fasting	Decrease serum IGFBP-3
Protein-restricted diet	Decrease serum IGFBP-3
Diabetes	Decrease serum IGFBP-3
Insulin	Increase serum IGFBP-3 at high dose (10 U/100 g per day)
Oestradiol	Increase IGFBP-3 production in osteoblast-like calvaria cells
Cortisol	Reduce IGFBP-3 production in rat osteoblastic cell line
Bombesin	Increase IGFBP-3 production in Swiss 3T3 fibroblast
Vasopressin	Increase IGFBP-3 production in Swiss 3T3 fibroblast
PDGF	Increase IGFBP-3 production in Swiss 3T3 fibroblast
EGF	Increase IGFBP-3 production in Swiss 3T3 fibroblast and human skin fibroblast
TGF- β	Increase IGFBP-3 production in human skin fibroblast, but inhibit IGFBP-3 production in porcine granulosa cells
Pregnancy	Increase in serum immunoreactive IGFBP-3, but decrease in serum IGFBP-3 detected by ligand blotting

GH and IGF-I

Serum IGFBP-3 level is increased in active acromegaly, reduced in GH-deficiency and raised by GH administration (Baxter & Martin, 1986b; Baxter & Martin, 1989c). In the rats, hypophysectomy causes a 4.5-fold reduction of serum IGFBP-3. Injection or infusion of GH for 8 days restores the serum IGFBP-3 levels and induces 150-200 KDa complex in hypox rats. Infusion of IGF-I for 7 or 8 days causes a marked increase in the serum IGFBP-3 in hypox rats, suggesting that IGF-I may mediate the effect of GH on IGFBP-3 synthesis in hypox rats (Clemmons *et al.*, 1989; Zapf *et al.*, 1989). However, infusion of IGF-I can not induce 150-200 KDa complex in hypox rats. It was suggested that GH, but not IGF-I, induces a component which is necessary in the formation of the 150-200 KDa complex. α -subunit could be a such component. A radioimmunoassay for measuring α -subunit was developed and the serum levels of α -subunit was found to be GH-dependent (Baxter, 1990). There was a 2.2-fold increase in serum α -subunit levels in acromegaly. In GH-deficient patients, serum levels of α -subunit was reduced to 30% of normal mean value. It is unknown at present whether IGF-I can induce serum α -subunit. If α -subunit is not induced by IGF-I, the critical dependence of α -subunit on GH may explain why formation of the 150-200 KDa complex is obligately dependent on GH which cannot be replaced by IGF-I.

The stimulating effect of IGF-I on IGFBP-3 production has been

demonstrated in an *in vitro* system. IGF-I increases the concentrations of IGFBP-3 in conditioned media from rat sertoli cells (Smith *et al.*, 1990), human endometrial carcinoma HEC-1B cells, human breast carcinoma MDA-231 cells (Camacho-Hubner *et al.*, 1991), normal and transformed human fibroblasts (Conover, 1991a). The IGF-I induced increase in IGFBP-3 concentrations in human fibroblasts was demonstrated to be a type 1 receptor-independent event by several evidences which include the α IR-3 antibody against IGF-I receptor did not block IGF-I effect (Conover, 1991a). In human fibroblasts, whereas IGF-I markedly increase the production of IGFBP-3, GH has little or no effect. This may indicate the possible indirect effect of GH on IGFBP-3 *in vivo*.

Nutrition and insulin

Serum IGFBP-3 levels was reduced by 18% after a 3 day fast (Blum & Ranke, 1990b). In rats with protein-restricted diet, serum IGFBP-3 levels were decreased by 34% and restored to normal by IGF-I infusion (Thissen *et al.*, 1991). It has been reported that serum IGFBP-3 levels were decreased in diabetic patients and streptozotocin-induced diabetic rats (Baxter & Martin 1986a; Zapf *et al.*, 1989; Graubert *et al.*, 1991). In poorly controlled diabetic patients there was a 40% decrease in serum IGFBP-3 levels compared to mean value of serum IGFBP-3 in normal subjects. In STZ-induced diabetic rats, serum IGFBP-3 levels decreased with the increased dosages of STZ. The levels of serum IGFBP-3 were only 7%

of the control levels in diabetic rats treated with a dose of 288 mg/kg body weight of STZ. In the rat which received similar dosage of STZ and received insulin (10 U/100 g per day) for 2 days, serum IGFBP-3 levels raised to 160% of untreated values. Low dose of insulin (3 U/100 g per day) treatment had little effect on serum IGFBP-3 in these animals. However, the same dose of insulin had significant effect in lowering the elevated IGFBP-1 levels in these diabetic rats. It seems that IGFBP-3 is less sensitive than IGFBP-1 to insulin status. IGFBP-3 fell more slowly than IGFBP-1 rose in diabetic rats with progressive severity of diabetes, and low-dose insulin treatment had no effect on IGFBP-3 but lowered IGFBP-1. Since IGFBP-3 has been shown to be IGF-I-dependent, the low levels of serum IGFBP-3 may result from the low levels of IGF-I in diabetic rats in which the GH secretion is decreased. Consistent with this hypothesis, infusion with IGF-I increased serum IGFBP-3 levels in diabetic rats (Zapf et al., 1989).

Other hormones

It has been reported that estradiol increased the amounts of IGFBP-3 in conditioned medium from osteoblast-like rat calvaria cells (Schmid et al., 1989a). Addition of IGF-I antibodies prevented the IGFBP-3 induction by estradiol, suggesting that IGF-I may mediate the effect of estradiol on IGFBP-3 production in these cells. Since estradiol was found to stimulate osteoblast replication via an IGF-I-dependent mechanism, the regulation of

IGFBP-3 by estradiol indicated that estradiol may control local IGF-I action not only by regulating synthesis of IGF-I but also by IGFBP. Contrary to estradiol, cortisol markedly reduced IGFBP-3 production in medium conditioned by a rat osteoblastic cell line (Schmid *et al.*, 1989b). DXM was reported to be able to inhibit IGF-binding activity in conditioned medium from Swiss 3T3 fibroblast cells. IGFBP-3 was believed to be a major component of IGFBP in this IGF-binding activity (Corps & Brown, 1991). In these 3T3 fibroblast cells, production of IGFBP-3 was stimulated by 50-150% by the bombesin, vasopressin, platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). Transforming growth factor- β (TGF β) and epidermal growth factor were shown to stimulate the production of IGFBP-3 in human skin fibroblast conditioned medium (Martin & Baxter, 1988; 1991). In contrast, TGF- β was reported to inhibit IGFBP-3 secretion in porcine granulosa cells (Mondschein *et al.*, 1990).

Pregnancy

In late pregnancy, serum IGFBP-3 levels measured by radioimmunoassay are elevated almost two-fold (Baxter & Martin, 1986b). In contrast, analysis of serum IGFBP-3 by Western ligand blotting shows extremely reduced levels of IGFBP-3 in serum from pregnant women (Guidice *et al.*, 1990; Hossenlopp *et al.*, 1990). Similar reduction of serum IGFBP-3 levels in pregnancy as detected by Western ligand blotting, was reported in rats (Davenport *et al.*,

1990) and in mice (Fielder *et al.*, 1990). The decreased serum IGFBP-3 levels was believed to be related to the protease produced during pregnancy (Davenport *et al.*, 1990; Fielder *et al.*, 1990; Guidice *et al.*, 1990; Hossenlopp *et al.*, 1990). This pregnancy-associated protease degrades IGFBP-3 to smaller fragments. The discrepancy between high levels of serum IGFBP-3 detected by radioimmunoassay and low levels of serum IGFBP-3 detected by Western ligand blotting in pregnancy could be explained by the possibility that radioimmunoassay detects not only intact IGFBP-3 but also degraded IGFBP-3 which loses the affinity for IGF ligand but maintains its immunoreactivity. The physiological effect of decreased IGFBP-3 in pregnancy is unknown. It was speculated that reduced IGFBP-3 may facilitate dissociation of the IGFs and, hence, increase IGF bioavailability in response to the demands of gestation and uterine growth. It was reported that although serum IGFBP-3 levels measured by Western ligand blotting was greatly reduced, the amounts of IGF-I and IGF-II in 150 KDa IGF-IGFBP complex were increased during late pregnancy (Gargosky *et al.*, 1991). The increase in 150 KDa IGF-I and IGF-II during late pregnancy indicates that IGFBP-3 levels should be increased since the 150 KDa complex is composed of IGFBP-3, IGF-I or IGF-II and α -subunit. It was proposed that IGFBP-3 stability or affinity for IGF radioligands is altered during the late pregnancy, and this may explain the failure of the Western ligand blotting to detect IGFBP-3. Recently Suikkari and Baxter (1992) demonstrated that IGFBP-3 in human pregnancy serum is functionally normal. Both the IGF

binding function and the α -subunit-binding function of IGFBP-3 are unaffected in pregnancy. A notable difference is that, IGFBP-3 in pregnancy serum is able to bind IGF-I but unable to bind iodo-IGF-I ($[^{125}\text{I}]$ -IGF-I or nonradioactive iodo-IGF-I), whereas IGFBP-3 in nonpregnancy serum binds IGF-I and iodo-IGF-I similarly (Suikkari & Baxter, 1991). This may be the reason for the under estimating of IGFBP-3 levels in pregnancy by radioligand binding methods. The inability of IGFBP-3 in pregnancy serum in binding to iodinated IGFs may result from the pregnancy protease, but this hypothesis has not been demonstrated yet. The physiological significance of the change in IGFBP-3 in pregnancy remains to be determined.

Biological activities

The reported biological activities of IGFBP-3 include inhibition and enhancement of IGF-I action depending on the experimental conditions. DeMellow and Baxter (1988) showed that co-incubation of IGF-I with IGFBP-3 resulted in inhibition of IGF-I-stimulated thymidine incorporation in human neonatal fibroblasts. The inhibition was complete when IGF-I and IGFBP-3 were equimolar. In contrast, preincubation of the cells with IGFBP-3 caused a potentiation of the subsequent IGF-I effect. A similar result was observed in bovine fibroblasts (Conover et al., 1990b) in which $[^3\text{H}]$ aminoisobutyric acid uptake was chosen as an evaluating parameter for the effect of IGF-I. In Conover's experiments, the potentiating effect of IGFBP-3 was found to be associated with

increased membrane-associated IGFBP-3 which resulted in the increase in IGF-I binding. It was suggested that soluble IGFBP-3 (co-incubation of IGF-I with IGFBP-3) inhibits IGF-I action by sequestering the IGF-I and preventing receptors interaction. However, the molecular mechanism for membrane-associated IGFBP-3 (preincubation) in potentiating the action of IGF-I is more obscure. Conover (1991c) showed that both glycosylated and nonglycosylated forms of IGFBP-3 potentiate IGF-I action in cultured bovine fibroblasts. This ability appears to relate to change of IGFBP-3 into a lower molecular form and to IGFBP-3-induced modifications in type I IGF receptor reactivity (Conover, 1992).

In contrast to above observations, Blum *et al.* (1989) reported that the IGF-I/IGFBP-3 complex stimulated [³H] thymidine incorporation in baby hamster kidney and human neonatal fibroblasts and there was no indication of IGFBP-3 binding to cells. They also showed that the hourly repeated addition of small amounts of free IGF-I to the cells stimulated more DNA synthesis compared to the addition of a single large concentration of IGF-I. They proposed that IGFBP-3 may act as a reservoir to supply continuously appropriate levels of IGF-I to the receptors. Constant low levels of IGF-I may well be more efficient in producing an optimal biological response since IGF-I receptor has been shown to be down-regulated by IGF-I. Consistent with this hypothesis, it was reported that the addition of IGFBP-3 prevented IGF-I induced

receptor down-regulation and cellular desensitization in bovine fibroblasts (Conover & Powell, 1991b). An IGFBP-3 purified from porcine follicular fluid was shown to inhibit estradiol and progesterone production as well as FSH-stimulated [³H] thymidine uptake in granulosa cells (Bicsak et al., 1990a). These inhibitions were believed to result from the sequestration of endogenously produced IGF-I by IGFBP-3 and direct interaction with the cell to elicit the inhibition, although how IGFBP-3 interacts with the cells is unknown.

In vivo, it has been shown that IGFBP-3 may prolong the half-life of IGF in the circulation. Zapf et al. (1986) showed that the half-lives of IGF-1 injected into normal rats and hypox rats in which IGFBP-3 was dramatically decreased were 4 hours and 20-30 min, respectively. Two IGF-I mutants, which have normal affinity for the IGF-I receptor but drastically reduced affinity for the IGFBP-3, have serum half-lives that are 4 times shorter than that of intact IGF-I in the rat (Cascieri et al., 1988b). It was suggested that the IGFBP-3/IGF/ α -subunit complex (150 KDa complex) in circulation may represent a storage form of IGFs. How does IGF release from 150 KDa complex and get access to tissues is unknown.

1.2.4 IGFBP-4

Mohan et al. (1989) purified a IGF-binding protein which was called hIGFBP-4 later on from human osteosarcoma cell conditioned

medium. This IGFBP-4 is different from the IGFBP-1, -2, and -3 in size, N-terminal sequence, and amino acid composition. Another group (Shimonaka *et al.*, 1989) isolated a novel IGFBP from adult rat serum. The N-terminal amino acid sequence of this protein is essentially identical to the hIGFBP-4. The complete primary structures of IGFBP-4 were subsequently obtained from the cDNA clones for rIGFBP-4 and hIGFBP-4 (Shimasaki *et al.*, 1990; LaTour *et al.*, 1990a). The deduced amino acid sequence of the cDNA revealed a signal sequence of 21 residues followed by a mature protein consisting of 233 amino acids for the rat, while the human homolog contains an additional 4 amino acid sequence. The predicted molecular mass of mature hIGFBP-4 is 26 KDa. hIGFBP-4 and rIGFBP-4 have 92% homology in amino acid sequences. Both hIGFBP-4 and rIGFBP-4 contain an additional two cysteines at the midportion of the molecules, while the locations of the remaining 18 cysteines are conserved as other IGFBPs. IGFBP-4 contains no RGD sequence, but a single potential N-linked glycosylation site. Human IGFBP-4 gene is located at chromosome 17 (Shimasaki *et al.*, 1991a), but both human and rat genes for IGFBP-4 have not been cloned and characterized yet. The gene expression of IGFBP-4 in rat is widespread and is highly active in the liver. The size of IGFBP-4 mRNA in rat tissues is about 2.6 Kb. hIGFBP-4 mRNA is expressed in human bone cells and skin fibroblasts. PTH increases both IGFBP-4 mRNA and protein levels in normal human bone cells, and the effect of PTH is mediated through a cAMP mechanism. IGF-I and IGF-II induce a decline in IGFBP-4 levels in the conditioned medium by

cell from the adult human fibroblast cell line N3652 (Neely & Rosenfeld, 1992). These changes do not require IGF binding to the type 1 or type 2 IGF receptor. Human serum IGFBP-4 levels were shown to decrease dramatically in pregnancy as detected by Western ligand blotting (Davies *et al.*, 1991). The IGFBP-2 and -3 were also reduced in pregnancy serum. The authors claimed that there are at least three proteases in pregnancy, each specific for an IGFBP and activates independently, since different inhibitions of the proteases have differential effects on each IGFBPs. In addition, these proteases do not cause complete degradation of IGFBPs. Instead, they functionally modify IGFBPs with a lower affinity for the IGFs to prevent their detection by ligand blotting. There is little information about the biological activities of IGFBP-4 available at present. Mohan *et al.* (1989) reported that IGFBP-4 could inhibit basal, IGF-stimulated proliferation of chicken calvarial cells, and serum-stimulated proliferation of cloned osteoblast cells. IGFBP-1 had no effect on proliferation of these cells. Erickson *et al.* (1992) reported that IGFBP-4 mRNA is present in adult rat ovaries, being localized almost exclusively to granulosa cells of atretic Graafian (antral) follicles. IGFBP-4 mRNA undergoes changes during the estrous cycle, with the highest levels of message occurring at 1000 h on estrus followed by a rapid fall to low levels during diestrous days 1 and 2. The activation of IGFBP-4 gene transcription in the granulosa cells of cohort Graafian follicles may result in their destruction, because the IGFBP-4 binds follicular fluid IGF-I,

rendering it inactive. A purified IGFBP from human colon adenocarcinoma cell conditioned medium, which has N-terminal 32 amino acid sequence identity with human IGFBP-4, was shown to inhibit the proliferation of the same cell line (Culouscou & Shoyab, 1991). Rosen et al. (1992) recently reported that the serum 24/25 KDa IGFBP levels were increased in elderly women with hip and spine fractures. This 24/25 KDa IGFBP was suspected as IGFBP-4. Since the serum immunoreactive PTH levels were increased in these patients and PTH was shown to be able to increase both IGFBP-4 mRNA and protein levels in normal human bone cells (LaTour et al., 1990b), the elevated serum 24/25 KDa IGFBP may be due to the increased serum PTH levels in these patients.

1.2.5 IGFBP-5

Shimasaki et al. (1991a) isolated a novel IGFBP which has a unique N-terminal amino acid sequence from adult rat serum and named it IGFBP-5. Using the information of N-terminal amino acid sequence of IGFBP-5, they performed polymerase chain reaction (PCR) to construct a hybridization probe and subsequently cloned rIGFBP-5 cDNA from rat ovary library and hIGFBP-5 cDNA from human placenta library. Kiefer et al. (1991a) designed primers according to amino acid sequences in the conserved regions of known human IGFBPs and performed PCR using human osteosarcoma cDNA as a template. One unique sequence of the PCR product was used to screen the osteosarcoma cDNA library, and full length of cDNA clone of hIGFBP-

5 was found. The deduced amino acid sequences of human and rat IGFBP-5 cDNAs revealed mature proteins of 252 amino acid residues. The calculated molecular weight of the mature proteins are 28,553 and 28,428 for human IGFBP-5 and rat IGFBP-5, respectively. The amino acid sequence homology between human and rat IGFBP-5 is very high (97%). Neither of the proteins contains N-linked glycosylation site and RDG sequence. The locations of 18 cysteines in both proteins are conserved as other IGFBPs. The rIGFBP-5 gene is expressed in many tissues, including kidney, adrenal, stomach, intestine, spleen, heart, lung, brain and liver. The expression is the highest in kidney among these tissues. The size of rIGFBP-5 transcript in Northern blot is 6 Kb. hIGFBP-5 gene is located on chromosome 5. The regulation and biological function of IGFBP-5 are not clear. An IGFBP preparation (containing IGFBP-5 and -6) purified from the conditioned medium of human osteosarcoma cell was shown to enhance IGF-I stimulated osteoblast mitogenesis (Andress *et al.*, 1991).

A novel IGFBP was purified from human bone extract (Bautista *et al.*, 1991). This protein has an apparent molecular mass of 29 KDa and has several fold higher affinity for IGF-II than for IGF-I. Nine amino acids in N-terminal amino acid sequence are identical to human IGFBP-5. It was shown to potentiate the proliferative actions of IGF-II on bone cells. Whether this protein represents a new IGFBP or a variant of IGFBP-5 remains to be determined.

1.2.6 IGFBP-6

IGFBP-6 was originally purified from human cerebrospinal fluid where this binding protein predominates (Roghani *et al.*, 1989). Subsequently, it was purified from the conditioned medium of an SV40-transformed human fibroblast cell line (Martin *et al.*, 1990), a human lung fibroblast cell line (Forbes *et al.*, 1990), serum (Zapf *et al.*, 1990; Shimasaki *et al.*, 1991b), and the conditioned medium of human osteosarcoma cells (Andress *et al.*, 1991). cDNAs for IGFBP-6 have been cloned from human osteosarcoma, human placenta cDNA libraries, and rat ovary cDNA library (Kiefer *et al.*, 1991b; Shimasaki *et al.*, 1991b). The deduced amino acid sequence revealed that human mature IGFBP-6 contains 213 amino acids with a calculated molecular weight of 22,610. Mature rat IGFBP-6 contains 201 amino acid, with a calculated molecular weight of 21,461. The amino acid sequence homology between human and rat IGFBP-6 is 72%. Human IGFBP-6 contains one potential N-linked glycosylation site near the C-terminal. N-linked glycosylation sites are not present in the rat IGFBP-6. A notable feature of human and rat IGFBP-6 is that they lack two and four of the 18 conserved cysteines, respectively, that are present in all other five IGFBPs. These missing cysteines result in the absence of the conserved Gly-Cys-Gly-Cys-Cys sequence which is present in all other IGFBPs in the N-terminal region of the molecule. Human IGFBP-6 was shown to preferentially bind to IGF-II compared to IGF-I (Martin *et al.*, 1990). The N-terminal differences from the other

IGFBPs may contribute to this preferential binding. An approximately 1.1 Kb transcript of IGFBP-6 was detected in adult human liver, HepG2 cells and human osteosarcoma. Human brain contains low levels of 1.1 Kb IGFBP-6 mRNA but contains a more of the 2.0 Kb IGFBP-6 mRNA which is also present in adult liver. In rat, a 1.3 Kb IGFBP-6 mRNA was found in many tissues, including testis, intestine, adrenal, kidney, stomach, spleen, heart, lung, brain and liver. The highest expression was found in the lung. Human IGFBP-6 gene is located on chromosome 12. The regulation and biological function of IGFBP-6 are unknown. However, the presence of IGFBP-6 in the cerebrospinal fluid and its selective affinity for IGF-II suggests a specific regulatory role of IGFBP-6 in the action of IGF-II in the nerve system.

Relationship among the six cloned IGFBPs

The amino acid sequence comparison shows that the six cloned IGFBPs are the distinct members of a family of related protein. Each of mature IGFBPs has 14-20 cysteines which are clustered in the N- and the C-terminal thirds of the molecules. Both human and rat IGFBP-4 contain 20 cysteines, whereas human and rat IGFBP-6 contain 16 and 14 cysteines, respectively. All other IGFBPs contain 18 cysteines. The positions of cysteines are highly conserved and may contribute to the formation of particular three dimensional structures. Based on amino acid sequence homologies, the IGFBPs can be divided into three portions. The conserved

regions are located at the N- and C-terminal portions, whereas the middle portions of the molecules are the most divergent. Both human and rat IGFBP-3, -4 as well as human IGFBP-6 contain N-linked glycosylation sites, whereas other IGFBPs do not. Only IGFBP-1 and -2 have the RGD sites. Human IGFBP-1 and -3 genes are located on the same chromosome but are separated genes. Other human IGFBP genes are located on different chromosomes. The most abundant of mRNAs for IGFBP-1, -2, -3, and -4 are detected in the liver, whereas the highest expression of IGFBP-5 and -6 are found in the kidney and the lung, respectively. Some characteristics of the six cloned IGFBPs are outlined in the Table 1-6.

1.3 Research Objectives

It is well known that GH deficiency, glucocorticoid excess and diabetes are associated with growth retardation, but the mechanisms involved are poorly understood. Since IGF-I plays an important role in the postnatal longitudinal growth and its action is modulated by the IGFBPs, possible alterations of IGF-I production and action due to changes of IGFBPs may contribute to the growth retardation seen in GH deficiency, glucocorticoid excess and diabetes. Decreased expression of IGF-I in GH deficiency was well documented but IGFBP-1 was considered to be GH independent by some investigators. Some clinical studies suggested that serum IGFBP-1 levels may correlate inversely with serum GH concentrations. Whether GH can regulate IGFBP-1 gene expression

Table 1-6. Some characteristics of six cloned IGFBPs

IGFBP	Molecular weight (calculated)	N-linked glycosylation sites	RGD number	Cysteine number	mRNA size (Kb)	Chromosome location
1 (human)	25,274	0	1	18	1.6	7
(rat)	29,600	0	1	18	1.6	
2 (human)	31,325	0	1	18	1.6-1.8; 4.4	2
(rat)	29,564	0	1	18	2.0	
3 (human)	28,700	3	0	18	2.5	7
(rat)		4	0	18	2.6	
4 (human)	25,978	1	0	20	2.3	17
(rat)		1	0	20	2.6	
5 (human)	28,553	0	0	18		5
(rat)	28,428	0	0	18	6.0	
6 (human)	22,610	1	0	16	1.1; 2.0	12
(rat)	21,461	0	0	14	1.3	

and at what step are unknown. In glucocorticoid excess, reported serum IGF-I levels are low, normal, or high. In addition, glucocorticoid excess is associated with the increase in some uncharacterized IGF inhibitors in serum. Before the studies of this thesis, there was no report describing the expression of IGF-I and IGFBPs in glucocorticoid excess conditions. In diabetes, it has been shown that serum IGF-I levels are decreased and IGF-I mRNA abundance in liver, lung, kidney and uterus is reduced. However, it is unknown whether the defect of IGF-I expression is general in diabetes, and there is no report on expression of IGFBPs in spontaneously diabetic rats.

To answer the above questions, a series of studies were conducted to reach the following specific aims:

1. To examine the GH regulation of IGFBP-1 expression and define the molecular mechanisms involved.
2. To examine the effects of DXM on expression of IGF-I, IGFBP-1 and IGFBP-3 genes and molecular mechanisms involved.
3. To examine the effects of diabetes and exogenous insulin on expression of IGF-I, IGFBP-1, -2, -3, and -4 genes.

Chapter 2: Materials and Methods

2.1 Materials

Restriction enzymes, DNA and RNA modifying enzymes were supplied by Pharmacia (Canada) Inc. (Baie d'Urfe, Que), Boehringer-Mannheim (Indianapolis, IN), and Promega Biotec. (Madison, WI). [α - ^{32} P]dCTP, [α - ^{32} P]CTP and [γ - ^{32} P]ATP were purchased from New England Nuclear (Lachaire, Que). ^{125} I-IGF-I was obtained from Amersham Canada (Oakville, Ont). Nick translation reagents were obtained from Amersham Canada (Oakville, Ont). 3'-end labelling kit was purchased from Boehringer-Mannheim (Indianapolis, IN). Oligo (dT)-cellulose was supplied by Sigma (St. Louis, MO). Nitrocellulose filter was obtained from Micron Separation INC. (Westboro, MA). Kodak X-Omat AR film was purchased from Eastman Kodak (Rochester, NY). Human pituitary GH was kindly provided by Dr. I. Worsley, Department of Physiology, University of Manitoba. DXM, STZ were obtained from Sigma (St. Louis, MO). Human insulin was purchased from Connaught Novo (Novo Industries, Toronto, Ont). Glucose oxidase system was supplied by Sigma (St. Louis, MO).

2.2 Animals

Specific protocol of animal studies will be described in appropriate chapters. In general, male Sprague-Dawley rats, 80-100

g body weight, 4 weeks of age, or female Sprague-Dawley rats, 75-100 g body weight, were obtained from the University of Manitoba breeding facility (Winnipeg, MB). Hypox male Sprague-Dawley rats, 75-120 g body weight were obtained from Charles River Canada (St. Constance, Que) and acclimatized in holding cages for approximately 1-2 weeks. Only animals which demonstrated less than 3 g weight gain/week were considered hypox. Prediabetic male Biobreeding/Worchester (BB/W) rats were obtained from the National Institutes of Health colony at the University of Massachusetts. Age-matched rats of a non-diabetes-prone strain of BB/W rats were obtained from the same source. Diabetes occurred at approximately 3 month of age in diabetes-prone BB/W rats.

2.3 Molecular probes

The cDNA and synthetic oligonucleotide probes used in this study are listed in Table 2-1. It is needed to mention that pNB29 is a cDNA encoding a constitutively expressed rat heat shock-like protein. In some experiments, pNB29 probe or a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe was used as a loading control. The sequences of the rat IGFBP-2, -3 and -4 oligonucleotides are shown in Table 2-1a. Oligonucleotides with sequences anticomplementary to nucleotides 614-673, 189-248 and 624-683 of the published sequences for rat IGFBP-2, -3 and -4 respectively (Brown *et al.*, 1989; Shimasaki *et al.*, 1989; Shimasaki *et al.*, 1990) were synthesized by DNA Synthesis Laboratory,

University of Manitoba (Winnipeg, MB). It has been shown that amino acid sequences of the six known IGFBPs are highly homologous in the regions located at the N- and C-terminal portions whereas the middle portions of the molecules are the most divergent (Shimasaki *et al.*, 1991). Therefore, the sequences of the rat IGFBP-2 and IGFBP-4 oligonucleotides were chosen from the divergent middle portion of the corresponding proteins. Although the sequence of the rat IGFBP-3 oligonucleotide was chosen from the N-terminal portion of rat IGFBP-3, this oligonucleotide probe appeared to be specific as indicated by the detecting of a single 2.6 Kb band in Northern blot.

2.4 Labelling of probes

A. Nick-translation: Gel-purified DNA insert and intact recombinant plasmid were labelled with [^{32}P] by nick-translation. In this method, the DNA polymerase I initiates DNA synthesis at free 3' OH groups, which are exposed as nicks in the unlabelled DNA. The nicks are generated in random location by a limited digest of the DNA to be labelled with DNase I. Nick-translation reaction was carried out as described by the supplier of the kit and using 70 μCi of 3,000 Ci/mmol [α - ^{32}P]dCTP. Usually 100-200 ng of DNA was used per reaction and specific activities of $1-5 \times 10^8$ cpm/ μg DNA were obtained. The unincorporated label was removed by chromatography of the nick-translation reaction over a 4.0 ml column of G100 sephadex equilibrated with TE pH 7.5.

Table 2-1. cDNA probes

Probes	Source	Length	Reference
IGF-I cDNA	Rat	521 bp	Murphy <i>et al.</i> , 1987a
IGFBP-1 cDNA	Rat	1486 bp	Murphy <i>et al.</i> , 1990
pNB29 cDNA	Rat	1477 bp	deToledo <i>et al.</i> , 1987
c-myc genomic DNA	Human	8300 bp	Dalla <i>et al.</i> , 1982
28S genomic DNA	Human	5025 bp	Gonzalez <i>et al.</i> , 1985
β -Actin cDNA	Chicken	2000 bp	Cleveland <i>et al.</i> , 1980
GAPDH cDNA	Rat	1241 bp	Tso <i>et al.</i> , 1985

Table 2-1a. The sequences of the rat IGFBP-2, -3 and -4 oligonucleotide probes

Probe	Sequence	Reference
IGFBP-2	5' CCCTCCGAGTGGTCATCCTCACTGTCTGCA ACCTGCTGTGGGGTGGCGCCCACGCGTCTC 3'	Brown <i>et al.</i> , 1989
IGFBP-3	5' GCACCAGCTCCGTGCACGCGGGCGCGGTGG GCGGAGGCGCGCACTGGGCCAGCGCACGCG 3'	Shimasaki <i>et al.</i> , 1989
IGFBP-4	5' GGGGCACAGGACGGGGTTCCTCCCGAGGTG TCCCCACAACCTTCATCTTGCTCCGATCTC 3'	Shimasaki <i>et al.</i> , 1990

B. End labelling: 5' end labelling was used for labelling the synthetic oligonucleotides. The enzyme T4 polynucleotide kinase will specifically transfer the gamma ^{32}P label from ATP to a 5' OH group of DNA. Usually 300 ng oligonucleotide was incubated at 37⁰ C for 1 hr with T4 polynucleotide kinase and [gamma- ^{32}P] ATP in the presence of a kinase buffer (500 mM Tris, pH 7.4, 100 mM MgCl_2 , 50 mM dithiotreitol (DTT), 10 mM spermidine). The reaction was stopped with 0.5 M EDTA, pH 8.0 (28 mM final). The unincorporated label was removed using DE-52 column and labelled oligonucleotide was eluted by 4 ml of high salt solution (10 mM Tris, pH 7.5, 500 mM NaCl, 0.2 mM EDTA).

2.5 Large scale plasmid DNA preparation

Plasmid DNA was prepared by using the method described by Maniatis et al. (1984) with modification. A single bacterial colony carrying the plasmid of interest was used to inoculate 5 ml of LB (Luria-Bertani) medium containing ampicillin. The bacteria was grown overnight in a shaking incubator at 37⁰ C. This 5 ml of overnight culture was added to 500 ml of LB medium containing ampicillin in a 2-liter flask. Incubate the culture for 2.5-3.0 hr at 37⁰ C with vigorous shaking (300 cycles/minute on a rotary shaker) until the growing bacterial cells had reached an optical density (O.D. 600) of 0.4-0.7. 2.5 ml of a solution of chloramphenicol (34 mg/ml in ethanol) was then added and the culture allowed to shake for a further 16 hr at 37⁰ C with vigorous

shaking. The broth was then centrifuged at 3,000 rpm (4⁰ C) for 10 min in PR7000 centrifuge (International Equipment Company, Needham Hts, MA). The supernatant was discarded and the bacterial pellet was then resuspended in 10 ml of lysis buffer containing lysozyme (25 mM Tris, pH 8.0, 10 mM EDTA, 0.9% glucose, 5 mg/ml lysozyme). The solution was transferred to 50 ml sterile screw-cap Oakridge tubes (Nalgene Co., Rochester, NY) and placed on ice for 15 min with occasional swirling and shaking of the tubes. Ten ml of 0.2 N NaOH, 0.2% SDS solution was added and incubated for 15 min on ice. Ten ml of 3 M sodium acetate pH 5.2 was added and the mixture was left on ice for 30 min. The tube was then centrifuged in a JA-20 rotor at 15,000 rpm for 30 min at 4⁰ C. The approximately 30 ml supernatant was transferred to a 50 ml cloning tube and the DNA precipitated with 0.6 volume of isopropanol. After incubating at room temperature for 20 min, the DNA was recovered by centrifugation at 3,000 rpm in PR7000 centrifuge for 20 min at room temperature. The pellet was then desiccated and redissolved in 6.0 ml of TE pH 7.5. Cesium chloride (CsCl, 6.6 g) and 0.5 ml ethidium bromide (10 mg/ml) were added the TE and tube contents mixed. The tube was then centrifuged at 2,000 rpm (20⁰ C) in PR7000 centrifuge for 10 min. The supernatant was transferred to a Beckman quick-seal Ti75 polyallomer centrifuge tubes. The Ti75 centrifuge tubes were filled with mineral oil, balanced and heat sealed. Centrifugation was at 55,000 rpm in a Beckman Ti75 rotor at 20⁰ C for 18 hr. After centrifugation, the lower plasmid band made visible by long wavelength ultraviolet light, was removed by an 18-

gauge needle fitted with 3 ml syringe and transferred to a 15 ml sterile polypropylene tube. It was then extracted with isoamyl alcohol 3 times or more to remove the ethidium bromide (EtBr). Two ml of sterile water was added followed by 2 volumes of cold ethanol and placed at -20° C overnight. The plasmid DNA was then centrifuged at 10,000 rpm (4° C) for 30 min, the pellet washed with 70% ethanol, desiccated and dissolved in TE pH 7.5.

2.6 RNA extraction and RNA blot hybridization

Two different RNA extraction procedures were used during the course of these studies. The first procedure was the guanidinium isothiocyanate-cesium chloride (CsCl) cushion method (Chirgwin J, 1979). In this method, the frozen tissue was homogenized (45-60 sec) using a polytron in a solution of 4 M guanidinium isothiocyanate, 0.005% sarkosyl, 0.025 M sodium citrate and 0.007% vol/vol β -mercaptoethanol (GITC). The ratio of 0.1-0.2 g tissue/ml GITC was usually used. The homogenate was then layered onto 3 ml of 5.7 M CsCl in 0.1 M EDTA (pH 7.5) in a Beckman Ti75 polyallomer tube and centrifuged at 25,000 rpm for 16-20 hours at 20° C. After the centrifugation, the RNA pellet was dissolved in TE buffer (pH 7.5) and precipitated with ethanol. The second procedure used for RNA extraction was a modified version of that described by Chomczynski and Sacchi (1987). Approximately one hundred minigram of frozen tissue was homogenized with 1 ml of GITC solution. Sequentially, 0.1 ml of 2 M sodium acetate, pH 4.0, 1 ml of phenol,

and 0.2 ml of chloroform-isoamyl alcohol mixture (49:1) were added to the homogenate, with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 sec and cooled on ice for 15 min. Sample was centrifuged at 10,000 g for 20 min at 4° C. After centrifugation, RNA present in the aqueous phase was transferred to a fresh tube, mixed with two volume of cold ethanol, and then placed at -20° C overnight to precipitate the RNA. Sedimentation at 10,000 g for 20 min was again performed and the resulting RNA pellet was dissolved in 0.3 ml of GITC solution, transferred into a 1.5-ml Eppendorf tube, and precipitated with two volume of cold ethanol at -70° C for 1 h. After centrifugation in an Eppendorf centrifuge for 20 min at 4° C the RNA pellet was resuspended in 75% ethanol, sedimented and dissolved in TE buffer (pH 7.5). The concentration of RNA was determined by spectrophotometer at O.D. A260.

Poly (A)⁺-enriched mRNA was isolated from total RNA by one cycle of oligo(dT) cellulose affinity chromatography (Aviv & Leder, 1972). This method relies on base pairing between the poly (A)⁺ residues at the 3' end of mRNAs and the oligo(dT) residues coupled to the cellulose column matrix. Poly(A)⁻ RNA species are not bound and are readily washed off the column. The bound poly(A)⁺ RNA is eluted by lowering the amount of salt in the column buffer.

The RNA was separated by electrophoresis in a 1% agarose/2.2 M formaldehyde gel containing 0.5 µg/ml EtBr in 1X 3-morpholino-

propanesulfonic acid buffer. The RNA gel was then photographed by ultraviolet transillumination and transferred to nitrocellulose filters (Thomas, 1980). In some experiments the abundance of specific mRNA in individual rat samples was quantitated by slot blot hybridization. For this purpose appropriate serial dilutions of RNA (5-40 μ g) to yield a linear densitometric signal were immobilized on nitrocellulose paper using a filtration vacuum manifold (Schleicher and Schuell, Keene, NH). The blot was baked for 2 h at 80⁰ C under vacuum or at 60⁰ C overnight. Nitrocellulose filters were prehybridized for at least 2 h at 42⁰ C in a solution containing 50% formamide, 20 mM NaH₂PO₄, pH 7, 4XSSC (1X = 0.15 M NaCl, 0.015 M sodium citrate), 2 mM EDTA, 4X Denhardt' solution (1X = 0.02% BSA, ficoll, and polyvinylpyrrolidone), 0.1% SDS and 100 μ g/ml sonicated, denatured salmon sperm DNA. Hybridization was performed at 42⁰ C overnight in the same solution for cDNA probe. For the oligonucleotide probes hybridization was performed at 42⁰ C overnight in a solution identical to that described above except that it contained 25% formamide. After hybridization the blot which was probed with cDNA, was washed initially at room temperature with 2 X SSC and 0.1% SDS for 15-30 min and finally washed for 30 min at 65⁰ C in 0.1 X SSC and 0.1% SDS. The membrane, which was hybridized with oligonucleotide probe, was washed at room temperature in 2 X SSC, 0.1% SDS for 15-30 min, followed by wash at 42⁰ C in the same solution for 15-30 min. The filters were also hybridized with 28s ribosomal probe or pNb29 probe as a control for RNA loading. Autoradiography was performed by exposing Kodak X-

Omat AR film (Eastman Kodak, Rochester, NY) to the nitrocellulose filters at -70° C in the presence of an enhancing screen for up to 7 days. Multiple exposures were obtained to ensure for linearity of autoradiographic signal. Autoradiographs were analyzed using a Bio-Rad video densitometer with a program 1DPACK (Bio-Rad, Mississauga, Ont). Each densitometric value came from a single scan.

2.7 Nuclear run-off assay

Liver was homogenized in 3 vol of buffer containing 0.32 M sucrose, 3 mM calcium chloride, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl (pH 8.0), 0.1 % Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1 mM spermidine. The homogenate was filtered through cheesecloth and centrifuged at 1000 X g for 10 min at 4° C. The crude nuclear pellet was homogenized in the same buffer containing 2 M sucrose and 5.0 mM magnesium acetate and layered over a cushion of 2 M sucrose in buffer and centrifuged at 80,000 X g for 1 h at 4° C. Nuclei, 5×10^7 , were suspended in 400 μ l of buffer containing 20% glycerol, 30 mM Tris-HCl (pH 8.0), 5 mM magnesium chloride, 0.05 mM EDTA, 3 mM DTT, 150 mM potassium chloride, and 0.5 mM each of ATP, GTP, and UTP. The nascent RNA chains were then elongated in the presence of 250 μ Ci of [^{32} P]CTP (800 Ci/mmol) at 28° C for 30 min. The incubation was terminated by the addition of RNase-free DNase (Promega Biotec, Madison, WI) and proteinase K (25 μ g/ml), and the RNA was extracted using the

guanidinium isothiocyanate-cesium chloride technique(Chirgwin et al, 1979). Nitrocellulose strips containing 15 μ g of immobilized, denatured plasmid DNA were incubated with 5×10^6 dpm/ml of labelled RNA in a solution containing 10 mM TES (pH 7.4), 10 mM EDTA, 0.2% SDS, and 0.3 M NaCl. After 36 h of hybridization at 65⁰ C the filters were initially washed in 2 X SSC at 65⁰ C for 1 h, followed by wash in 2 X SSC with 10 μ g/ml RNase A at 37⁰ C for 30 min and wash in 2 X SSC at 37⁰ C for 1 h. As a control for nonspecific hybridization, pGEM-3 DNA was included, while pABB (28S ribosomal probe, Gonzalez, et al., 1985) , pc-myc (Dalla et al., 1982), and pACTIN (Cleveland et al., 1980) served as a positive control. Quantitation of RNA was determined by densitometric scanning of autoradiographs after subtraction of nonspecific background hybridization and normalization for pABB signals.

2.8 IGF-I radioimmunoassay

IGF-I was measured in acid extracts of serum (Daughaday et al, 1980) from individual rats by a nonequilibrium radioimmunoassay using antisera distributed by the National Hormone and Pituitary Program (Baltimore, MD). Prior to radioimmunoassay the sera were extracted by acid ethanol to separate IGF-I from IGFBPs. To 100 μ l of a mixture of 87.5% ethanol and 12.5% 2 N HCl (vol/vol) in 1.5-ml polypropylene Microfuge tubes, 25 μ l of serum was added with thorough mixing. After standing for 30 min at room temperature, the tubes were centrifuged at 10,000 rpm in an Eppendorf centrifuge

for 5 min at room temperature. Supernatant (25 μ l) was removed and transferred to a fresh polypropylene tube, and 10 μ l of 0.885 M Tris base was added and mixed. After neutralization, the extracts were subjected to radioimmunoassay. Iodinated trace was obtained from Amersham Canada Ltd., and a serum pool derived from 10 normal male rats (250-300 g body weight) was used as a standard. Results were expressed as units per ml where 1 U is equivalent to 940 ng human recombinant IGF-I (Amgen Biologicals, Thousand Oaks, CA). The sensitivity of the assay in our laboratory is 0.015 U/ml with a within-assay variation of 9 %. All samples in which a comparison was made were analyzed in the same assay.

2.9 Immunoblotting

A polyclonal antisera raised against a synthetic peptide, encoding amino acids 155-173 of the predicted sequence of rat IGFBP-1 (Murphy *et al.*, 1990) coupled to keyhole limpet hemocyanin was used. Serum samples were analyzed on a 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. Nitrocellulose paper was incubated overnight at 4⁰ C with a 1 in 1,000 dilution of antisera in Tris-buffered saline containing 0.05% Tween 20 and 0.1% BSA. Alkaline phosphatase conjugated goat anti-rabbit immunoglobulin was used to visualize the first antibody (GIBCO/BRL, Burlington, Ont).

2.10 Ligand blotting

Two types of assays as described by Unterman *et al.* (1989) and Hossenlopp *et al.* (1986) were used. Firstly total available serum IGF-I binding activity was quantified by a dot-blot assay. Serial dilutions (in 150 mM NaCl, 20 mM Tris, pH 7.4) of sera from individual rats were immobilized on nitrocellulose paper using a filtration vacuum manifold (Schleicher and Schuell, Keene, NH, USA). In second type of assay, insulin-like growth factor-binding proteins were separated by size. Serum samples pooled from the various treatment groups were boiled, resolved on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose paper. In both types of assay, nitrocellulose membranes were sequentially blocked with 3% (v/v) Nonidet P-40, 10 g/L BSA, and 0.1% (v/v) Tween 20 in 150 mM NaCl, 10 mM Tris, pH 7.4, then incubated overnight at 4⁰ C with 300,000 cpm/ml [¹²⁵I] IGF-I (Amersham Canada, Oakville, Ont) in 10 g/L BSA, 0.1% Tween 20, 150 mM NaCl, 10 mM Tris-HCl pH 7.4, and 0.5 mg/ml of sodium azide. After incubation, membranes were washed 2 times in 150 mM NaCl, 10 mM Tris-HCl pH 7.4, 0.1% Tween 20 at 4⁰ C for 15 min each time, followed by wash 3 times in 150 mM NaCl, 10 mM Tris-HCl pH 7.4 at 4⁰ C for 15 min each time. Blot was dried and then exposed to Kodak X-Omat AR film at -70⁰ C for up to 7 days. Autoradiographs were analyzed by densitometry as described in the chapter 2.6.

2.11 Determination of serum glucose, glycosylated haemoglobin

Serum glucose concentrations were measured as described by the

supplier using a glucose oxidase system (Sigma, St, Louis, MO). In briefly, 25 μ l of serum was diluted with 500 μ l of water. Five ml of combined enzyme-colour Reagent solution containing glucose oxidase, peroxidase and O-dianisidine dihydrochloride was added and mixed thoroughly. Samples were incubated at room temperature for 45 min. At the end of incubation period, absorbance (A) at 425-475 nm was read and the glucose concentration was calculated by comparing to the glucose standard. Glycosylated haemoglobin was determined using a Kit (Pierce, Rockford, IL).

2.12 Statistical analysis

For comparison between treatment groups, individual rat RNA samples or RNA samples from pooled tissue were treated as independent observations. The initial slope of the linear part of the relationship between the amount of total RNA applied and the OD area, as determined by densitometry of slot-blot autoradiograms, was used to compare control and treatment groups. The mean slope of the appropriate control group was arbitrarily attributed a value of 100% or 1 and the ratio of the slope obtained for each RNA sample to this mean was determined. Significant differences between treated and control rats were determined for various parameters using Student's t-test.

Chapter 3: Transcriptional regulation of rat IGFBP-1 expression by GH

3.1 Introduction

GH has been clearly demonstrated to be able to stimulate IGF-I gene expression, and thereby exert its growth-promoting effects (Sara & Hall, 1990). In addition, GH may regulate IGFBPs and therefore modulates the actions of IGF-I. It has been shown that IGFBP-3 expression is regulated by GH. In hypox rats, serum IGFBP-3 levels are reduced. Injection or infusion of GH can restore the serum IGFBP-3 levels and induce 150-200 KDa complex in hypox rats. The stimulating effect of GH on IGFBP-3 production may be mediated by IGF-I, because IGF-I can markedly increase the serum IGFBP-3 levels in hypox rats and stimulate the production of IGFBP-3 in many cells *in vitro* while GH has little or no effect in an *in vitro* system (Clemmons *et al.*, 1989; Zapf *et al.*, 1989; Conover, 1991a). The effect of GH on IGFBP-2 is not clear. Although hepatic IGFBP-2 mRNA levels and serum IGFBP-2 concentrations are elevated in hypox rats (Ooi *et al.*, 1990; Orłowski *et al.*, 1990b), GH treatment alone cannot suppress the elevated hepatic IGFBP-2 mRNA levels (Margot *et al.*, 1989; Ooi *et al.*, 1990). IGFBP-1, in contrast to IGFBP-3, was thought to be GH independent (Baxter & Cowell, 1987a; Forbes *et al.*, 1988). Some clinical studies showed that serum IGFBP-1 levels are elevated in GH-deficiency patients and reduced in patients with acromegaly (Drop *et al.*, 1984; Pova *et al.*, 1984b; Busby *et al.*,

1988b; Hall *et al.*, 1988), suggesting that IGFBP-1 may be inversely regulated by GH. However, the effect of GH on IGFBP-1 gene expression has not been reported. The present study was undertaken to examine the gene expression of IGFBP-1 in hypox and pituitary-intact rats, and the effect of GH on the IGFBP-1 gene expression in hypox rats.

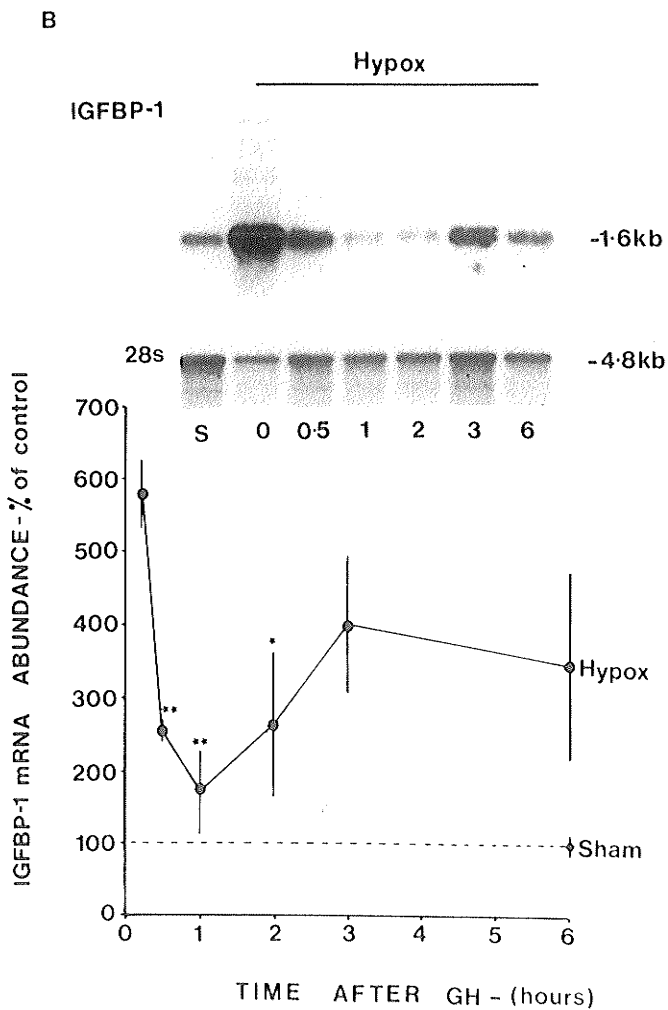
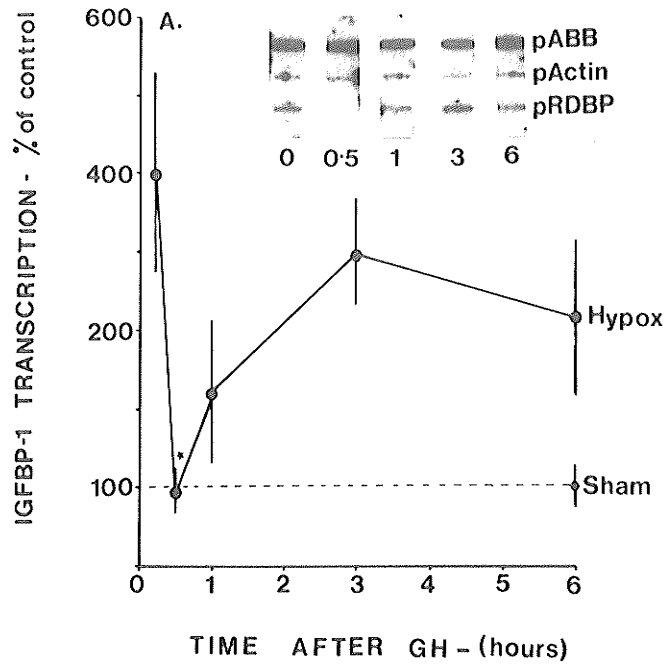
3.2 Experimental design

Male hypox Sprague-Dawley rats, 80-100 g body weight and 3 weeks of age, were acclimatized in holding cages for approximately 2 weeks. Age-matched sham-operated rats were used as control. Hypox rats were killed at various times after a single i.p. injection of 100 μ g/100 g body wt human pituitary GH (3 IU/mg).

3.3 Results

A significant increase (5.8-fold) in hepatic IGFBP-1 mRNA abundance was observed in hypox rats compared to that in pituitary-intact rats. I next examined the effects of GH on hepatic IGFBP-1 transcription and IGFBP-1 mRNA abundance. In hypox rats, transcription of IGFBP-1 was significantly increased (4-fold) compared to that in pituitary-intact sham-operated rats (Fig. 3-1A). The magnitude of increase in IGFBP-1 transcription in hypox rats was similar to that of increase in IGFBP-1 mRNA abundance. GH administration rapidly reduced IGFBP-1 transcription. Thirty

Fig. 3-1. The effects of GH on hepatic IGFBP-1 gene expression in hypox rats. A. The effect of GH on IGFBP-1 transcription. The insert shows a representative experiment where nuclei from untreated and GH treated hypox rats have been used. Data from four separate experiments have been plotted below. Data have been expressed as a percentage of IGFBP-1 transcription in sham-operated control rats. B. A representative Northern blot of hepatic RNA from sham-operated rats (S) or hypox rats at various times after the administration of GH. Ten micrograms of poly (A)⁺ RNA per lane were loaded. The filters have been hybridized with a IGFBP-1 cDNA and, subsequently, with pABB (28S ribosomal probe) as a control for gel loading. In the lower part of B data from three separate experiments have been quantitated and represent the mean $\pm$ SEM. IGFBP-1 mRNA abundance has been normalized for the loading control and has been plotted as a percentage of that in the sham-operated control rats. ★, $p < 0.05$; ★★, $p < 0.005$ (for the difference between GH-treated and untreated hypox rats, respectively).



minutes after GH administration, IGFBP-1 transcription was not significantly different from that in pituitary-intact sham-operated rats. There was a gradual increase in IGFBP-1 transcription over the subsequent 5 h after GH injection. Similarly, IGFBP-1 mRNA abundance decreased after GH administration; however, the pattern of decline and recovery was slightly delayed compared to the data obtained in the *in vitro* transcription assay (Fig. 3-1B).

3.4 Discussion

The results of this study clearly showed that IGFBP-1 gene expression is inversely regulated by GH. I believe this is the first report in which regulation of IGFBP-1 by GH occurring at mRNA levels has been demonstrated. Furthermore, I clearly proved that the acute GH inhibits transcription rate of IGFBP-1 gene. Results from the colleagues in our laboratory showed that an increase in the abundance of a 30 KDa IGFBPs which presumptively present IGFBP-1 was detected in both hepatic extracts and serum from hypox rats. When hypox rats were treated with GH for 8 days, a decrease in this 30 KDa IGFBP from hepatic extracts was seen. These data suggest that the change in IGFBP-1 mRNA levels in our experimental condition may result in the relevant change in IGFBP-1.

Indeed our results *in vivo* cannot prove that the inhibitory effect of GH on IGFBP-1 is direct. It is possible that the effects of GH are mediated by other mechanisms. Insulin has been

consistently reported to inhibit IGFBP-1 production both *in vivo* and *in vitro* (Suikkari *et al.*, 1988; 1989; Lewitt & Baxter, 1989; Conover & Lee, 1990a; Singh *et al.*, 1990; Powell *et al.*, 1991; Unterman *et al.*, 1991). Also GH has been shown to stimulate pancreatic insulin gene expression and release (Sandler *et al.*, 1987; Formby *et al.*, 1988). One possible reason for the inhibitory effect of GH on IGFBP-1 gene expression is that, GH stimulates insulin production and release, then high insulin level inhibits IGFBP-1 gene expression. It has been reported that the low levels of IGFBP-1 in acromegaly were unrelated to mean GH but were inversely related to elevated fasting insulin levels (Holly *et al.*, 1991). In another study in which GH was given on alternate days, the serum levels of both IGF-I and insulin were significantly higher in GH-treated than in placebo-treated women during the 11-day follow-up period (Tapanainen *et al.*, 1991). These results suggest that long term elevated GH increases serum IGF-I and insulin which results in low levels of IGFBP-1. However, the effect of acute GH on production of IGFBP-1 may not be secondary to the elevated levels of insulin. A rapid decrease in IGFBP-1 gene transcription rate and mRNA levels after GH treatment argues strongly against the view that the effect of acute GH on IGFBP-1 gene expression is mediated by insulin. In a study *in vitro* (Unterman *et al.*, 1991), a significant fall of the abundance of IGFBP-1 mRNA in rat H4IIE hepatoma cells began at 2 h (not at 1 h) after incubation with 100 nM insulin, while in our study the fall of IGFBP-1 mRNA abundance after GH injection occurred at much

earlier time points: significantly fall at 0.5 h and reached the lowest level at 1 h after GH injection. If the effect of acute GH on hepatic IGFBP-1 mRNA is mediated by insulin, one should expect that decreased IGFBP-1 gene expression after GH treatment starts at a time after the insulin effect takes place. The results to the contrary suggest that the effects of acute GH on hepatic IGFBP-1 mRNA is unlikely mediated by an increase in insulin levels. A report, which is consistent with our results, came from the same study of Tapanainen *et al.* (1991) in human. They showed that serum IGFBP-1 levels decreased significantly during the first 4 h after acute GH administration, while serum levels of insulin did not change during the same period. In another study, octreotide which is a somatostatin analogue was used to treat acromegalic patients (Ezzat *et al.*, 1991). After one month treatment with octreotide, an injection of 100 μ g of octreotide maximally attenuated GH to 18% of control levels, and IGF-I to 46% of control levels after 4 h. Serum IGFBP-1 levels were increased 1.5-fold after 2 h and 3-fold after 4 h, while serum insulin levels did not change during this period. These observations suggest a non-insulin-mediated mechanism of the acute GH effect on IGFBP-1.

Unterman *et al.* (1991) showed that treatment with 1 μ M rat GH for 3 h or 24 h did not alter the abundance of IGFBP-1 mRNA in rat H4IIE hepatoma cells. However, these results still could not exclude the possibility of the direct inhibitory effect of GH on IGFBP-1 gene expression. In my *in vivo* study, the levels of

hepatic IGFBP-1 mRNA reached the lowest point at 1 h, but began to rise at 2 h and reached control (hypox) levels at 3 h after the injection of GH. While in Unterman's *in vitro* study, IGFBP-1 mRNA levels were measured at 3 h or 24 h after the addition of GH and they did not examine the IGFBP-1 mRNA levels at earlier time points. Therefore, the possibility of change in IGFBP-1 mRNA levels at earlier time points after the incubation with GH can not be excluded. Another explanation for the negative results of Unterman is that the rat H4IIE hepatoma cells may have lost GH receptors and therefore do not response to GH. Undoubtedly, a suitable system and a detailed study *in vitro* are needed to demonstrate whether GH has direct effects on IGFBP-1 gene expression.

The observations reported here not only demonstrated that IGFBP-1 gene expression is enhanced in the hypox rat but also prove that GH is able to acutely down-regulate IGFBP-1 gene expression at the transcriptional level. These results clearly indicate that IGFBP-1 expression is not GH-independent in the rat.

Chapter 4: Regulation of Expression of IGF-I and IGFBPs by Dexamethasone

4.1 Introduction

Glucocorticoid excess, whether iatrogenic or endogenous, inhibits somatic growth in both man and experimental animals (Blodgett *et al.*, 1956; McArthur *et al.*, 1972; Loeb, 1976). The long acting glucocorticoids are particularly potent in this regard and limit their therapeutic usefulness in children. The mechanisms whereby glucocorticoids excess exerts this effect are unclear. In the adult human, glucocorticoid treatment has been found to be associated with attenuated GH responses to a number of pharmacological and physiological stimuli which include GH-releasing hormone (GHRH) (Frantz *et al.*, 1969; Nakagawa *et al.*, 1969; Takeda *et al.*, 1974; Hotta *et al.*, 1988). However, both potentiating and inhibitory effects of glucocorticoids on stimulated GH release have been reported, depending on the duration of pituitary exposure (Casanueva *et al.*, 1988; Burguera *et al.*, 1990). Furthermore, a recent report from Miell *et al.* (1991) showed that basal GH levels significantly increase after dexamethasone treatment in adult man. In children, there was no change in serum GH concentration in relation to corticosteroid therapy (Morris *et al.*, 1968). In the rat, glucocorticoid treatment enhances the pituitary GH response to GHRH both *in vitro* and *in vivo* (Vale *et al.*, 1983; Wehrenberg *et al.*, 1983). In

addition, the glucocorticoid, dexamethasone has been shown to stimulate GH production in cultured rat pituitary tumour cells by increasing the GH gene transcription rate (Martial *et al.*, 1977; Yu *et al.*, 1977; Spindler *et al.*, 1982). These results suggest that the effect of glucocorticoid on somatic growth is unrelated to the inhibitory action on pituitary GH release. The observation that the administration of GH can not restore normal growth in the children with glucocorticoid excess (Ward *et al.*, 1966; Morris *et al.*, 1968) supports the hypothesis that the effect of glucocorticoid excess may be related to impairment of GH action rather than GH concentration. Glucocorticoid excess is also known to inhibit bone mineralization, and collagen formation (Daughaday & Mariz, 1962; Silbermann & Maor, 1978). An important factor involved in the mechanisms whereby glucocorticoid excess retards growth may be the impairment of either the production or the actions of IGF-I. Earlier studies demonstrated that glucocorticoids inhibited sulphate incorporation into cartilage both *in vivo* and *in vitro* (Bostrom *et al.*, 1964; Phillips *et al.*, 1975; Tessler & Salmon, 1975; Keret *et al.*, 1976). Although IGF activity was consistently reported to be low, circulating IGF levels have been reported to be low, normal, or high in patients with Cushing's syndrome or in those receiving glucocorticoid therapy (Van der Brande *et al.*, 1974; Elders *et al.*, 1975; Furlanetto *et al.*, 1977; Thoren *et al.*, 1981; Gourmelen *et al.*, 1982). These differences may, in part, result from the different assay techniques used: bioassay, receptor assays, protein binding

and immunoassays. Gourmelen *et al.* (1982) have shown in a study of children and adolescents with Cushing's syndrome accompanied by growth retardation, that circulating IGF-I levels were not low and there was no significant difference in IGF-I levels before and after treatment of Cushing's syndrome despite resumption of growth. They concluded that the stunted growth associated with hypercorticism could not be attributed to IGF deficiency. Unterman and Phillips (1985) have shown that glucocorticoid administration is associated with an increase in circulating somatomedin inhibitors and a consequent steroid-induced fall in net somatomedin activity. They have suggested these steroid-induced inhibitors may explain the impaired growth observed in children with hypercorticism. However, these inhibitors have not been completely purified and characterized. The relationship of the IGF inhibitors to the IGFBPs remains to be determined.

These observations seem to imply that growth retardation seen in glucocorticoid excess results from the impaired action of the IGF caused by uncharacterized IGF inhibitors, rather than IGF production. Recent studies have emphasized the importance of local tissue IGF-I acting as an autocrine or paracrine fashion in the growth response (D'Ercole *et al.*, 1984). Furthermore, the IGF-I is complexed with IGFBPs in circulation and tissue fluids, and the IGF-I actions are believed to be modulated by these IGFBPs. The present study was undertaken to investigate the possibility that the potent glucocorticoid, DXM may have an effect on expression of

IGF-I and IGFBPs in rats.

4.2 Experimental design

Male Sprague-Dawley rats, 80-100 g body wt, 4 weeks of age, were used. Hypox male rats, 100-120 g body wt were acclimatized in holding cages for approximately 1-2 weeks. For studies in which hypox rats were used, groups of five rats were killed 6 h after a single intraperitoneal (i.p.) injection of 100 μ g/100 g body wt of human pituitary GH. Control hypox rats received an i.p. injection of an equivalent volume of saline and were killed 6 h later. This time was chosen since it was the earliest time point at which an increase in abundance of IGF-I mRNA can be clearly detected in the tissues studies. Hypox rats were pretreated with varying doses of DXM or vehicle (15% ethanol, 85% PBS). DXM was injected i.p. 3 h before GH administration, such that rats received either 1, 6, or 60 μ g/100 g body wt. For studies of the effects of DXM on IGF-I gene expression in pituitary intact rats, groups of six rats received a single i.p. injection of DXM: 1, 6, 60, or 360 μ g/100 g body wt or vehicle. These animals were killed 9 h after DXM injection, a time point comparable to that used in the hypox rats study. The protocol of treatment in the study of effects of DXM on IGF-I gene expression is outlined in Fig. 4-1. In addition, a group of five hypox rats were given DXM only 360 μ g/100 g body wt and killed 9 h later. For studies of the effects of DXM on IGFBP-1 and -3 gene expression in pituitary intact rats, groups of four or

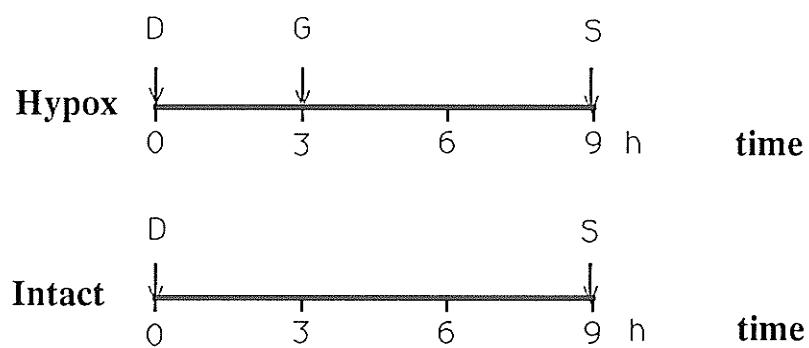


Fig. 4-1. Protocol of treatment in the study of effects of DXM on IGF-I gene expression. Arrows labelled D, G and S denote times of DXM treatment, GH treatment and death, respectively.

five rats were injected i.p. with DXM, 6 μ g/100 g body wt, and killed at various times: 1-24 h. In a separate study, groups of four or five rats were killed 1 h after a single injection of varying doses of DXM, 0.1-60 μ g/100 body wt. Control rats received an equivalent volume of vehicle (15% ethanol and 85% PBS). In the chronic study groups of five rats received daily injections for 6 days of vehicle containing 0, 1, 6, or 60 μ g of DXM. Animals were allowed food ad libitum, weighed on alternate days, and killed 9 h after the final injection on day 6.

4.3 Results

4.3.1 DXM inhibits GH-induction of IGF-I gene expression in hypox rats and reduces IGF-I mRNA levels in the pituitary intact rats

The effect of increasing amounts of DXM on GH induction of IGF-I mRNA in hepatic, renal, and pulmonary tissues of the hypox rat is shown in Fig. 4-2. In each tissue, an increase in IGF-I mRNAs was seen in the hypox rats that received GH, with the most dramatic response apparent in the liver. Pretreatment of hypox rats with DXM diminished the response seen in each of these tissues. DXM had no apparent differential effect on the relative abundance of the different sized IGF-I transcripts. In Fig. 4-3 the data obtained from slot blot analysis was quantified. Administration of GH increased IGF-I mRNA abundance approximately

Fig. 4-2. Northern blot analysis of poly (A)⁺ RNA from hypox rats treated with GH and DXM. Rats received a single injection of GH, 100 µg/100 g body wt, or an equivalent volume of vehicle. Ten micrograms of poly (A)⁺ RNA extracted from pooled tissue were analyzed. The upper panel depicts the pattern of hybridization obtained with rat IGF-I cDNA while the lower panel shows hybridization of the same filter with a rat GAPDH cDNA probe or a 28S ribosomal probe as a control for gel loading. The size of the major groups of IGF-I transcripts is shown.

LIVER

-7.0kb

IGF-I

GAPDH

LUNG

IGF-I

GAPDH

KIDNEY

IGF-I

28s

GH

-

+

+

+

+

0

0

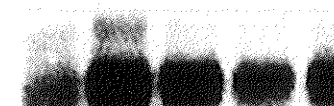
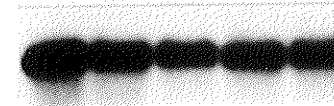
1

6

60

DEXAMETHASONE

$\mu\text{g}/100\text{g B.W.}$



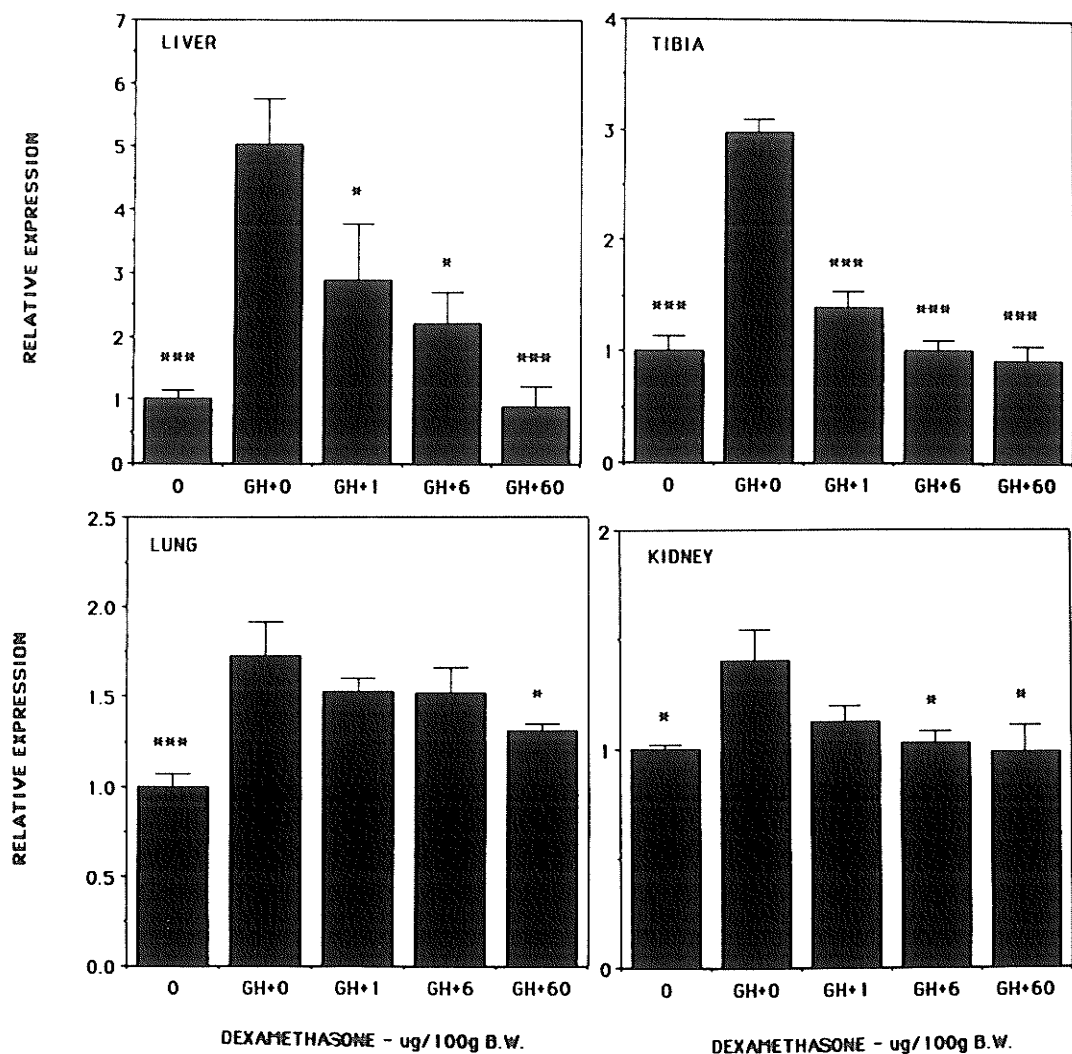


Fig. 4-3. The effect of DXM on GH-induced increase in IGF-I mRNA abundance in various tissues of the hypox rat. The relative abundance of IGF-I mRNA in total RNA extracted from individual rats was quantitated by slot-blot hybridization and has been expressed relative to hypox controls. The data represent the mean $\pm$ SEM, $n = 5$. The significant difference from the mean of the GH-treated hypox rats (column 2) is shown as *, **, and ***, which represent $p < 0.05$, $p < 0.01$, and $p < 0.005$, respectively.

5-, 3-, 2-, and 1.5-fold compared to hypox controls in the liver, proximal tibia, lung, and kidney respectively. As little as 1 μ g DXM/100 g body wt significantly reduced GH induction of IGF-I mRNA in the liver and the proximal tibia. Higher doses of DXM were required to reduce IGF-I mRNA abundance in the kidney and lung of GH-treated hypox rats. Of the various tissues examined the order of sensitivity to DXM was liver > tibia > kidney > lung. DXM alone, when administered in high doses (360 μ g/100 g body wt), caused a modest reduction in the low levels of IGF-I mRNA detected in the untreated hypox rat liver ($44.5 \pm 4.8\%$ of hypox controls, $p < 0.005$). The abundance of IGF-I mRNA in other tissues of the hypox rats was too low to allow for any firm conclusions concerning the effects of DXM on IGF-I mRNA in the untreated hypox rat. There was close agreement between the data obtained from the Northern blot analysis (Fig. 4-2) and that obtained by quantitating RNA abundance by slot-blot hybridization (Fig.4-3).

A significant increase in IGF-I concentration, approximately 2-fold, was apparent in sera from hypox rats killed 6 h after GH injection (Fig. 4-4). The lowest dose of DXM used, 1 μ g/100 g body wt, did not reduce the concentration of IGF-I in the serum despite the significant inhibition of GH-induced increase in hepatic IGF-I mRNA abundance. Hypox rats that received the higher doses of DXM had serum IGF-I levels not significantly different from the control hypox rats.

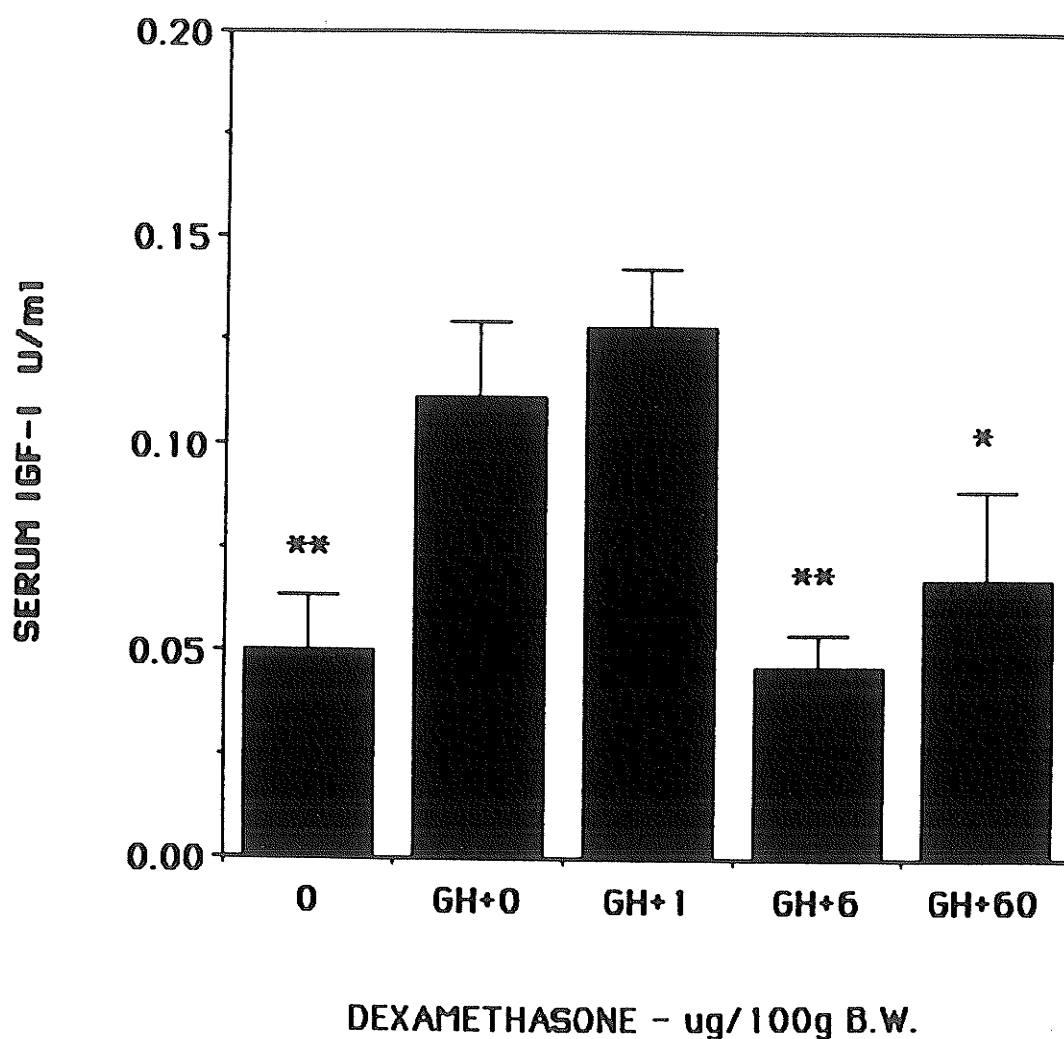


Fig. 4-4. The effects of DXM and GH on serum IGF-I concentrations in the hypox rat. The data represent the mean $\pm$ SEM, $n = 5$. The significant difference from the mean of the GH-treated hypox rats (column 2) is shown as * and **, which represent $p < 0.05$ and $p < 0.005$, respectively.

The effect of DXM on steady state IGF-I mRNA abundance was examined in pituitary-intact rats which were killed 9 h after varying doses of DXM. Fig. 4-5 shows a Northern blot of poly(A)⁺ RNA pooled from liver, lung, and kidney tissues of rats treated with differing amounts of DXM. The higher doses of DXM appeared to reduce IGF-I mRNA abundance in all tissues examined. Data obtained from slot-blot hybridization are shown in Fig. 4-6. A significant reduction in IGF-I mRNA was seen in all of the tissues examined, with the most marked effect apparent in the tibia. The kidney appeared to be less sensitive to DXM compared to the other tissues. In the liver there was a suggestion that DXM had a biphasic effect, with low and high doses having less effect than the intermediate dose.

The effect of DXM on serum IGF-I concentrations in pituitary-intact rats is shown in Fig. 4-7. In contrast to the effect of DXM on tissue IGF-I mRNA abundance, no significant reduction in serum IGF-I was seen in DXM-treated rats. Rather, a significant increase in serum IGF-I was apparent in the pituitary intact rats which received the lowest and highest doses of DXM (Fig. 4-7).

The effect of chronic administration of DXM on body wt gain, serum IGF-I concentration, and hepatic and proximal tibial IGF-I mRNA abundance is shown in Fig. 4-8. The lowest dose of DXM, 1 μ g/day, had no significant effect on body wt gain; however, the higher doses of DXM significantly retarded weight gain (Fig. 4-8A).

Fig. 4-5. The Northern blot analysis of poly (A)⁺ RNA from pituitary-intact rats treated with DXM. Ten micrograms of poly (A)⁺ RNA extracted from pooled tissue were analyzed. The upper panel depicts the pattern of hybridization obtained with rat IGF-I cDNA, while the lower panel shows hybridization of the same filter with a rat GAPDH cDNA probe or a 28S ribosomal probe as a control for gel loading. The size of the major groups of IGF-I transcripts is shown.

LIVER

-7.0kb

IGF-I

-1.8

-1.0

GAPDH

LUNG

IGF-I

GAPDH

KIDNEY

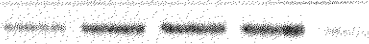
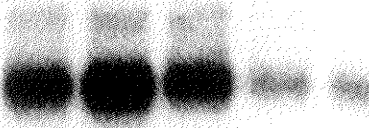
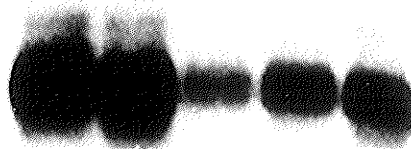
IGF-I

28s

0 1 6 60 360

DEXAMETHASONE

$\mu\text{g}/100\text{g B.W.}$



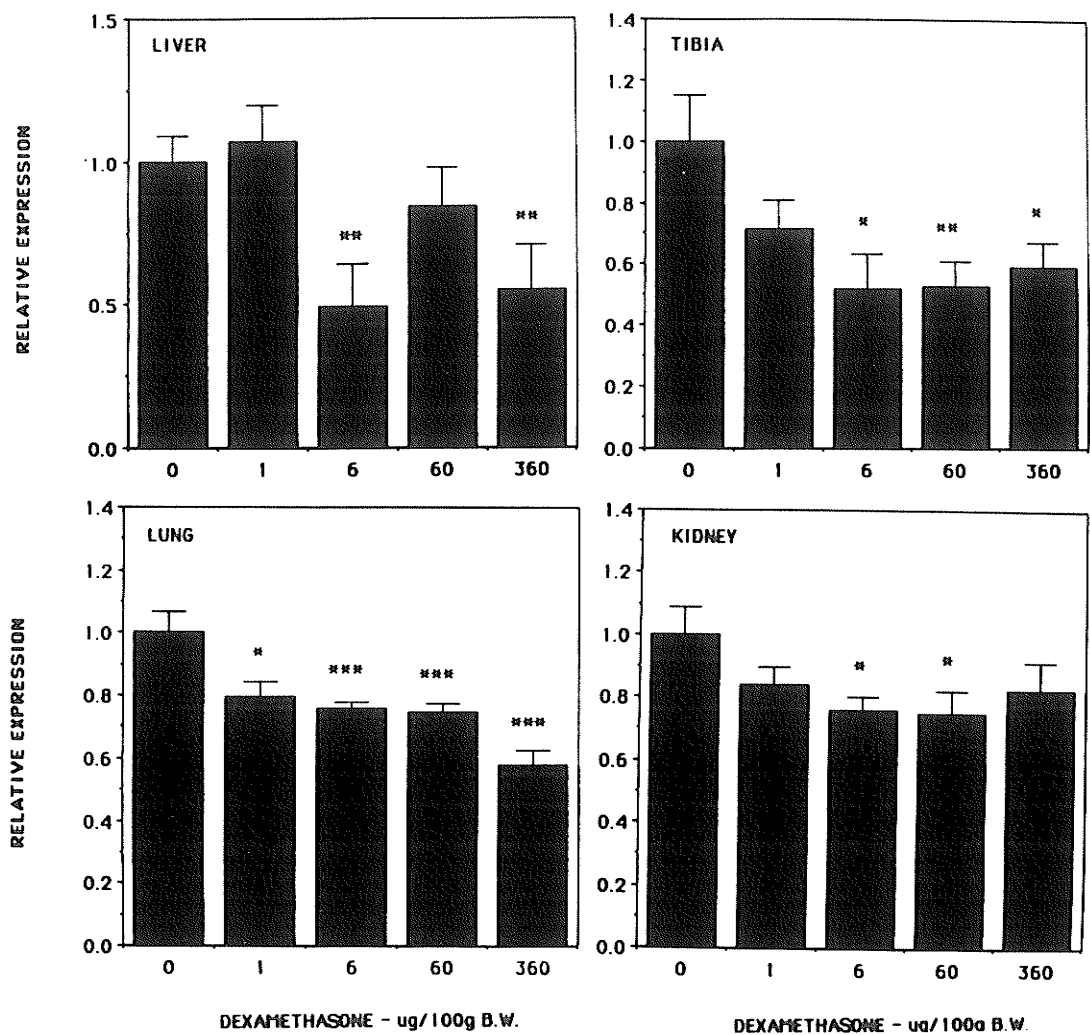


Fig. 4-6. The effect of DXM on steady state IGF-I mRNA abundance in various tissues of pituitary-intact rats. The relative abundance of IGF-I mRNA in total RNA extracted from individual rats was quantitated by slot-blot hybridization and has been expressed relative to control rats. The data represent the mean $\pm$ SEM, $n = 6$. The significant difference from the mean of the untreated control rats (column 1) is shown as *, **, and ***, which represent $p < 0.05$, $p < 0.01$, and $p < 0.005$, respectively.

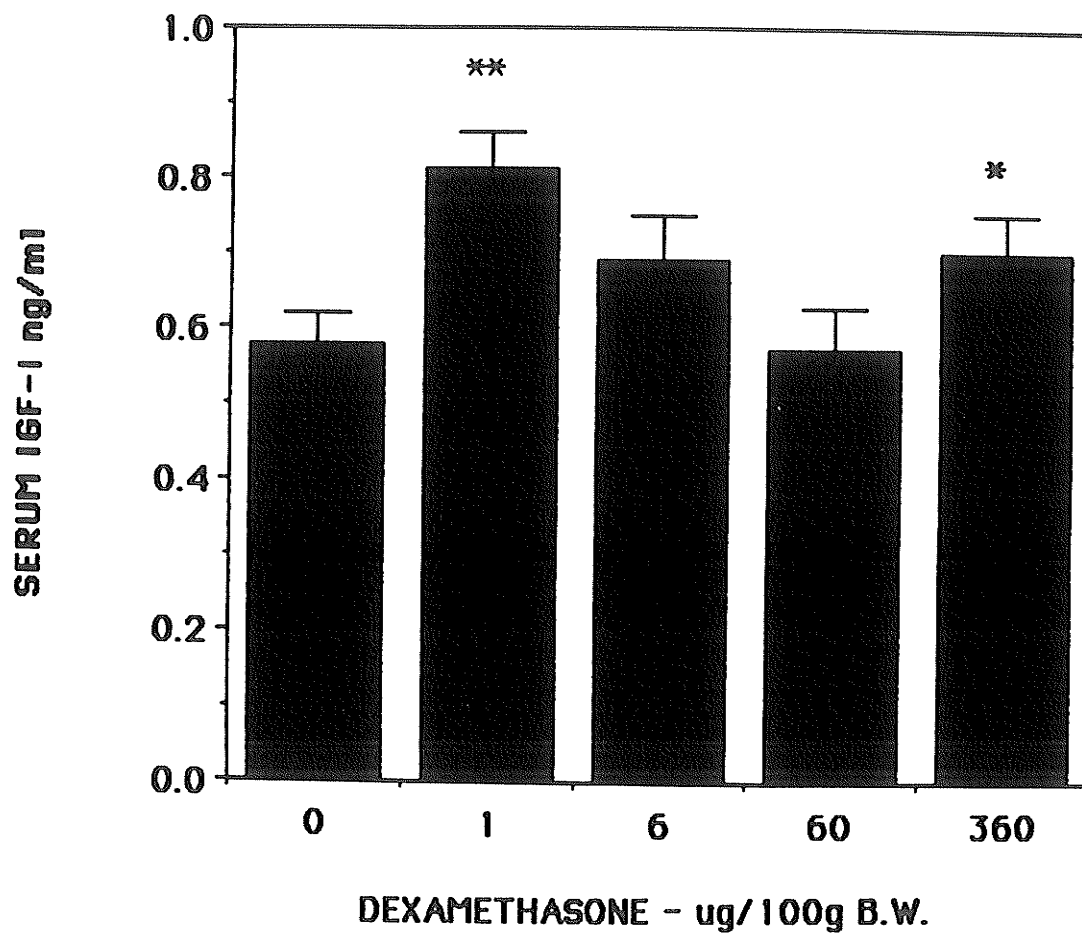
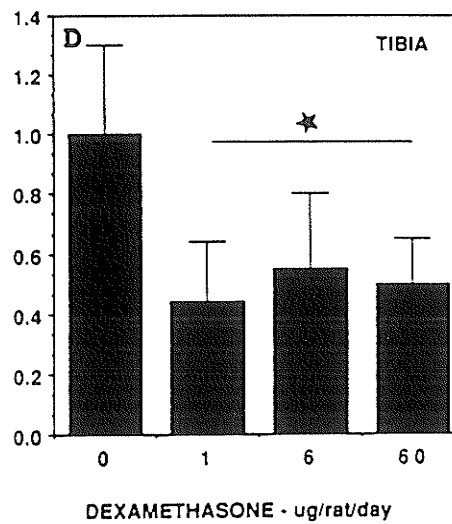
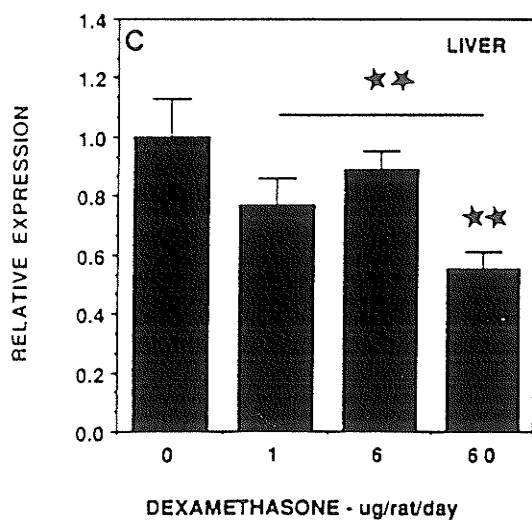
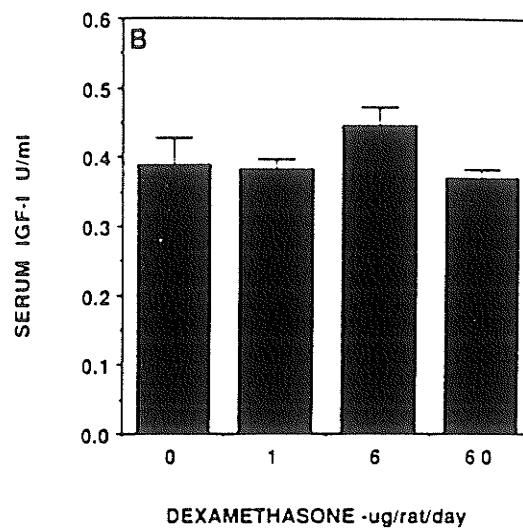
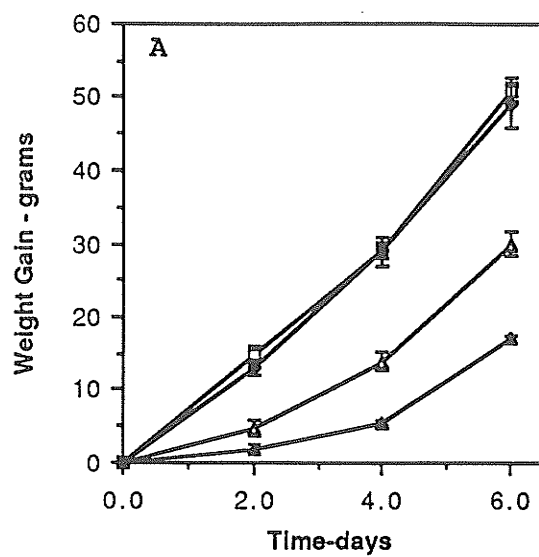


Fig. 4-7. The effects of DXM on serum IGF-I concentrations in the pituitary intact rats. The data represent the mean $\pm$ SEM, $n = 6$. The significant difference from the mean of the untreated rats (column 1) is shown as $\star$ and $\star\star$, while represent $p < 0.05$ and $p < 0.005$, respectively.

Fig. 4-8. The effect of chronic administration of DXM on body wt gain, serum IGF-I concentration, hepatic and proximal tibial IGF-I mRNA abundance in pituitary-intact rats. Panel A depicts the increase in body wt over the 6-day period in control rats ($\square$), and rats treated with 1 ($\diamond$), 6 (Δ), and 60 ($\blacktriangle$) μ g DXM/day. Panels B, C, and D show the serum IGF-I concentrations and hepatic and tibial IGF-I mRNA abundance, respectively, in each group of rats on day 6. All data represent the mean $\pm$ SEM, n = 5. The significant difference between treated groups and controls is shown as $\star$ and $\star\star$, which represent $p < 0.05$ and $p < 0.01$, respectively. The bar shows the significant difference between all rats that received DXM and the control group of rats.



The change in body weight may not well reflect the change in growth in some circumstances because body weight may also be influenced by certain factors such as change in fat content. Since growth retardation including a decrease in body weight in glucocorticoid treated rats is well documented, the measurement of body weight in this study still is an important and convenient way to determine the growth of DXM-treated rats. Serum IGF-I was not significantly different in any of the treatment groups (Fig. 4-8B). A dose-dependent decrease in hepatic and tibial IGF-I mRNA abundance was seen in rats that received DXM (Fig. 4-8, C and D). When the entire group of DXM-treated rats were considered, both hepatic and tibial IGF-I mRNA levels were significantly reduced compared to vehicle-treated controls ($p < 0.01$ and $p < 0.05$, respectively). However, because of the large scatter and small number of animal studied, the mean IGF-I mRNA levels in any individual group of DXM-treated rats did not differ significantly from control rats except in the case of hepatic IGF-I mRNA abundance in the rats which received the highest dose of DXM (Fig. 4-8C).

4.3.2 DXM increases hepatic IGFBP-1 mRNA abundance and serum IGFBP-1 concentrations in the rats

In the studies of the effect of DXM on the gene expression of IGF-I, DXM was shown to be able to inhibit GH induction of IGF-I expression in various tissues in the hypox rats. In addition, DXM at a slightly higher dose was able to retard growth and reduce

steady-state IGF-I mRNA levels in a variety of tissues. However, in these studies, despite significant growth retardation and reduced hepatic and peripheral tissue IGF-I mRNA concentrations, serum IGF-I levels were not markedly reduced. These observations have led me to speculate that DXM may have some effect on the circulating IGF binding proteins. Therefore, an investigation of the effect of DXM on the gene expression of IGFBPs was conducted. As the first step, the serum IGF total binding activity after the treatment of DXM was measured by the dot-blot ligand blotting assay. There was a dose-dependent increase in serum total IGF binding activities after the acute or chronic treatment of DXM (Fig. 4-9, A and B). Thereafter, whether IGFBP-1 and IGFBP-3 contribute to these elevated IGF binding activities was examined.

The effect of a single injection of DXM, 6 $\mu\text{g}/100$ g body wt, on hepatic IGFBP-1 mRNA is shown in Fig. 4-10. A significant increase in IGFBP-1 mRNA abundance (2.24 ± 0.25 , $p < 0.05$) was apparent 1 h after DXM administration. This was followed by a slow decline to basal levels. The dose-dependence of the DXM effect was shown in Fig. 4-11. A dose-dependent increase in IGFBP-1 mRNA abundance was seen with as little as 1 $\mu\text{g}/100$ g body wt, resulting in a significant increase. In these acute experiments no detectable increase in IGFBP-1 was detected by immunoblotting of serum samples (data not shown).

To determine whether the effects of DXM on IGFBP-1 mRNA

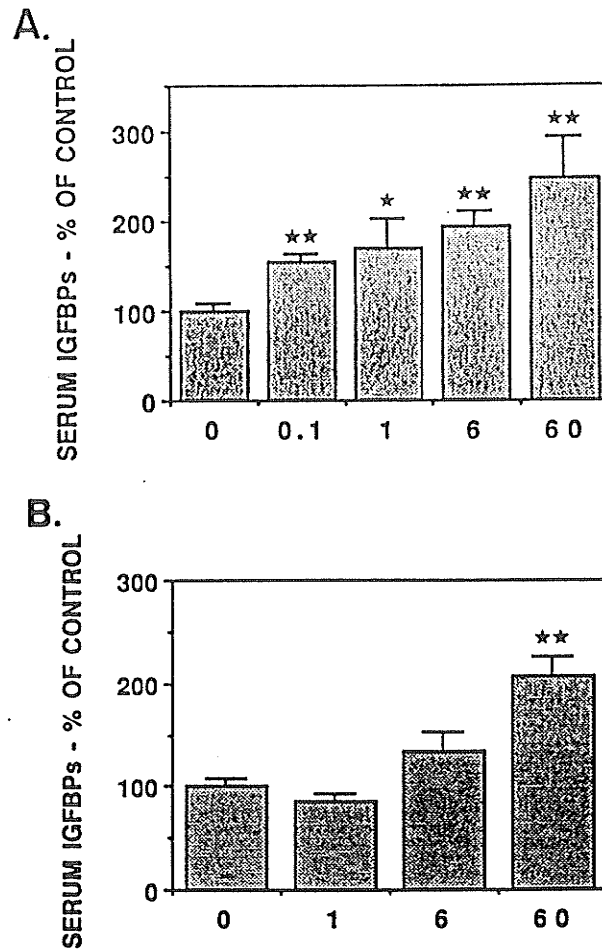
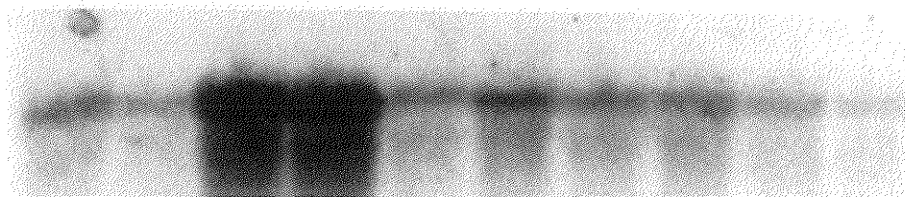


Fig. 4-9. The dose-dependent induction of serum total IGF binding activities by DXM. ^{125}I -IGF binding activities of sera from individual rats, four or five rats per group, has been quantitated using a dot-blot assay. The data represent the mean $\pm$ SEM. In panel A data from each group of rats killed 1 h after a single injection of varying amounts of DXM have been expressed as a percentage of the untreated control rats. In panel B, data from each group of rats killed after 6 days of treatment with varying amounts of DXM have been expressed as a percentage of the untreated control rats. *, ** $p < 0.05$ and $p < 0.01$, respectively, for the difference between treated and control rats.

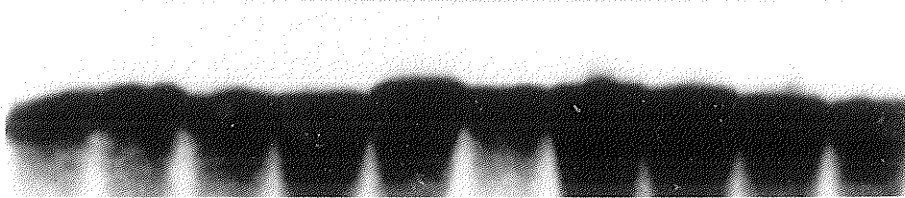
Fig. 4-10. The time course of induction of hepatic IGFBP-1 mRNA following DXM administration. Panel A depicts a representative Northern blot of total RNA (80 μ g per lane) extracted from rats at various times after a single injection of 6 μ g/100 g body wt of DXM. The pattern of hybridization obtained with a rat IGFBP-1 cDNA is shown in the upper autoradiogram. As a control for gel-loading the pattern of hybridization obtained with a rat GAPDH cDNA probe is depicted in the lower autoradiogram. In this Northern blot RNA samples from two individual rats for each time point were analyzed. In panel B data from four or five rats per time point have been quantitated by densitometry and IGFBP-1 mRNA abundance has been expressed relative to the time 0 control rats which have arbitrarily been attributed a value of 1 U. The data represent the mean $\pm$ SEM. $\star p < 0.05$.

A.

IGFBP-1



GAPDH



0 0 1 1 3 3 6 6 12 12

B.

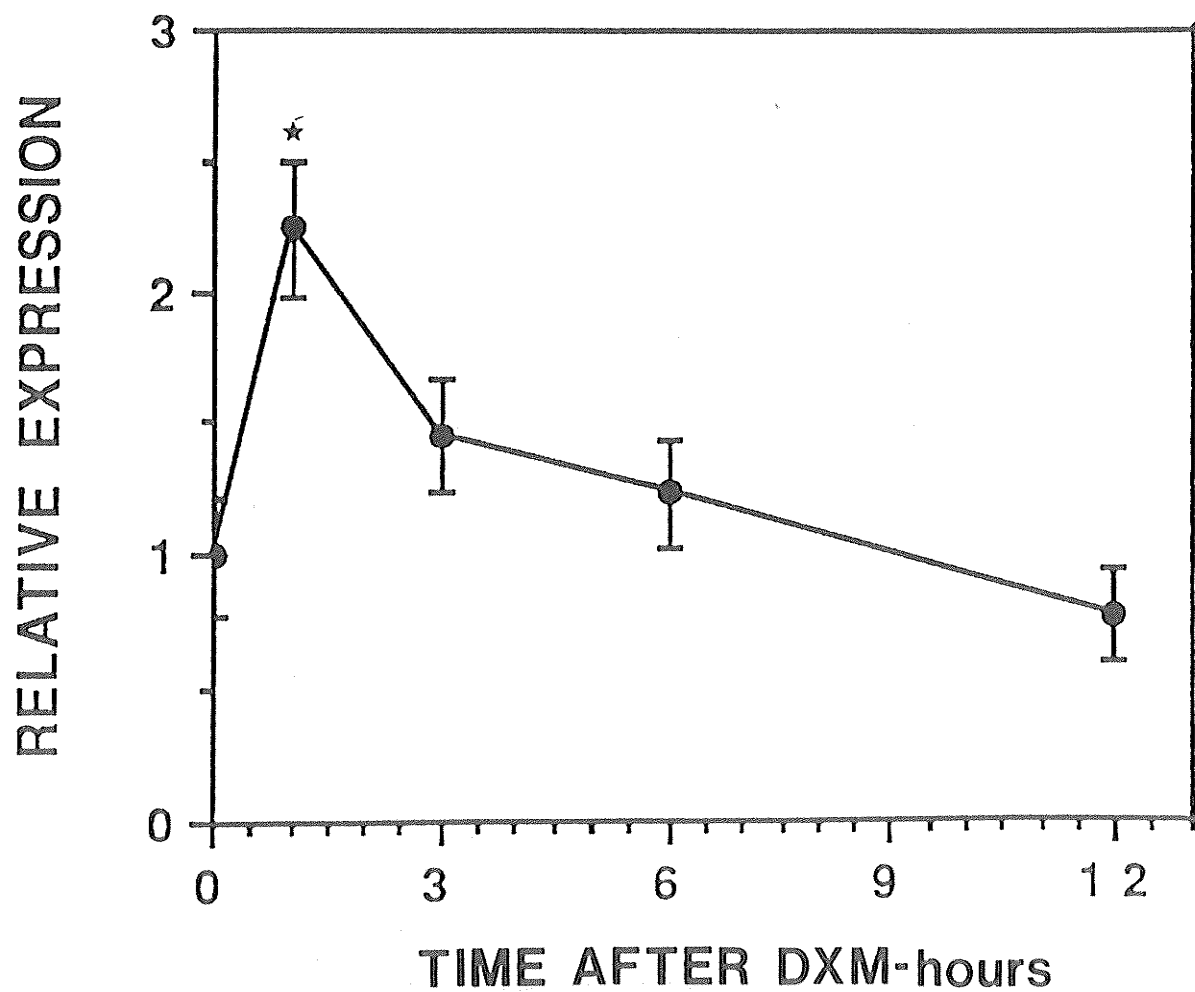
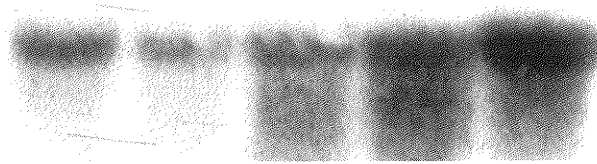


Fig. 4-11. The effect of DXM on hepatic IGFBP-1 mRNA abundance. Panel A depicts a representative Northern blot of total RNA (80 μ g per lane) extracted from rats killed 1 h after administration of varying amounts of DXM. The pattern of hybridization obtained with a rat IGFBP-1 cDNA is shown in the upper autoradiogram. As a control for gel-loading the pattern of hybridization obtained with the Nb-29 cDNA is depicted in the lower autoradiogram. In panel B data from four or five rats per treatment group have been normalized for the loading control and IGFBP-1 mRNA abundance has been expressed relative to the untreated control rats which have arbitrarily been attributed a value of 1 U. The data represent the mean $\pm$ SEM. $\star$ $p < 0.05$; $\star\star$ $p < 0.01$.

A.

IGFBP-1

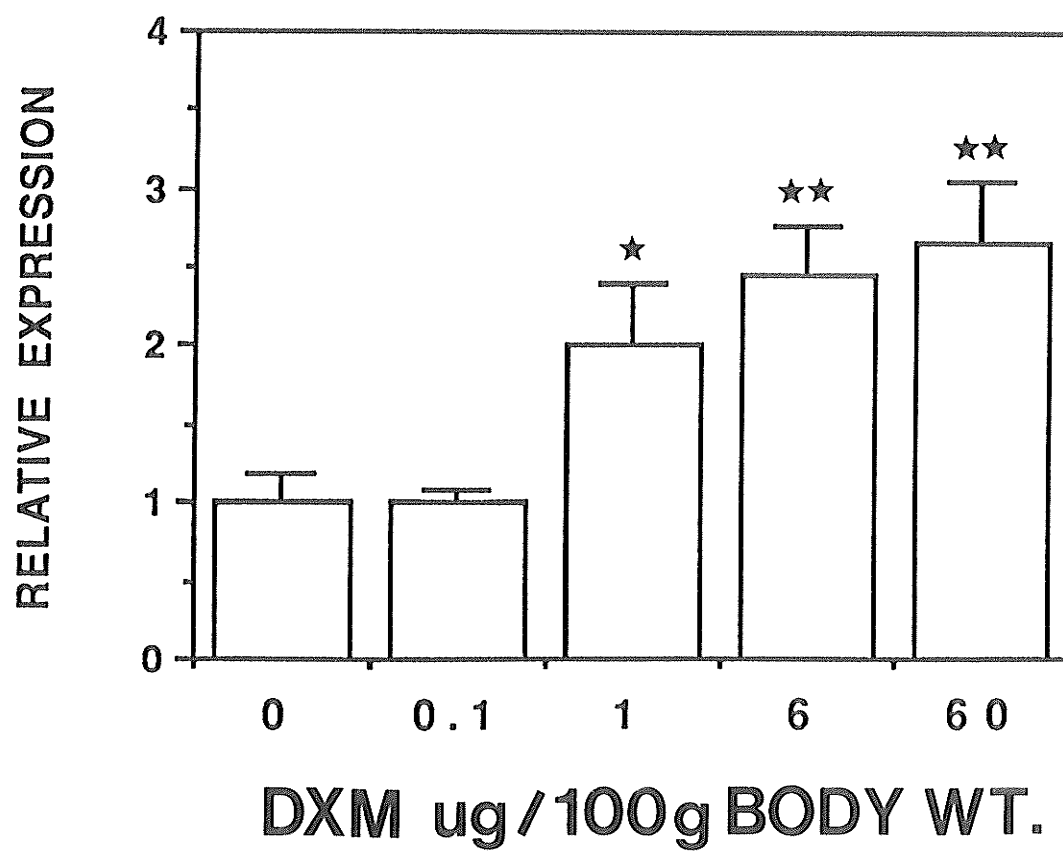


Nb-29



0 0.1 1 6 60

B.

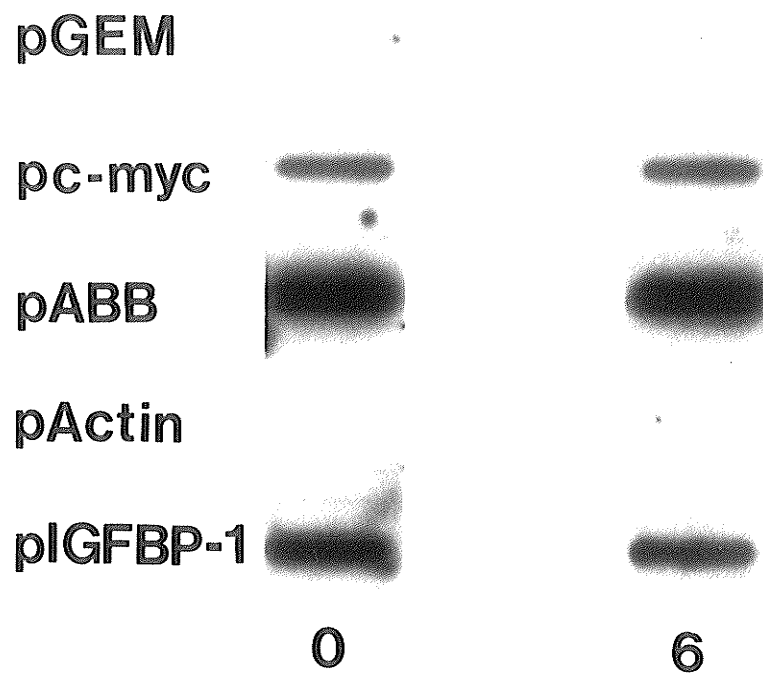


abundance was due to enhanced transcription I measured transcription rate in hepatic nuclei from rats which had been pretreated for 1 h with 6 μ g/100 g body wt DXM and in untreated rats. In four separate experiments there was no significant difference in IGFBP-1 transcription rate in DXM treated rats compared to untreated control rats. The results of one representative experiment are shown in Fig. 4-12. Although actin signals in panel A of Fig. 4-12 were not clearly seen, faint bands of actin were observed in the original autoradiograph. More clear signals of actin were seen in other nuclear runoff experiments (e.g. in the insert of Fig. 3-1). In general, transcription rate of actin detected in rat liver is relatively low compared with that of c-myc, pABB or IGFBP-1 in our experiment condition.

The chronic effects of DXM on hepatic IGFBP-1 mRNA were examined in rats which received daily injections of DXM. A dose-dependent increase in hepatic IGFBP-1 mRNA was seen; however, the fold induction was considerably greater than that seen in the acute experiments (Fig. 4-13). When serum from these rats was analyzed by immunoblotting using an antisera raised against a synthetic peptide, a doublet of approximately 30 kDa was detected. The doublet was detected even in the presence of reducing agents and may represent IGF-1 bound and free IGFBP-1. The smaller of these proteins demonstrated the most marked increase in response to DXM administration (Fig. 4-14). When quantitated by densitometry after subtraction of the background the fold increase in immunoreactive

Fig. 4-12. The effect of DXM on IGFBP-1 transcription. Hepatic nuclei were prepared from untreated rats and rats killed 1 h after administration of DXM. Panel A shows the results of a representative experiment. In panel B, data from four experiments have been plotted. Transcription rate has been quantitated by densitometry of autoradiograms and has been expressed relative to the untreated control rats which have arbitrarily been attributed a value of 1.0. The data represent the mean $\pm$ SEM.

A.



B.

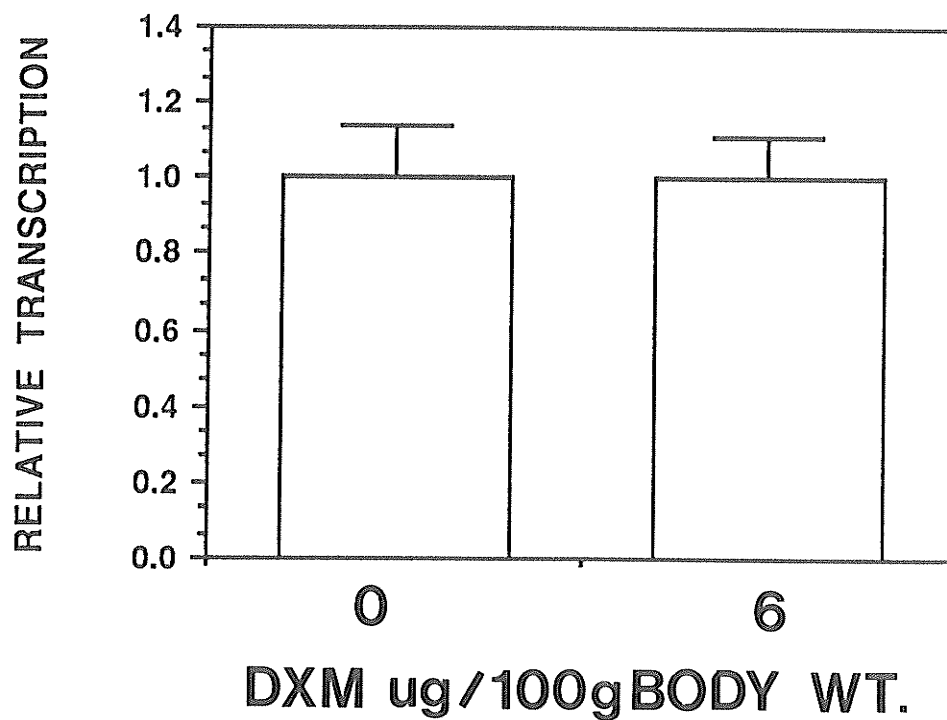
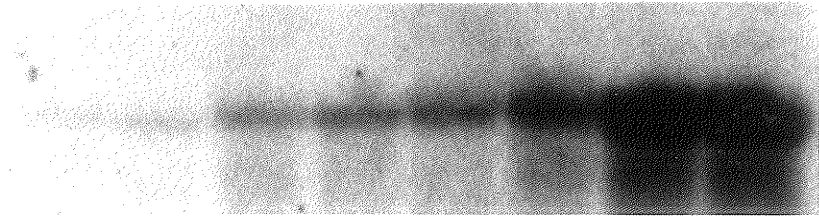


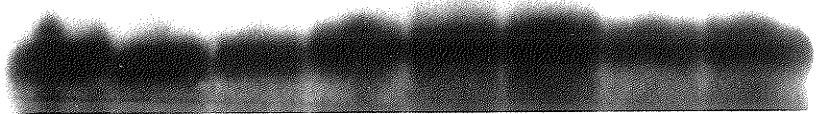
Fig. 4-13. The effects of chronic administration of DXM on hepatic IGFBP-1 mRNA abundance. Panel A depicts a representative Northern blot of total RNA (80 μ g per lane) extracted from rats killed after 6 days of treatment with varying amounts of DXM. The pattern of hybridization obtained with a rat IGFBP-1 cDNA is shown in the upper autoradiogram. As a control for gel-loading the pattern of hybridization obtained with the Nb-29 cDNA is depicted in the lower autoradiogram. In panel B data from five rats per treatment group have been normalized for the loading control and IGFBP-1 mRNA abundance has been expressed relative to the untreated control rats. The data represent the mean $\pm$ SEM. $\star$ $p < 0.05$; $\star\star$ $p < 0.01$.

A.

IGFBP-1



Nb-29



0 0 1 1 6 6 60 60

B.

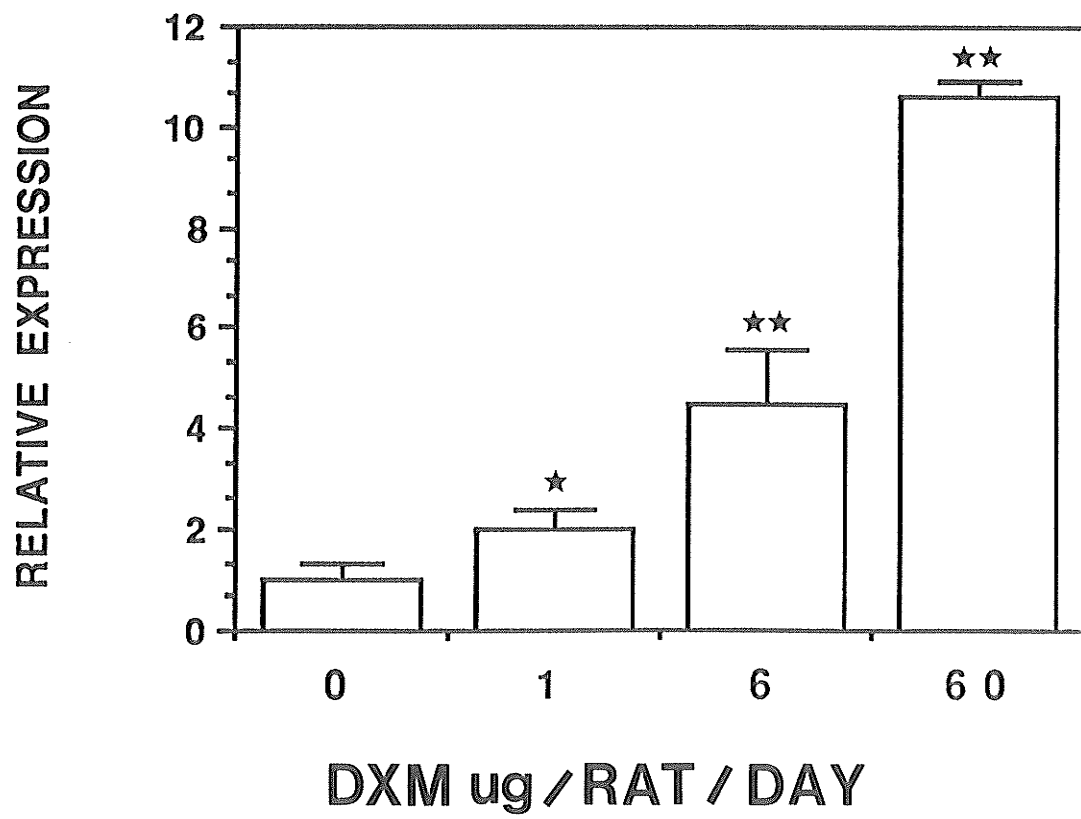
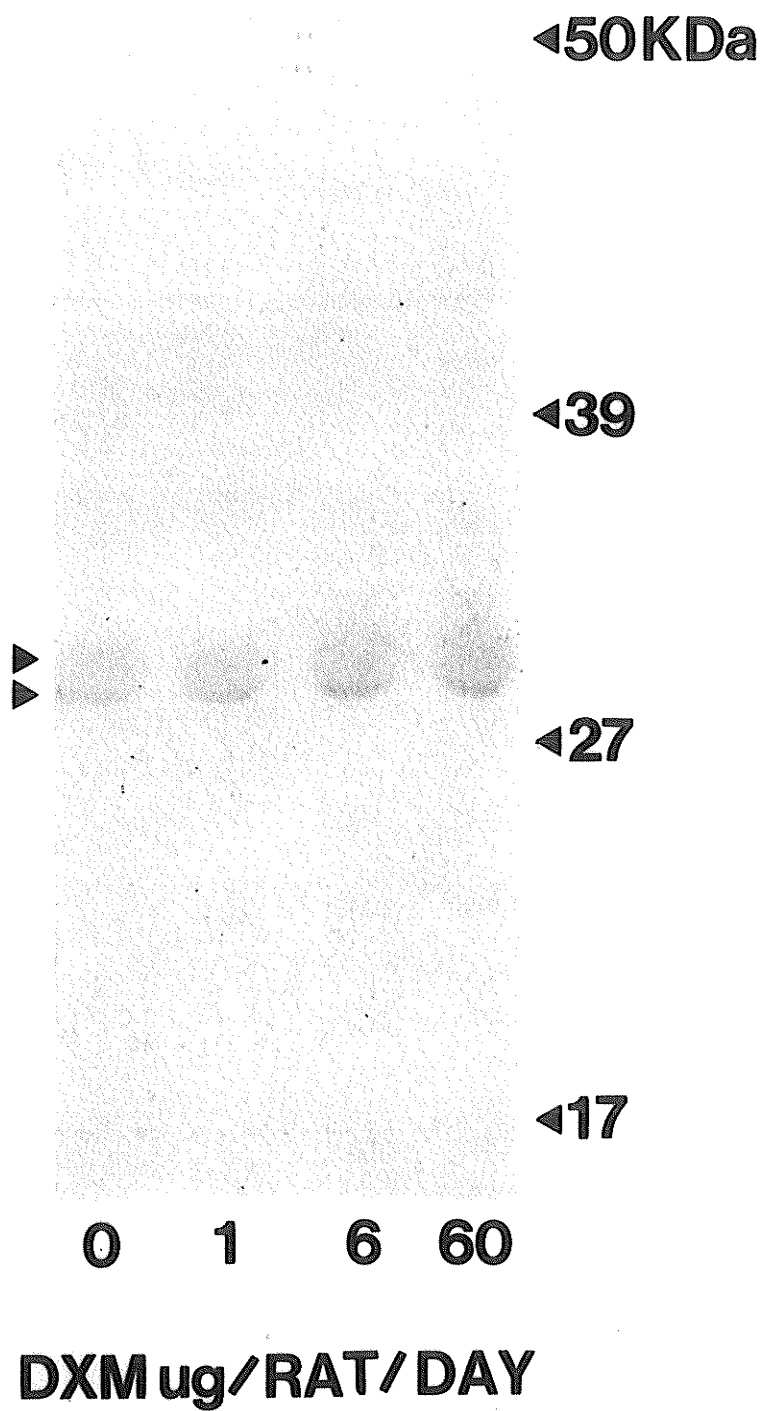


Fig. 4-14. The effect of chronic DXM administration on serum IGFBP-1 concentration. A Western blot of serum pooled from each treatment group is shown. Equivalent volumes of serum (9 μ l per lane) were loaded. A doublet of approximately 30 kDa was detected using antisera directed against a synthetic peptide. The size was determined by comparison with protein molecular mass markers.



was 2.27, 3.71, and 4.53 for pooled serum from rats chronically treated with 1, 6, and 60 μ g of DXM, respectively. This increase in serum IGFBP-1 abundance was consistently seen when individual rat samples were analyzed.

4.3.3 DXM enhances hepatic IGFBP-3 mRNA abundance and serum IGFBP-3 concentrations in the rats

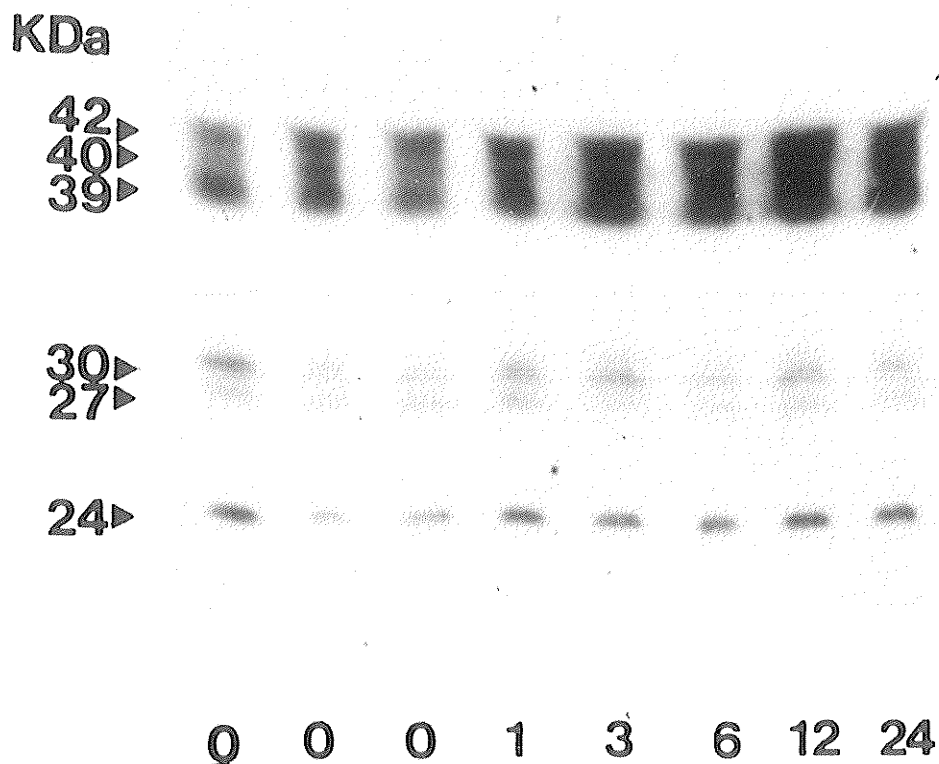
When sera from control rats were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and ligand blotting, six bands were detected. These proteins had apparent molecular masses of 42, 40, 39, 30, 27 and 24 kDa. The 42, 40 and 39 kDa binding proteins represent IGFBP-3 which is known to be glycosylated and consequently poorly resolved by SDS-PAGE (Yang et al., 1989). IGFBP-3 constituted the majority of the serum ^{125} I-IGF-I binding capacity and accounted for $62.8 \pm 7.3\%$ of the total densitometric signal in my studies when ligand blot autoradiograms were quantitated. The 30 kDa binding protein which may represent IGFBP-1 represent less than 10% of the total serum ^{125} I-IGF-I binding capacity. The 27 kDa binding protein has been shown to be glycosylated and correspond to an amino-terminal fragment of IGFBP-3 (Zapf et al., 1988; Yang et al., 1989). The nature of the 24 kDa binding protein is unclear. It may represent IGFBP-4, an unidentified binding protein species, or a mixture of several components of binding proteins. IGFBP-5, which calculated molecular weight is 28,553, may be located in the region of 27-30

KDa. However, IGFBP-5 has not been identified in ligand blot yet. Each of six bands detected in serum from control rats was also present in sera from DXM-treated rats. It has been reported that ligand blotting analysis may underestimate the amount of IGFBP-3 (Bicsak et al., 1990b). Heating of samples prior to serum IGFBP-1 electrophoresis increases the amount of the IGFBP-3 detected by ligand blotting but still cannot completely dissociate binding protein from IGF-IGFBP complex. It was suggested that using the approach of ligand blotting together with immunoblotting provides a more complete analysis of IGFBPs than either method used alone. Since the antibody against rat IGFBP-3 is not available, I used ligand blotting method alone (with heating of sample) to detect serum IGFBP-3.

The effect of a single injection of DXM, 6 μ g/100 g body wt, on serum IGFBP-3 is shown in Fig. 4-15. An increase in the 42, 40 and 39 kDa binding proteins was apparent 1-3 h after DXM administration. Using an antisense oligonucleotide probe, hepatic IGFBP-3 was detected as a single 2.6 kb band on Northern blots. A representative Northern blot of hepatic RNA from rats killed at various times after DXM treatment is shown in Fig. 4-16. A significant increase in IGFBP-3 mRNA abundance was apparent as early as 1 h after a single injection of DXM (Fig. 4-16B). IGFBP-3 mRNA levels reached a peak at 3 h (1.9 ± 0.2 fold, $p < 0.005$) and declined to normal levels in 6-12 h after DXM administration.

Fig. 4-15. The time course of induction of serum IGFBPs following DXM administration. Panel A depicts a ligand blot of serum from rats at various times after a single injection of 6 $\mu\text{g}/100$ g body wt of DXM. Pooled serum samples (9 μl per lane) were analyzed in a 12% SDS-PAGE and transferred to nitrocellulose for ligand blotting with ^{125}I -IGF-I. Three pooled serum samples which came from three separated control groups are analyzed. The apparent molecular weights were determined by comparison with protein molecular weight standards. In panel B, the abundance of 42, 40 and 39 kDa proteins has been quantitated by densitometry and expressed as a percentage of the time 0 control rats.

A.



B.

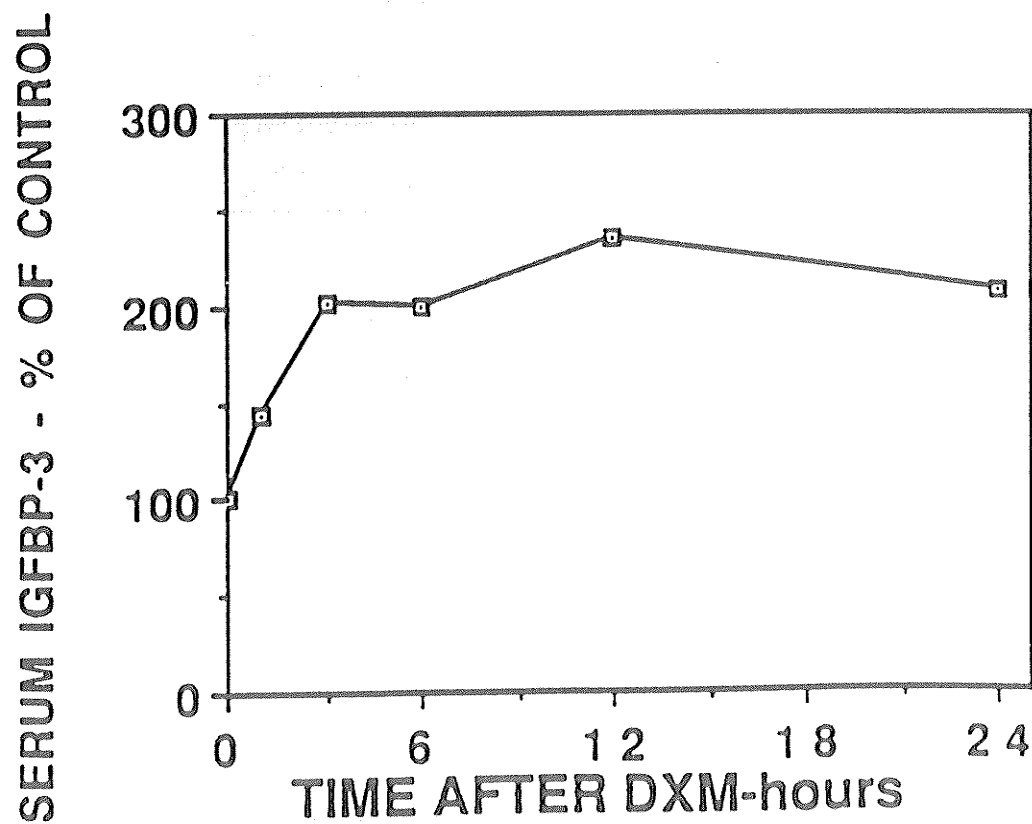
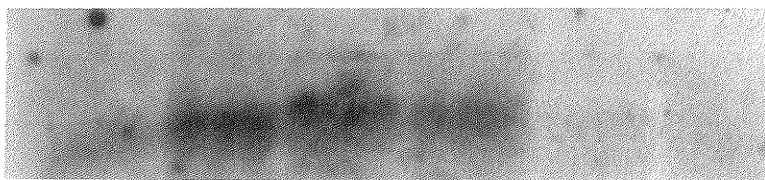


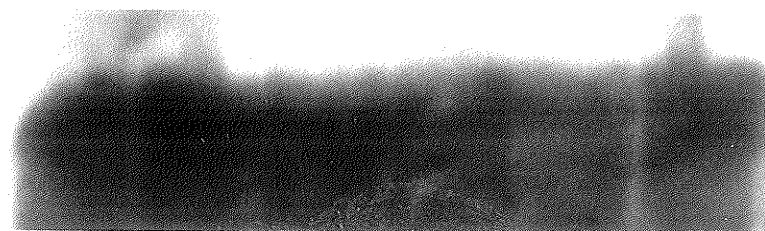
Fig. 4-16. The time course of induction of hepatic IGFBP-3 mRNA following DXM administration. Panel A depicts a representative Northern blot of total RNA (80 μ g per lane) extracted from rats killed at various times after a single injection of DXM (6 μ g/100 g body wt). The pattern of hybridization obtained with an antisense oligonucleotide probe is shown in the upper autoradiogram. As a control for gel loading the pattern of hybridization obtained with a 28S ribosomal probe is depicted in the lower autoradiogram. In panel B data from four or five rats per time point have been quantitated by densitometry and IGFBP-3 mRNA abundance has been expressed as a percentage of the time 0, control rats. The data represent the mean $\pm$ SEM. $\star$, $\star\star$ $p < 0.05$ and $p < 0.005$, respectively, for the difference between treated and control rats.

A.

IGFBP-3



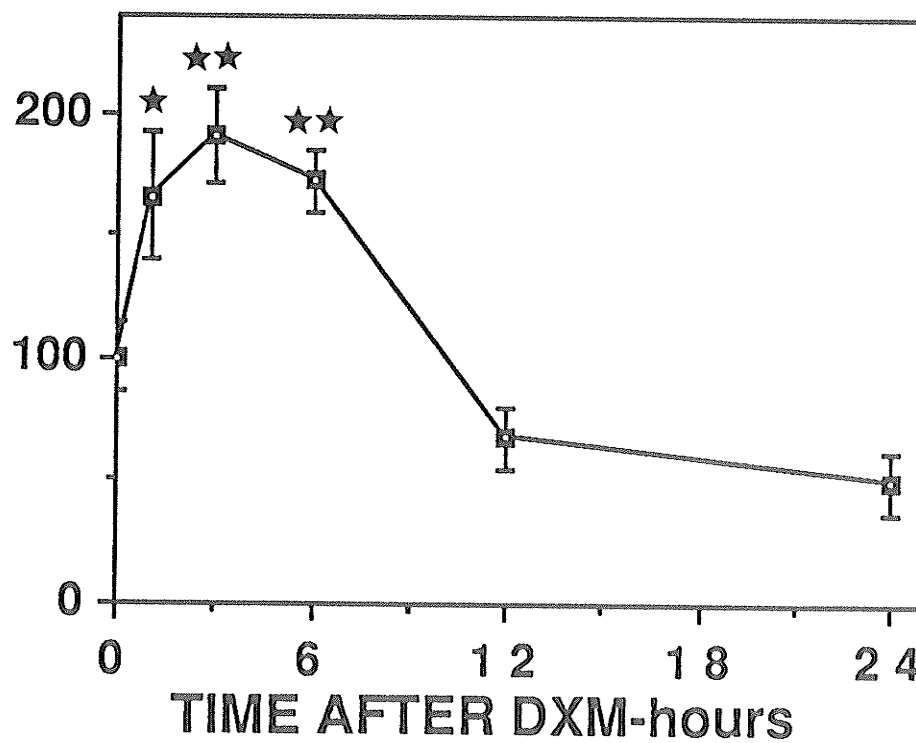
28s



0 1 3 6 12 24

B.

IGFBP-3 mRNA - % OF CONTROL



The dose dependence of the DXM effect on IGFBP-3 was examined in Fig. 4-17. A dose-dependent increase in serum IGFBP-3 was observed with as little as 0.1 μ g/100 g body wt (Fig. 4-17, A and B). The most dramatic increase was seen in the 39 kDa binding protein. Smaller increases were also consistently seen in the 42 and 40 kDa binding proteins. When IGFBP-3 mRNA was quantitated in rats which received varying doses of DXM a significant increase was observed only with 6 μ g/100 g body wt (Fig. 4-17C).

The chronic effects of DXM on serum IGFBP-3 levels and hepatic IGFBP-3 mRNA abundance were also examined. In sera from animals treated chronically with varying amounts of DXM, there was a dose-dependent increase in IGFBP-3, as determined by ligand blotting of pooled sera (Fig. 4-18, A). An increase in IGFBP-3 mRNA abundance was also observed in hepatic tissue from rats treated chronically with DXM (Fig. 4-18B).

4.4 Discussion

In this study I tested the hypothesis that glucocorticoid-induced growth retardation is mediated by the decrease in the IGF-I gene expression and the increase in the expression of IGFBPs.

The first part of our study clearly demonstrates that DXM inhibits GH induction of IGF-I expression in the hypox rats and reduces steady-state IGF-I mRNA levels in pituitary-intact rats.

Fig. 4-17. The dose-dependent induction of IGFBP-3 expression by **DXM**. In panel A sera (9 μ l per lane) pooled from rats killed 1 h after injection of varying amounts of DXM were analyzed by SDS-PAGE and ligand blotting. The apparent molecular weights of the major binding proteins were determined by comparison with protein molecular standards. In the panel B., the abundance of the 42, 40 and 39 kDa proteins have been quantitated by densitometry. In panel C, the relative abundance of IGFBP-3 mRNA has been determined in hepatic total RNA extracted from individual rats, four or five rats per group. In each case data have been normalized for the loading control and expressed as a percentage of the untreated control rats. The data represent the mean $\pm$ SEM. $\star p < 0.05$ for the difference between treated and control rats.

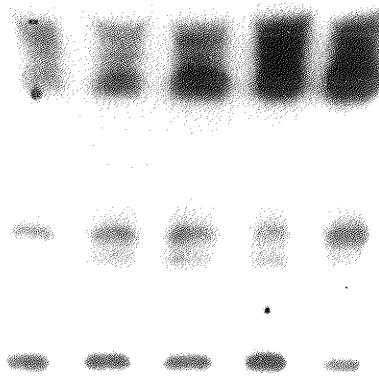
A.

KDa

42▶
40▶
39▶

30▶
27▶

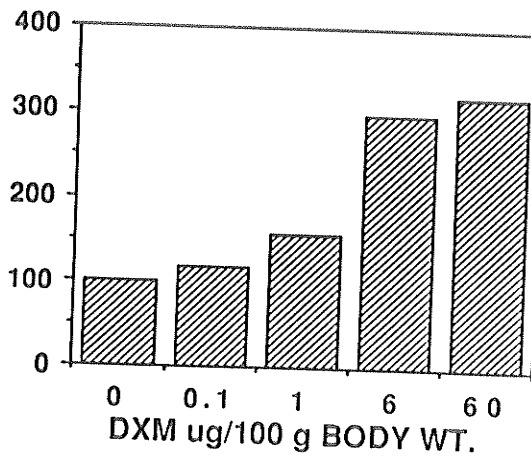
24▶



B.

SERUM IGFBP-3 - % OF CONTROL

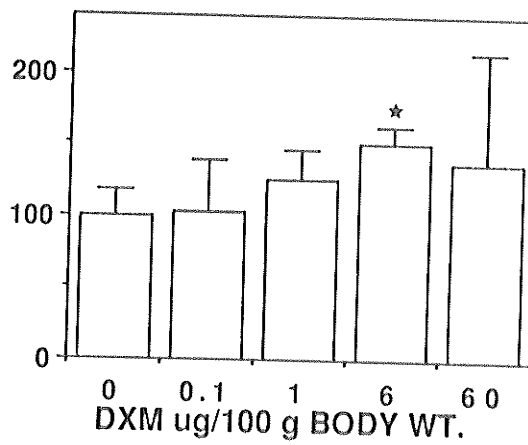
0 0.1 1 6 60



C.

IGFBP-3 mRNA - % OF CONTROL

0 0.1 1 6 60



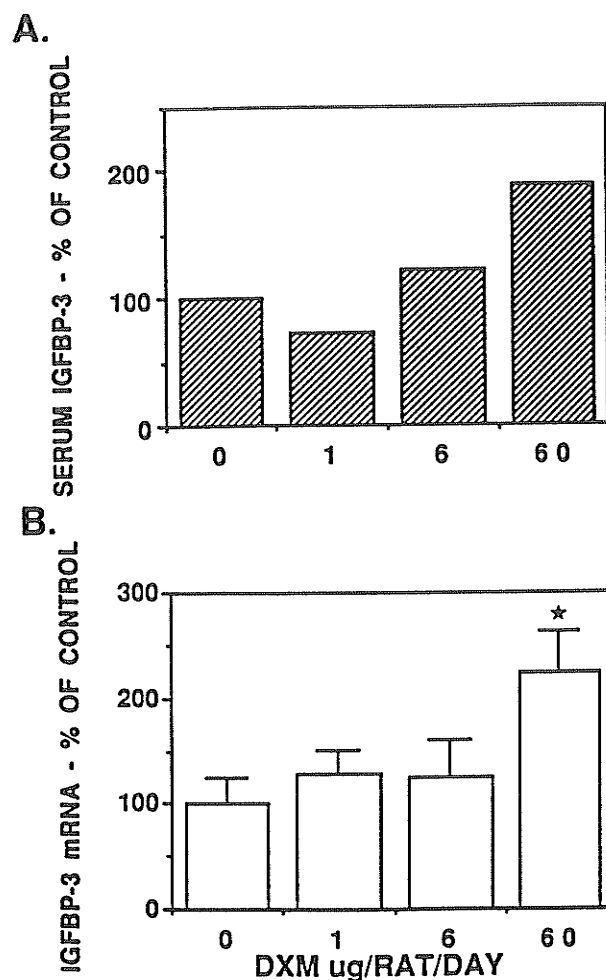


Fig. 4-18. The effects of chronic administration of DXM on IGFBP-3 expression. In panel A, equivalent volumes (9 μ l) of pooled serum samples were analyzed by SDS-PAGE and ligand blotting. The 42, 40 and 39 kDa binding proteins corresponding to IGFBP-3 were quantified by densitometry. In panel B, the relative abundance of hepatic IGFBP-3 mRNA was determined. The data represent the mean $\pm$ SEM. In each case data have been expressed as a percentage of untreated control rats. $\star$ $p < 0.05$ for the difference between treated and control rats.

A significant inhibition was seen at the lowest dose used, 1 μ g/100 g body wt with almost complete inhibition seen at higher doses of DXM. Of the tissues examined the liver and proximal tibia (including the tibia epiphysis) appeared to be particularly sensitive to DXM. Similar data were obtained when DXM was administered to pituitary-intact rats with a reduction of IGF-I mRNA abundance apparent in all tissues examined. In the case of the pituitary-intact rat, higher doses of DXM were required than in the hypox rat. A report from Binoux *et al.* (1982) showed that the IGF-I synthesis by liver explants in organ culture was reduced by glucocorticoid. During the course of this study, Adamo *et al.* (1988) reported that DXM reduces steady state IGF-I mRNA in cultured rat neuronal and glial cells. These *in vitro* studies suggest that the glucocorticoids may have a direct inhibitory effect on IGF-I gene expression. At present, there is no data to show whether DXM effects on IGF-I gene transcription or on IGF-I mRNA stability. Further studies are needed to address this question.

In addition to the reduced tissue IGF-I mRNA abundance, the GH-induced rise in serum IGF-I in hypox rats was significantly inhibited by the higher doses of DXM. Surprisingly, little effect of DXM on serum IGF-I was seen in the pituitary-intact rat. The discordance between serum IGF-I and hepatic IGF-I mRNA abundance was particularly obvious in the pituitary-intact rat that received DXM chronically. A similar discordance was also seen in GH-treated

hypox rats that received the lowest dose of DXM, suggesting that DXM had complex and varying actions on the synthesis, secretion, or clearance of IGF-I. A number of possible explanations could account for this marked discordance between hepatic IGF-I mRNA levels and serum IGF-I concentrations. First, at the time I conducted this study I used an in-tube acid-ethanol extraction method (Daughaday *et al.*, 1980) to remove binding proteins before radioimmunoassay for IGF-I. It is possible that method used was not effective enough, and therefore the results of serum IGF-I by radioimmunoassay were interfered by residual binding proteins, particularly in the case of pituitary-intact rats which had relatively high amounts of total IGFBPs compared to hypox rats. It is believed now that column chromatography at low pH is a more effective way to remove binding protein. Second, glucocorticoids may alter translation and secretion of IGF-I in such a way that IGF-I mRNA abundance no longer reflects IGF-I protein synthesis. Finally, glucocorticoids may effect production of IGF binding proteins and thereby change the metabolic clearance rate of IGF-I. IGFBP-3 has been shown to prolong the half life of IGF-I (Zapf *et al.*, 1986; Cascieri *et al.*, 1988b). Therefore, change in IGFBP-3 after DXM administration would alter the clearance rate of IGF-I and consequently the serum IGF-I concentrations. The observation that IGFBP-3 is markedly reduced in hypox rats compared to pituitary-intact rats (Zapf *et al.*, 1989) may account for the different serum IGF-I response to DXM treatment seen in the hypox and pituitary-intact rat. Although serum IGF-I concentrations were

not decreased after the DXM in pituitary-intact rats, circulating IGF-I levels may not reflect tissue IGF-I levels under this circumstance. Because the majority of IGF-I is bound to binding proteins that may be regulated by DXM, the distribution of the IGF-I in circulation and tissues may be altered. Regardless of the explanation for the discordance between serum IGF-I concentrations and tissue IGF-I mRNA levels, our data clearly demonstrated that, at least in the hypox rats, glucocorticoid antagonism of the growth-promoting activity of GH involves impaired IGF-I expression in both hepatic and nonhepatic tissues. Furthermore, in pituitary-intact rats, DXM, at slightly higher doses, inhibited body wt gain and hepatic and tibial IGF-I expression. The inhibition of the production of IGF-I by DXM may be one of the mechanisms underlying the growth retardation seen in glucocorticoid excess.

Since I failed to observe the predicted decrease in circulating IGF-I levels in DXM-treated pituitary-intact rats, I speculate that DXM may have opposing effects on serum IGFBPs in these rats. First I examined the effect of DXM on serum total IGF binding activity by ligand blotting. Administration of DXM caused a time- and dose-dependent increase in the serum total IGF binding activity. I further analyzed whether IGFBP-1 and -3 expression are responsible for this increase in serum total IGF binding activity. In this study, I have not examined the effects of DXM on IGFBP-2, -4, -5 and -6 expression in detail. The cDNAs for rat IGFBP-4, -5 and -6 were cloned recently. My preliminary results using specific

oligonucleotide probes suggested that DXM increases renal IGFBP-5 mRNA abundance in dose-dependent manner whereas there was no change in hepatic IGFBP-4 mRNA abundance after the treatment of DXM. IGFBP-2 expression is high in fetal liver but rapidly decreases to very low levels after birth (Ooi *et al.*, 1990). In addition, IGFBP-2 is not detected in adult rat serum (Yang *et al.*, 1989). It was reported that treatment with DXM to 4-day-old rats for 4 days resulted in a 50% decrease in hepatic IGFBP-2 mRNA abundance (Orlowski *et al.*, 1990b). A more detailed work on the effects of DXM on IGFBP-1 and -3 expression was done in this study.

In both acute and chronic experiments, DXM at doses as low as 1 μ g/100 g body wt increased hepatic IGFBP-1 mRNA abundance. The effect of DXM appeared to be cumulative since after repeated injections the levels of IGFBP-1 mRNA were considerable greater than after a single injection particular when one considers that the chronically treated rats were killed 9 h after the last injection. Although no increases in serum IGFBP-1 concentrations were detected in acute experiments, the increased IGFBP-1 mRNA detected in the chronic experiments was translated into protein since increased IGFBP-1 levels were apparent when serum was analyzed by immunoblotting. Since no significant effect of DXM on IGFBP-1 transcription was detected, the effect of DXM on IGFBP-1 mRNA abundance appeared to be post-transcriptional. I chose to examine the effect of DXM on transcription 1 h after DXM administration, a time when IGFBP-1 mRNA abundance was

significantly increased. I cannot exclude the possibility that an increase in transcription may have been detected at earlier time points. It is not clear from my studies whether the effects of DXM on hepatic IGFBP-1 mRNA levels are direct. However, some *in vitro* studies from Orlowski et al. (1990a) and Unterman et al. (1991) suggest that DXM may have direct effects on IGFBP-1 gene expression. In Orlowski's study, incubation with 10^{-6} M DXM for 48 h increased the amount of IGFBP-1 in conditioned medium by approximately 10-fold, and increased the IGFBP-1 mRNA abundance by 7-fold. DXM caused an increase in half-life of IGFBP-1 mRNA (from 1.7 h to 2.5 h). They claimed that this small difference in half-life of mRNA cannot account for the 10-fold increase in steady state levels of IGFBP-1 mRNA, suggesting that DXM stimulate the IGFBP-1 gene transcription rate. This conclusion is different from ours that DXM did not increase the IGFBP-1 transcription rate, and is inferential since transcription rate was not measured directly in their study. The discrepancy may be due to the difference in the experimental models. In addition, their results could not exclude the possibility that DXM may regulate the IGFBP-1 gene expression at other post-transcriptional locus, e.g. the processing or transport of IGFBP-1 nuclear transcripts, rather than at transcription level. In contrast to the results of stimulating effect of DXM on IGFBP-1 gene expression, Lewitt and Baxter (1989) reported that DXM inhibited production of IGFBP-1 in human fetal liver explants. This difference in response may result from a species difference or a difference between fetal and adult tissue.

The physiological role of IGFBP-1 is not completely defined. In most *in vitro* studies, IGFBP-1 has been shown to inhibit the actions of the IGF-I (Ritvos *et al.*, 1988; Rutanen *et al.*, 1988; Frauman *et al.*, 1989; Burch *et al.*, 1990). Of these studies, the most physiologically relevant are the observations of Burch *et al.* (1990), that IGFBP-1 inhibits growth in chicken pelvic cartilage. There are some reports that IGFBP-1 enhances IGF-I action (Elgin *et al.*, 1987; Clemmous & Gardner, 1990). The discrepancy of biological activities of IGFBP-1 is not well understood. It may be due to the differences in preparation of IGFBP-1, experimental conditions, target cells or different forms of IGFBP-1. The inhibitory action of IGFBP-1 was thought to compete with receptors for IGF binding and therefore inhibit binding of IGF-I to cell receptors. If IGFBP-1 is able to inhibit IGF-I action, then the enhanced expression of this protein may be one component of the growth retardation which accompanies glucocorticoid excess.

Since serum IGFBP-1 only accounts for small proportion of serum total IGFBPs, and IGFBP-3 is a major component of serum IGFBPs (accounted for 62.8% of the total densitometric signal in my Western-ligand blotting), I next examined the effects of DXM on IGFBP-3 gene expression. The rat IGFBP-3 is a protein of 265 amino acids with four potential N-linked glycosylation sites (Shimasaki *et al.*, 1989). Since antisera directed against rat IGFBP-3 is not available, I used ligand blotting method to detect rat IGFBP-3 in serum. The 39-42 KDa IGF binding proteins in rat serum have been

identified as IGFBP-3, differing only in the degree of glycosylation.

Administration of DXM caused a time- and dose-dependent increase in IGFBP-3 mRNA and in the serum IGFBP-3. This was unexpected, because the IGFBP-3 appears to be IGF-I dependent. It has been reported that IGF-I enhances IGFBP-3 production both *in vitro* and *in vivo* (Clemmons *et al.*, 1989; Zapf *et al.*, 1989; Smith *et al.*, 1990; Camacho-Hubner *et al.*, 1991; Conover, 1991a). Under the same conditions used here, I have shown that DXM decreases hepatic IGF-I mRNA steady state levels. Thus, it would seem unlikely that the effects of DXM on IGFBP-3 expression are mediated via IGF-I. The effects of DXM on IGFBP-3 expression appear to involve a different mechanism since inhibition of GH-signal transduction in the liver should result in a decrease rather an increase in hepatic IGFBP-3 expression. Corps and Brown (1991) reported that DXM inhibited IGF-binding activity in conditioned medium from Swiss 3T3 fibroblast cells, and the IGFBP-3 was believed to be a major component of this IGF-binding activity. This inhibitory effect of DXM on IGFBP-3 *in vitro* is contrary to the stimulating effect of DXM on IGFBP-3 *in vivo*. This difference may be due to the difference in experimental system or may suggest that the stimulating effects of DXM on IGFBP-3 expression is not direct. Further studies are required to understand the mechanisms underlying the stimulating effect of DXM on IGFBP-3 expression *in vivo*.

The reported biological activities of IGFBP-3 include inhibition and enhancement of IGF-I action depending on the experimental conditions. DeMellow and Baxter (1988) showed that preincubation of human neonatal fibroblasts with IGFBP-3 caused a potentiation of the subsequent IGF-I stimulated thymidine incorporation in the cells. In contrast, co-incubation of IGF-I with IGFBP-3 resulted in inhibition of the IGF-I effect. It was suggested that IGFBP-3 inhibits IGF-I action by sequestering the IGF-I and preventing receptors interaction. If the function of IGFBP-3 is to limit the action of the IGFs then enhanced expression of this binding protein together with IGFBP-1 may reduce bioavailability of IGF-I. This together with the DXM-induced inhibition of IGF-I expression may be responsible in part for the pronounced growth retardation which is characteristic of glucocorticoid excess.

Chapter 5: Expression of IGF-I and IGFBPs in diabetic rats

5.1 Introduction

It has been known for a long time that impaired growth is a characteristic of insulin-dependent diabetes in both man and experimental animals (Pond, 1970; Phillips & Young, 1976; Phillips & Orawski, 1977). Poorly controlled diabetes in children is associated with failure to thrive, delayed puberty, reduced growth velocity and short stature (Birbeck, 1972). In the rats, diabetes causes a decrease in body weight gain or even loss of body weight. The mechanisms responsible for this growth retardation are not fully understood. Diabetes mellitus in the rat, whether occurring spontaneously or after STZ administration, results in decreased pituitary GH synthesis (Gonzalez & Jolin, 1985) and suppressed GH secretion with loss of high amplitude secretory pulses (Tannenbaum 1981a; Tannenbaum *et al.*, 1981b). A combined defect involving increased hypothalamic somatostatin and decreased GH-releasing factor influence accounts for this decreased pituitary GH synthesis and secretion (Olchovsky *et al.*, 1990). However, this mechanism alone does not appear to explain growth retardation, since serum GH levels are normal or elevated rather than decreased in human diabetes associated with growth retardation (Hansen & Johansen, 1970; Winter *et al.*, 1980; Press *et al.*, 1984). Furthermore, diabetic rats are relatively unresponsive to exogenous GH, suggesting that GH receptor and/or postreceptor defects may play a

role in the growth retardation which accompanies poorly controlled diabetes (Phillips & Orawski, 1977; Baxter *et al.*, 1980; Maes *et al.*, 1986). Baxter *et al.* (1980) reported that the numbers of hepatic GH receptors were reduced in rats one week after the onset of severe ketotic, STZ-induced diabetes. In non-ketotic, STZ-induced diabetic rats, the resistance to GH action is due to post-receptor defects because GH binding has been found to be normal 1 week after STZ injection (Maes *et al.*, 1986). A permissive role for insulin in GH-signal transduction has been demonstrated (Daughaday *et al.*, 1976; Scott & Baxter, 1986; Griffen *et al.*, 1987; Salamon *et al.*, 1989) and may be mediated by the modulation of hepatic GH receptors since insulin has been shown to reverse the decrease in GH binding on liver microsomal membranes from diabetic rat (Baxter *et al.*, 1980).

IGF-I is an important growth factor in postnatal longitudinal growth and is thought to be the major mediator of the growth promoting actions of GH. A number of studies have shown that circulating IGF-I levels are low in diabetes both in human and rats (Winter *et al.*, 1979; Blethen *et al.*, 1981; Tamborlane *et al.*, 1981; Maes *et al.*, 1983; 1986). It has been demonstrated that IGF-I mRNA abundance is diminished in hepatic, pulmonary, renal, and uterine tissues from diabetic rats (Goldstein *et al.*, 1988; Murphy *et al.*, 1988b; Fagin *et al.*, 1989b). This defect in IGF-I gene expression may contribute to the low circulating IGF-I levels. Since IGF-I gene expression is ubiquitously present in tissues and

IGF-I is believed to be able to act at the sites of its production, the important roles of autocrine and paracrine effects of IGF-I have been emphasized. However, whether the defect of IGF-I gene expression is general in diabetes is unknown.

In addition to the reduced circulating levels of IGF-I in diabetic rats, inhibitors of IGF action are present in increased amounts in serum from diabetic rats, resulting in an overall decrease in serum IGF activity when measured in a cartilage bioassays (Phillips *et al.*, 1979; Taylor *et al.*, 1987). The nature and character of these inhibitors have not been clarified. Since some of these IGF inhibitors are similar in size to low molecular weight IGF binding proteins, and the profile of change in IGF inhibitor activity under some metabolic conditions strongly resembles the profile of change in IGFBP-1, it is speculated that some of these IGF inhibitors may actually represent IGFBP-1. Studies in human subjects have established that circulating IGFBP-1 levels are inversely correlated with plasma insulin concentration (Busby *et al.*, 1988b). In addition, Unterman *et al.* (1989) have shown that serum IGFBP-1 levels is increased in diabetic rats. However, little is known of the effects of diabetes on mRNA levels of IGF binding proteins when I was conducting this research work. Therefore we examined the abundance of IGF-I and IGFBP-1 mRNAs in various tissues in STZ-induced diabetic rats. Also, I examined the levels of hepatic IGFBP-1, -2, -3 and -4 mRNAs and serum IGFBP-1 and -3 levels in the spontaneously diabetic Biobreeding/Worcester

(BB/W) rats.

5.2 Experimental design

Female Sprague Dawley rats, 85-100 g body wt, were used. For induction of diabetes a single i.p. injection of STZ, 65 mg/kg body wt was administered to rats which had been fasted overnight. Control rats received an equivalent volume of citrated buffered saline, pH 3.0. STZ-treated rats, which had serum glucose less than 200 mg/dl at the time of sacrifice, were excluded. Using this criterion, approximately 28% of the STZ-treated rats were excluded from the study. The mean serum glucose level at the time of sacrifice in diabetic rats was 672.5 ± 33.5 mg/dl. None of the STZ-treated rats died prematurely. Diabetic rats were killed on day 7, 32 and 91 post STZ administration. Age-matched, non-diabetic rats were killed as controls because of the age-related changes in IGF-I and IGF binding proteins. In a separate experiment the effects of insulin on IGF-I and IGFBP-1 mRNA abundance in diabetic rats were assessed. For these studies diabetic rats, 32 days post STZ administration, were randomized into two groups. Prior to initiation of treatment, each rat was bled via the tail vein for serum glucose determination. One group of 6 diabetic rats was injected twice daily with human insulin (3 U/rat). The other group of 6 diabetic control rats were injected twice per day with an equivalent volume of phosphate-buffer. Animals were allowed food and water ad libitum. Rats were weighed

and killed 6 h after the final injection on the morning of the 7th day of treatment.

Another model of diabetic rat used in these studies was spontaneously diabetic rat. The BB/W rat provides an excellent animal model of autoimmune diabetes. The development of autoimmune insulinitis in this model results in the destruction of pancreatic β -cells. Prediabetic male BB/W rats and age-matched rats of a non-diabetes-prone strain of BB/W rat were used. After the onset of diabetes, at approximately 3 months of age, small doses of protamine-zinc insulin 0.5-1 unit/day, were administered to prevent ketosis and death. This dose of insulin maintains blood glucose levels between 270 and 450 mg/dL. Diabetic rats were killed 1 or 3 months after the onset of diabetes and received no insulin for at least 16 h before death. Age-matched, non-diabetic rats were killed as controls. To assess the effects of good metabolic control on the IGFBPs, one group of diabetic rats (3 months after the onset of diabetes) received twice-daily insulin injections for 3 weeks to achieve aglycosuria and normoglycaemia. In this intensive insulin treatment group, individually adjusted doses of insulin (3-6 unit/day) were administered to rats maintained in metabolic cages. The dosage of insulin was adjusted according to the previous 24-h urinary-glucose excretion. These rats were killed within 4 h of the morning insulin injection.

5.3 Results

5.3.1 Expression of IGF-I in the STZ-induced diabetic rats

The effects of STZ-diabetes on body weight gain and serum glucose are outlined in Table 5-1. In each group of diabetic rats the mean body weight gain was significantly reduced compared to age-matched, non-diabetic control rats ($p < 0.0005$). The serum glucose concentration was significantly elevated as early as 7 days post STZ administration and the difference between diabetic and non-diabetic rats increased with increasing duration of diabetes. The relative abundance of IGF-I mRNA in various tissues at each of the time points is shown in Fig. 5-1 and Table 5-1a. In diabetic rats on day 7 after STZ administration, a decrease in IGF-I mRNA was observed in the lung and diaphragm ($p < 0.05$ and $p < 0.01$ respectively). Although changes of similar magnitude were observed in other tissues, these changes did not achieve statistical significance. At 32 days post STZ-administration the difference between diabetic and non-diabetic rats in terms of IGF-I mRNA abundance in the kidney, diaphragm and muscle was statistically significant. A more dramatic decrease in IGF-I mRNA was seen in diabetic rats 91 days after STZ administration. In most of the tissues examined, there was a trend towards a decrease in IGF-I mRNA abundance with increasing duration of diabetes. This was over and above the age-related decrease in tissue IGF-I mRNA observed in the non-diabetic age-matched control rats (data not shown). Representative autoradiograms of Northern blots are shown in Fig. 5-2. In each tissue there was good agreement between IGF-I mRNA

Table 5-1. Effects of STZ-induced diabetes on body weight, body weight gain and serum glucose

Day ^a		Body weight ^b (g)	Body weight gain ^c (g)	Serum glucose ^d (mg/dL)
7 days				
Control	(6)	147.2±4.6	44.3±1.7	145.1±5.5
Diabetic	(6)	128.7±8.3	25.4±2.7*	429.4±41.3*
 32 days				
Control	(10)	214.4±4.5	112.1±2.4	120.3±5.1
Diabetic	(8)	173.0±14.3	71.2±10.0*	615.8±30.2*
 91 days				
Control	(10)	294.9±10.0	188.5±7.4	121.1±2.4
Diabetic	(15)	217.8±7.8	114.8±5.1*	799.9±29.8*

All data represent mean ± SEM. The number in parentheses indicate the number of animals per group.

^a Number of days after STZ administration.

^b Body weight at the time of death.

^c Body weight gain over the period of the study.

^d Serum glucose at the time of death

* indicates $p < 0.0005$ for the difference between diabetic and age-matched non-diabetic control rats.

Fig. 5-1. The effect of STZ-induced diabetes on IGF-I mRNA abundance in various tissues of the rat. The relative tissue abundance of IGF-I mRNA at 7, 32 and 91 days after STZ administration was quantitated by slot-blot hybridization and has been expressed as a percentage of the age-matched, non-diabetic control rats. The data represent the mean $\pm$ SEM for 3 to 6 samples per groups. Significant difference between diabetic and non-diabetic rats is indicated as *, ** and ***, which indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. Li, liver; K, kidney, Lu, lung; D, diaphragm; M, quadracep femoris; H, heart; F, adipose tissue.

RELATIVE IGF-I mRNA ABUNDANCE

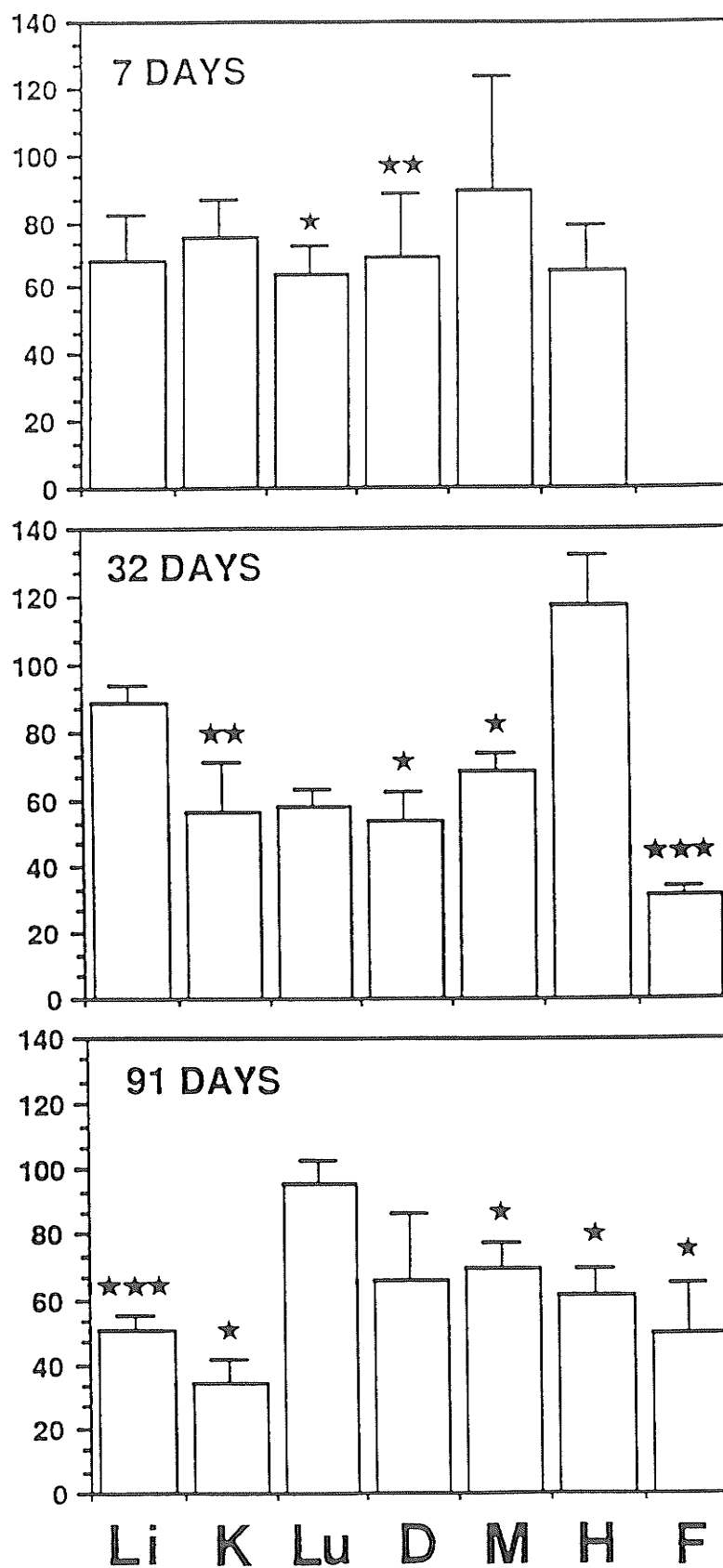


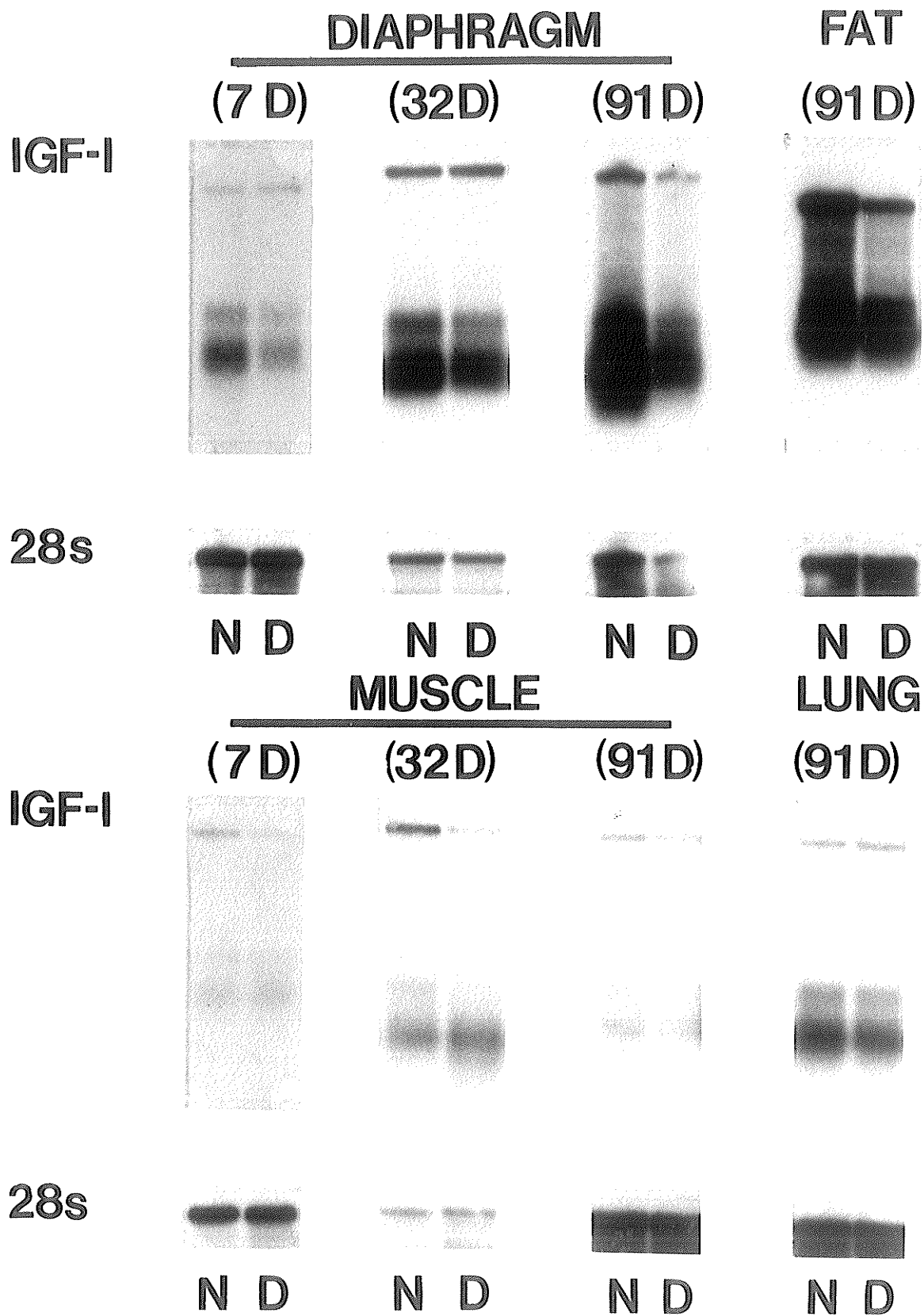
Table 5-1a. The relative values of IGF-I mRNA abundance in various tissues of STZ-induced diabetic rats and age-matched non-diabetic control rats

Tissue	7 days	32 days	91 days
liver			
Control	1.00±0.31 (6)	1.00±0.09 (5)	1.00±0.09 (5)
Diabetic	0.68±0.14 (6)	0.88±0.05 (5)	0.51±0.04 (5) §
Kidney			
Control	1.00±0.18 (6)	1.00±0.08 (6)	1.00±0.26 (5)
Diabetic	0.75±0.12 (6)	0.56±0.15 (5) δ	0.34±0.07 (6) φ
Lung			
Control	1.00±0.13 (6)	1.00±0.21 (6)	1.00±0.04 (5)
Diabetic	0.64±0.08 (6) φ	0.58±0.05 (4)	0.96±0.07 (6)
Diaphragm			
Control	1.00±0.09 (5)	1.00±0.12 (4)	1.00±0.25 (5)
Diabetic	0.69±0.19 (5) δ	0.53±0.09 (3) φ	0.66±0.20 (5)
Muscle			
Control	1.00±0.18 (5)	1.00±0.11 (5)	1.00±0.09 (6)
Diabetic	0.89±0.34 (4)	0.68±0.05 (5) φ	0.69±0.07 (5) φ
Heart			
Control	1.00±0.26 (6)	1.00±0.08 (5)	1.00±0.13 (6)
Diabetic	0.65±0.13 (6)	1.17±0.15 (4)	0.61±0.07 (6) φ
Fat			
Control		1.00±0.03 (4)	1.00±0.16 (5)
Diabetic		0.31±0.02 (3) §	0.49±0.15 (5) φ

All data represent mean ± SEM. The number in parentheses indicate the number of animals per group.

φ, δ, and § indicate p<0.05, p<0.01 and p<0.001, respectively, for the difference between diabetic and age-matched non-diabetic control rats.

Fig. 5-2. Representative autoradiograms of Northern blots demonstrating IGF-I mRNA abundance in tissues from diabetic and non-diabetic rats. In each case the upper panel depicts the pattern of hybridization obtained with a rat IGF-I cDNA while the lower panel shows hybridization of the same filter with a ribosomal probe (28s). RNA was extracted from diabetic (D) rats killed 7 (7D), 32 (32D) or 91 (91D) days after STZ administration and from age-matched, non-diabetic rats (N). Ten micrograms of poly (A)⁺ RNA per lane were loaded.



levels as determined by Northern blot analysis and slot-blot hybridization. Each of the major IGF-I mRNA transcripts appeared to be similarly reduced in RNA extracted from diabetic animals. When all Northern blots were considered, there are no consistent differences between diabetic and non-diabetic rats in terms of the hybridization signal obtained with the 28S or pNB29 probes. The relationship between serum glucose and hepatic IGF-I mRNA abundance is shown in Fig. 5-3. There was no significant correlation between serum glucose and hepatic IGF-I mRNA ($R=0.24$, $p=NS$).

5.3.2 Expression of IGFBP-1 gene in the STZ-induced diabetic rats

In contrast to the decrease in IGF-I mRNA, an increase in IGFBP-1 mRNA was seen in the liver (2-9 fold) and kidney (1.6-2.2 fold) in all groups of diabetic rats (Fig. 5-4). IGFBP-1 mRNA abundance increased with increasing duration of diabetes. In contrast an age related decrease in IGFBP-1 mRNA was observed in the non-diabetic rats (data not shown). Autoradiograms of representative Northern blots are shown in Fig. 5-5. IGFBP-1 mRNA was also detectable at much lower levels in a number of other tissues including diaphragm, uterus and brain. In both the uterus and the diaphragm IGFBP-1 mRNA abundance appeared to be increased in diabetic rats (data not shown). As shown in Fig. 5-6, a highly significant correlation between serum glucose and hepatic IGFBP-1

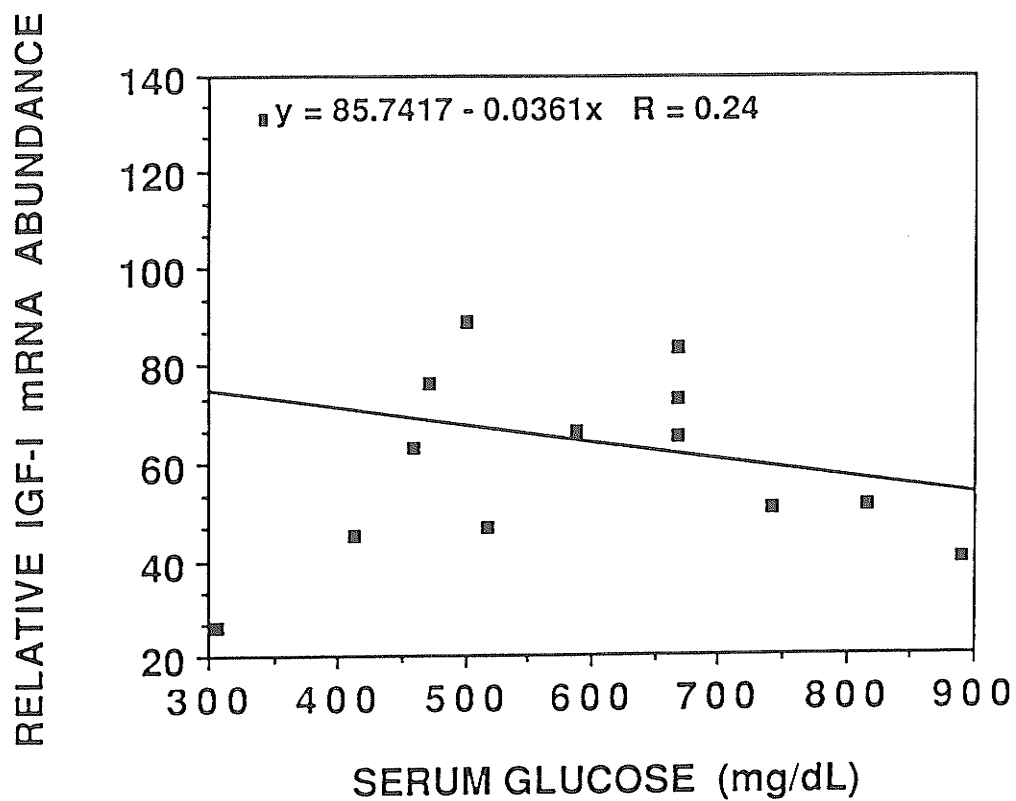


Fig. 5-3. Correlation between serum glucose and hepatic IGF-I mRNA abundance. Data is derived from individual rats at various times after STZ-administration.

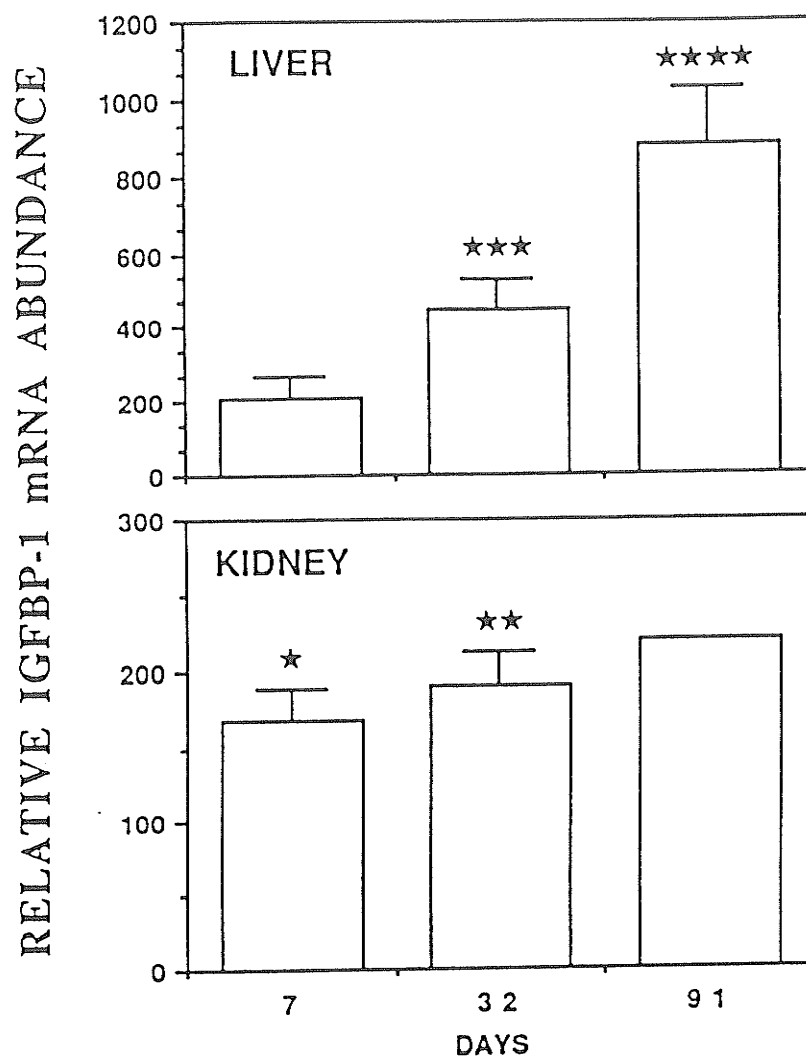
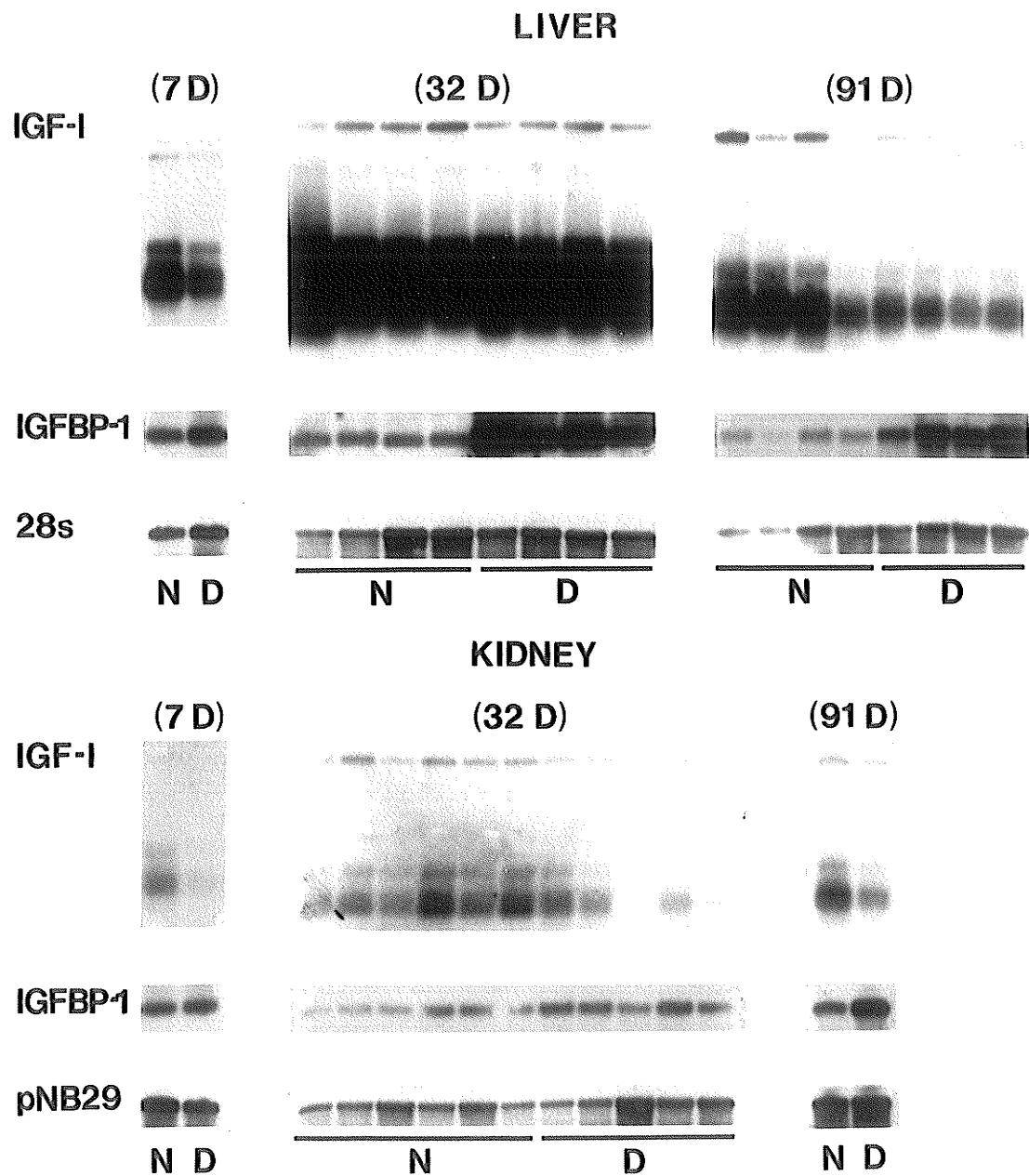


Fig. 5-4. The effect of STZ-induced diabetes on IGFBP-1 mRNA abundance in the liver and kidney. The relative tissue abundance of IGFBP-1 mRNA at 7, 32 and 91 days after STZ administration was quantitated either by slot-blot or Northern blot hybridization and has been expressed as a percentage of the age-matched, non-diabetic control rats. The data represent the mean $\pm$ SEM for 3 to 6 per group except in the case of the 91 day kidney, which represents a single observation on a pooled sample. Significant difference between diabetic and non-diabetic rats is indicated as *, **, *** and **** which represent $p < 0.05$, $p < 0.01$, $p < 0.005$ and $p < 0.0005$, respectively.

Fig. 5-5. Northern blot analysis of hepatic and renal RNA from diabetic rats at various times after STZ administration. Ten micrograms of poly (A)⁺ RNA extracted from individual (liver) or pooled tissue (kidney) were analyzed. The upper panel depicts the pattern of hybridization obtained with a rat IGF-I cDNA, the middle panel shows the hybridization obtained with a rat IGFBP-1 cDNA while the lower panel shows hybridization of the same filter with a ribosomal probe (28s) or a cDNA probe for a rat heat shock-like protein (pNB29).



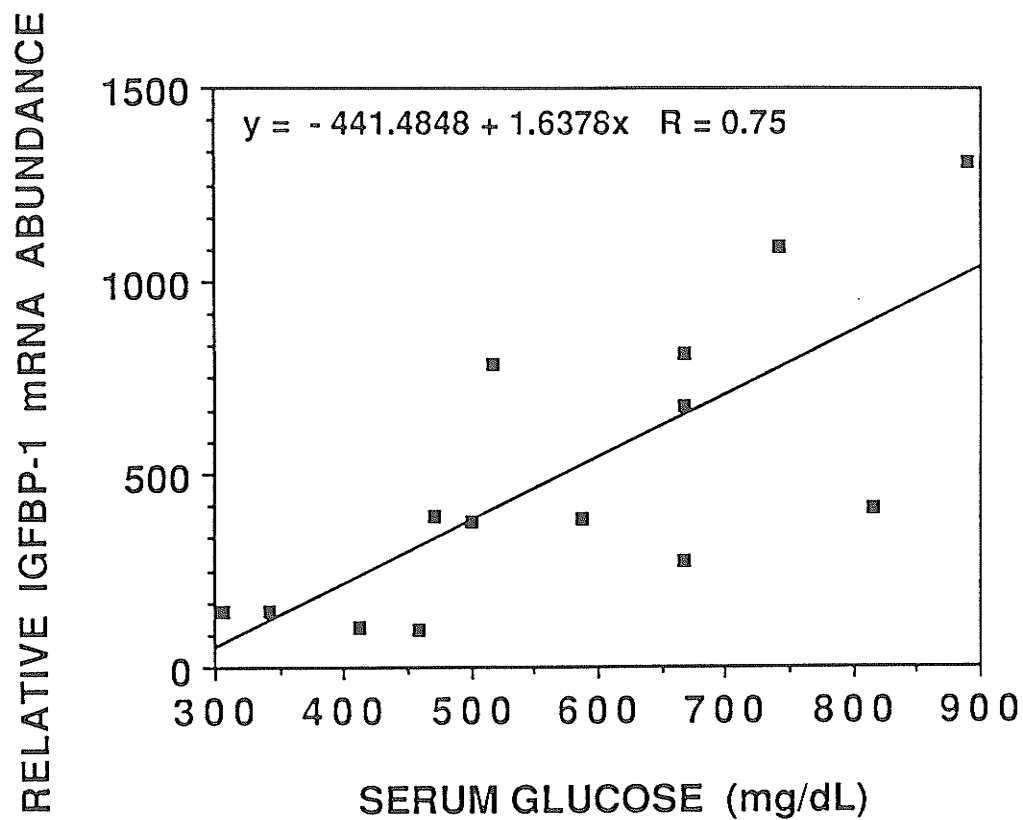


Fig. 5-6. Correlation between serum glucose and hepatic IGFBP-1 mRNA abundance. Data is derived from individual rats at various times after STZ-administration.

mRNA was observed ($R = 0.75$, $p < 0.001$). There was no significant correlation between hepatic IGF-I mRNA and IGFBP-1 mRNA ($R = 0.25$, $p = \text{NS}$).

5.3.3 Effect of insulin on IGF-I and IGFBP-1 mRNA abundance in STZ-induced diabetic rats

In a separate experiment the effect of insulin on IGF-I and IGFBP-1 mRNA abundance was examined. Diabetic rats, 32 days after STZ administration, were treated for 7 days with insulin. The insulin-treated diabetic rats had a mean serum glucose concentration at the time of death which was not significantly different from non-diabetic rats (164 ± 28 versus 101 ± 3 mg/dL). Treatment of diabetic rats with insulin resulted in an increase in hepatic and renal IGF-I mRNA. However, the changes observed were of small magnitude and did not achieve statistical significance (Fig. 5-7). In contrast administration of insulin to diabetic rats resulted in a significant decrease in renal IGFBP-1 mRNA (Fig. 5-7).

5.3.4 Gene expression of IGFBP-1, -2, -3, and -4 in the spontaneously diabetic BB/W rats and the effects of intensive insulin treatment on these IGFBPs

The mean body weight, blood glucose concentration and glycosylated haemoglobin in the various treatment groups are shown

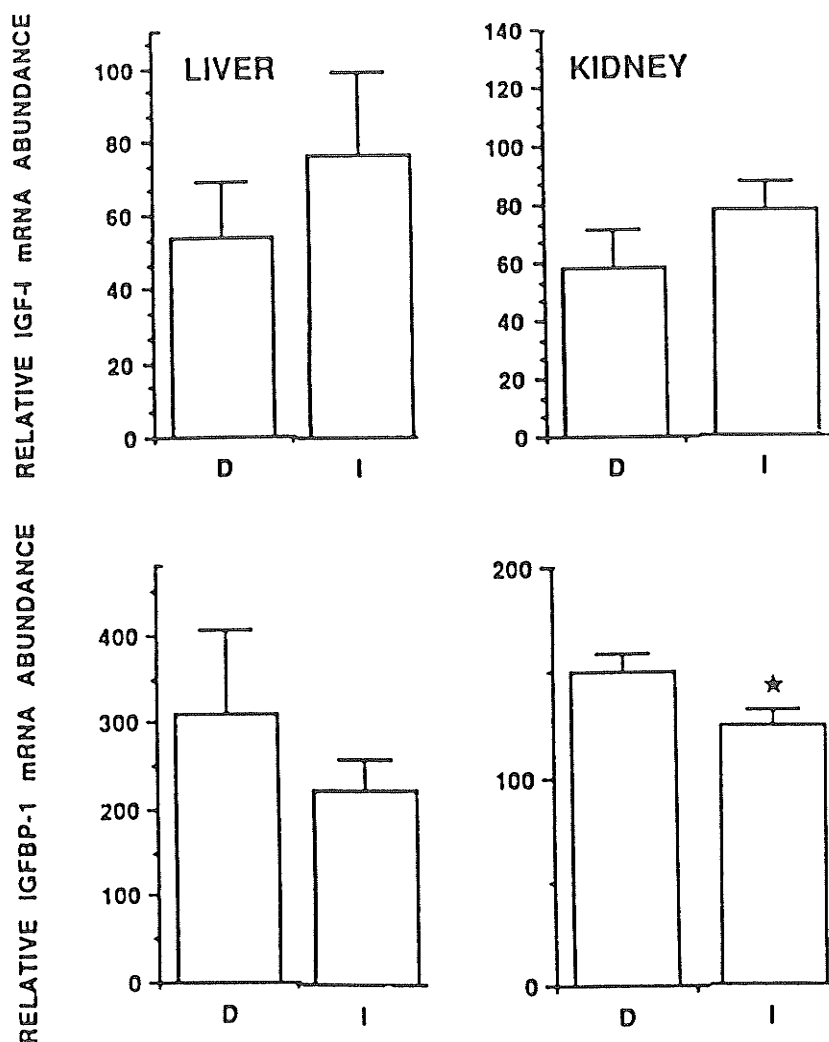


Fig. 5-7. The relative tissue abundance of IGF-I and IGFBP-1 mRNAs in diabetic rats following treatment with insulin. Abundance of IGF-I and IGFBP-1 mRNAs were quantitated by slot-blot hybridization and have been expressed as a percentage of the age-matched, non-diabetic control rats. The data represent the mean $\pm$ SEM for 6 rats per group. The significant difference ($p < 0.05$) between diabetic rats (D) and insulin-treated diabetic rats (I) is indicated by the asterisks.

in Table 5-2. In each group of diabetic rats, the mean body weight gain was significantly reduced compared with age-matched, non-diabetic control rats. The serum glucose concentration and glycosylated haemoglobin levels were significantly elevated in diabetic rats. Intensive insulin treatment normalized these biochemical parameters and partially restored body weight in diabetic rats.

The effects of diabetes and insulin on hepatic levels of IGFBP-1 mRNA are shown in Fig. 5-8. A significant increase in IGFBP-1 mRNA was seen 1 month and 3 months after the onset of diabetes (5.96 ± 1.92 -fold, $p < 0.05$ and 4.78 ± 0.67 -fold, $p < 0.0005$ respectively). The levels of hepatic IGFBP-1 mRNA in diabetic rats were quite variable with one diabetic rat having very high levels of IGFBP-1 mRNA, about 50-fold greater than control animals. This animal was deleted from the calculation of mean data for the group. The rest of the rats, which had been diabetic for 1 month, had IGFBP-1 mRNA levels two- to ten-fold above controls. The amount of IGFBP-1 mRNA did not appear to increase with increasing duration of diabetes and the levels of hepatic IGFBP-1 mRNA were comparable in rats diabetic for 1 and 3 months. Intensive insulin treatment for 3 weeks normalized the IGFBP-1 mRNA levels in diabetic rats. No significant increase in serum IGFBP-1 in diabetic rats was detected by immunoblotting experiments (data not shown).

Table 5-2. Effects of spontaneous diabetes on body weight, serum glucose and glycosylated haemoglobin in control rats and rats diabetic for 1 and 3 months. Values are means $\pm$ SEM for six rats per group.

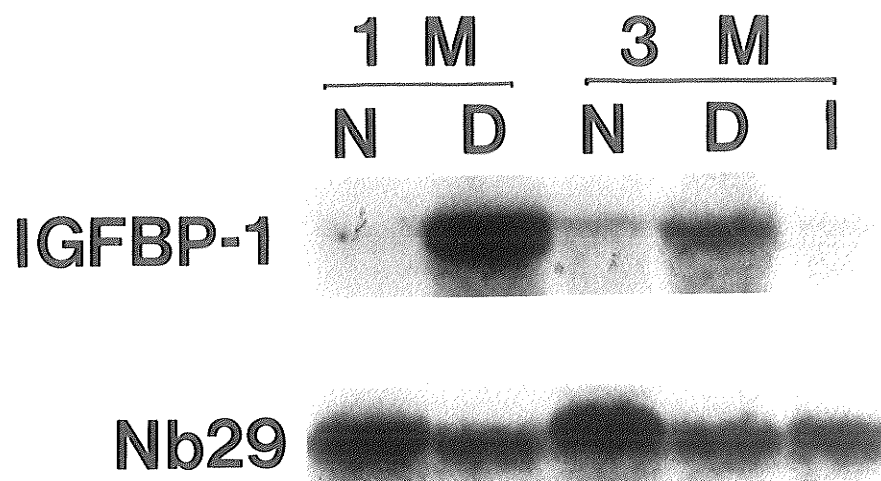
	Body weight (g)	Serum glucose (mg/dL)	Glycosylated haemoglobin (%)
1 Month			
Control	432.3 $\pm$ 6.0	42.0 $\pm$ 5.9	4.41 $\pm$ 0.06
Diabetic	321.2 $\pm$ 6.8**	252.2 $\pm$ 48.5**	9.46 $\pm$ 0.54**
3 Months			
Control	440.8 $\pm$ 25.0	102.7 $\pm$ 8.3	5.44 $\pm$ 0.34
Diabetic	391.0 $\pm$ 16.7*	405.4 $\pm$ 45.4**	8.45 $\pm$ 1.54*
Insulin	414.7 $\pm$ 9.3	76.0 $\pm$ 21.6‡	4.95 $\pm$ 0.98†

* $p < 0.05$, ** $p < 0.005$ compared with control group; † $p < 0.05$,

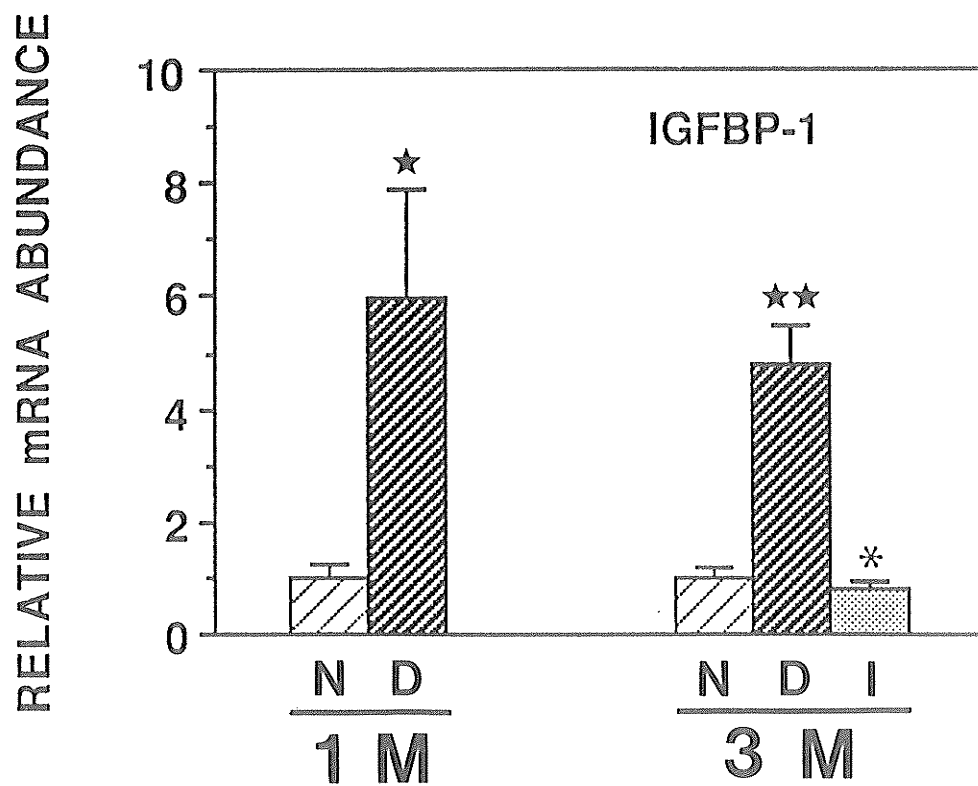
‡ $p < 0.005$ compared with the untreated diabetic groups of rats.

Fig. 5-8. The effects of spontaneous diabetes and insulin treatment on hepatic IGFBP-1 mRNA abundance. (A) A representative Northern blot of total RNA (80 μ g per lane) extracted from non-diabetic (N), diabetic (D) and diabetic rats treated with intensive insulin for 3 weeks (I). Rats were killed 1 month (1 M) or 3 months (3 M) after the onset of diabetes. The pattern of hybridization obtained with a rat IGFBP-1 cDNA is shown in the upper autoradiogram. As a control for gel loading, the pattern of hybridization obtained with Nb29 cDNA is shown in the lower autoradiogram. (B) Data from six rats per group were quantitated by densitometry and were normalized for the loading control. IGFBP-1 mRNA levels were expressed relative to those from the age-matched non-diabetic rats which were attributed an arbitrary value of 1. The data represent means $\pm$ SEM. * $P < 0.05$, ** $P < 0.0005$ compared with the non-diabetic rats. For the 3 month group, the difference between diabetic rats and insulin-treated diabetic rats was significant (+ $P < 0.0005$).

A.



B.

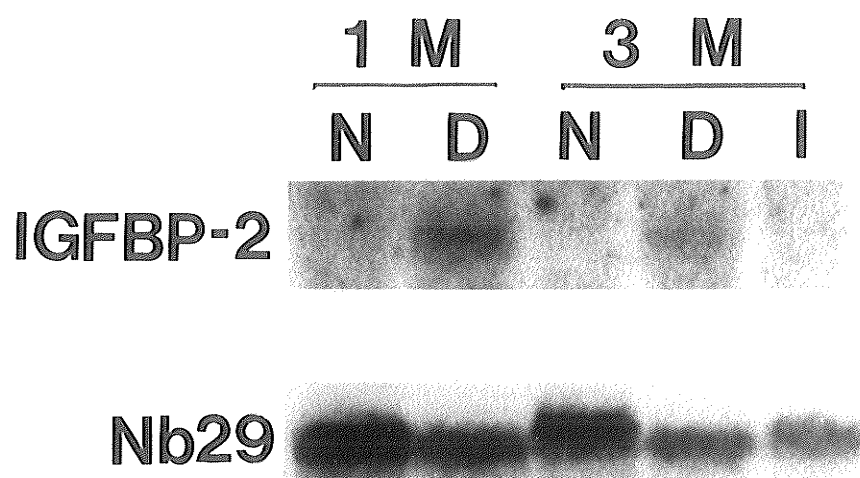


The effects of diabetes and insulin on the amount of hepatic IGFBP-2 mRNA are shown in Fig. 5-9. A dramatic increase (32.6-fold, $p < 0.01$) in hepatic IGFBP-2 mRNA was observed in rats diabetic for 1 month. IGFBP-2 mRNA levels decreased with increasing duration of diabetes and, after 3 months of diabetes, IGFBP-2 mRNA levels were approximately half that seen in rats 1 month after the onset of diabetes (14.5 ± 9.8 -fold versus 32.6 ± 10.8 -fold). Intensive treatment of diabetic rats with insulin resulted in a decrease in hepatic IGFBP-2 mRNA levels.

In contrast to the increase in hepatic IGFBP-1 and IGFBP-2 mRNA, a significant decrease in hepatic IGFBP-3 mRNA (Fig. 5-10) was seen in rats diabetic for 1 month (54.6% of control, $p < 0.0005$) and in those diabetic for 3 months (64.6% of control, $p < 0.005$). Intensive treatment with insulin for 3 weeks did not restore the IGFBP-3 mRNA levels in diabetic rats. Ligand blotting was used to quantitate changes in various binding proteins present in the circulation (Fig. 5-11). Consistent with the reduced IGFBP-3 mRNA level, a significant decrease in 39-42 kDa binding proteins was observed in rats diabetic for 1 month (51.4% of control, $p < 0.05$; Table 5-3). This group of binding proteins, by analogy with data derived from human serum, represents IGFBP-3. There was no significant difference in the amount of the 24 kDa binding protein, as detected by ligand blotting. The amount of 27-30 kDa binding protein was reduced in rats diabetic for 1 month and 3 months compared with the control group (Table 5-3).

Fig. 5-9. The effects of spontaneous diabetes and insulin treatment on hepatic IGFBP-2 mRNA abundance. (A) A representative Northern blot of total RNA (80 μ g per lane) extracted from non-diabetic (N), diabetic (D) and diabetic rats treated with intensive insulin for 3 weeks (I). Rats were killed 1 month (1 M) or 3 months (3 M) after the onset of diabetes. The pattern of hybridization obtained with a rat IGFBP-2 antisense oligonucleotide probe is shown in the upper autoradiogram. As a control for gel loading, the pattern of hybridization obtained with Nb29 cDNA is shown in the lower autoradiogram. (B) The mean $\pm$ SEM. IGFBP-2 mRNA levels for six rats per group were measured by densitometry, normalized for the loading control, and expressed relative to those from the age-matched non-diabetic rats. * $P < 0.01$ compared with the non-diabetic rats.

A.



B.

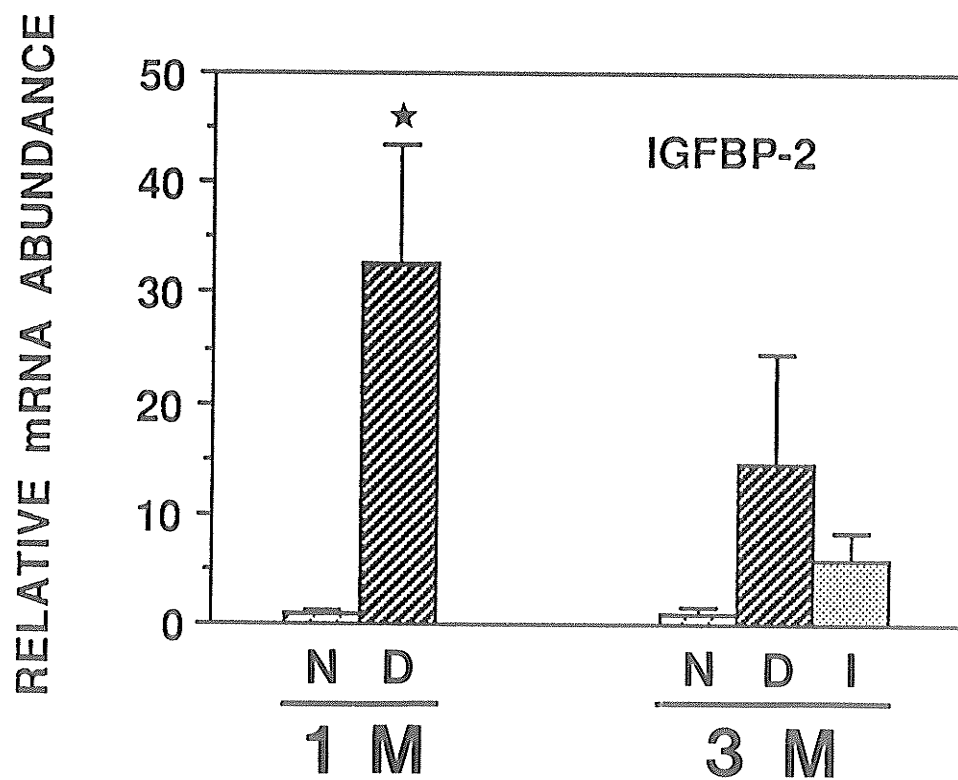
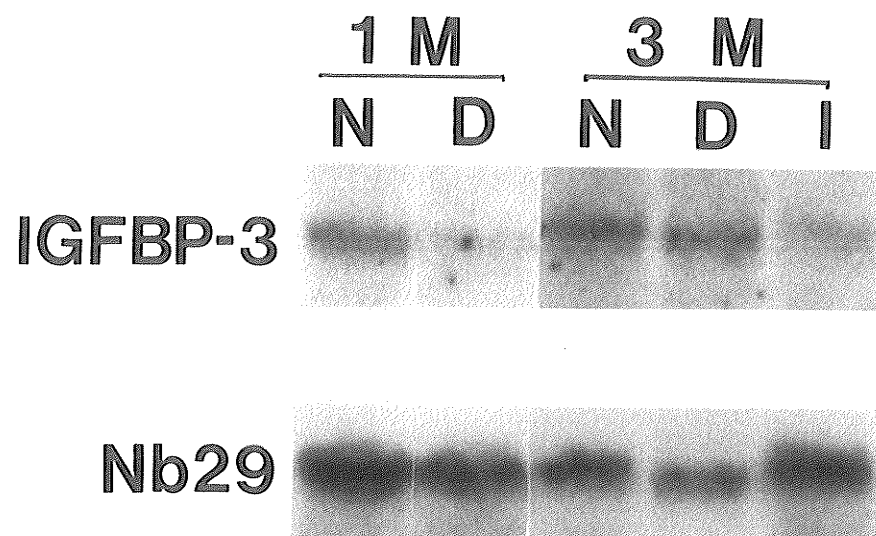


Fig. 5-10. The effects of spontaneous diabetes and insulin treatment on hepatic IGFBP-3 mRNA abundance. (A) A representative Northern blot of total RNA (80 μ g per lane) extracted from non-diabetic (N), diabetic (D) and diabetic rats treated with intensive insulin for 3 weeks (I). Rats were killed 1 month (1 M) or 3 months (3 M) after the onset of diabetes. The pattern of hybridization obtained with a rat IGFBP-3 antisense oligonucleotide probe is shown in the upper autoradiogram. As a control for gel loading, the pattern of hybridization obtained with Nb29 cDNA is shown in the lower autoradiogram. (B) The mean $\pm$ SEM. IGFBP-3 mRNA levels for six rats per group were quantitated by densitometry, normalized for the loading control, and expressed relative to those from the age-matched non-diabetic rats. * $P < 0.005$, ** $P < 0.0005$ compared with the non-diabetic rats.

A.



B.

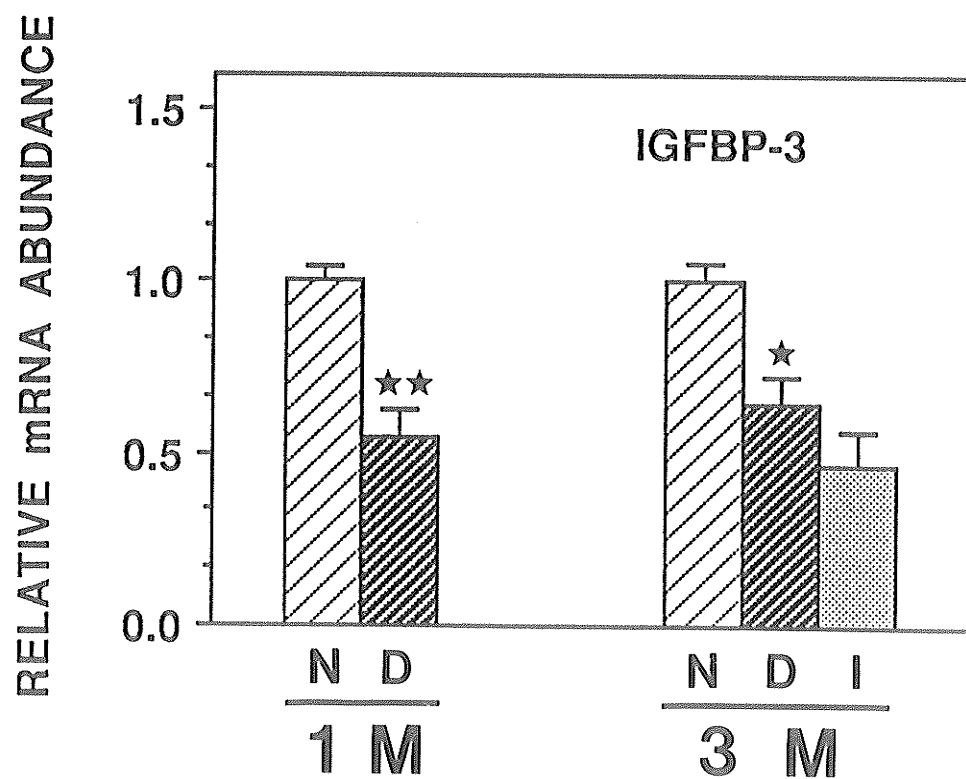


Fig. 5-11. Ligand blot of serum from diabetic and non-diabetic rats. Pooled serum samples (9 μ l per lane) from each group were analyzed in a 12 % SDS-PAGE and transferred to nitrocellulose for ligand blotting with ^{125} I-IGF-I. Rats were killed 1 month (1 M) or 3 months (3 M) after the onset of diabetes. The apparent molecular masses were determined by comparison with protein molecular mass markers.

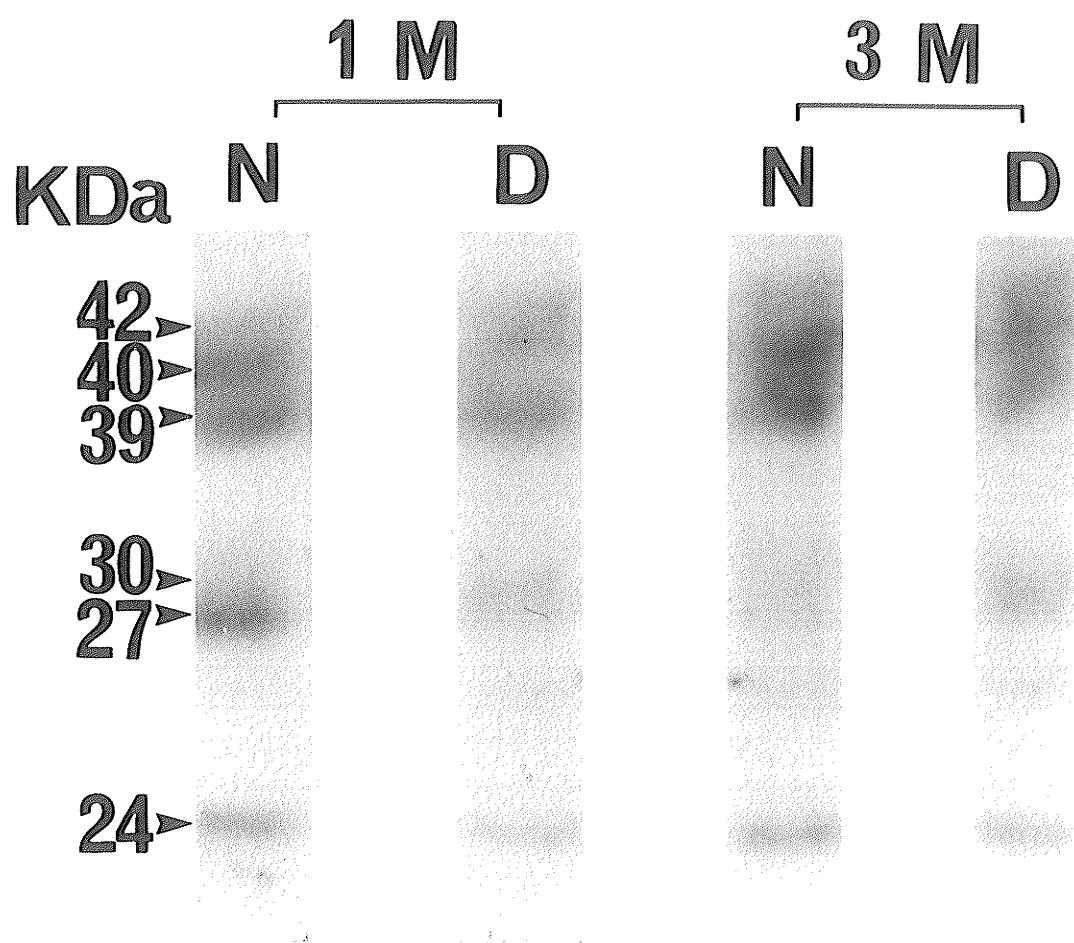


Table 5-3. Densitometric quantitation of serum IGFBPs in control rats and rats diabetic for 1 and 3 months. Equivalent volumes of individual rat serum samples (9 μ l per lane) were loaded on SDS-PAGE gels. The data, expressed in arbitrary units, are means $\pm$ SEM of serum samples from five or six individual rats

	Non-diabetic rats	Diabetic rats
IGFBP†		
1 month		
39-42 KDa	1.03 $\pm$ 0.18	0.53 $\pm$ 0.13*
28-30 KDa	0.69 $\pm$ 0.12	0.32 $\pm$ 0.06*
24 KDa	0.12 $\pm$ 0.05	0.13 $\pm$ 0.03
3 months		
39-42 KDa	0.91 $\pm$ 0.13	0.66 $\pm$ 0.16
28-30 KDa	0.36 $\pm$ 0.07	0.27 $\pm$ 0.07
24 KDa	0.17 $\pm$ 0.07	0.14 $\pm$ 0.05

* p < 0.05 compared with control rats.

† ^{125} I-labelled IGF-I was used in ligand blotting experiments and the resulting autoradiograms were quantitated by densitometry.

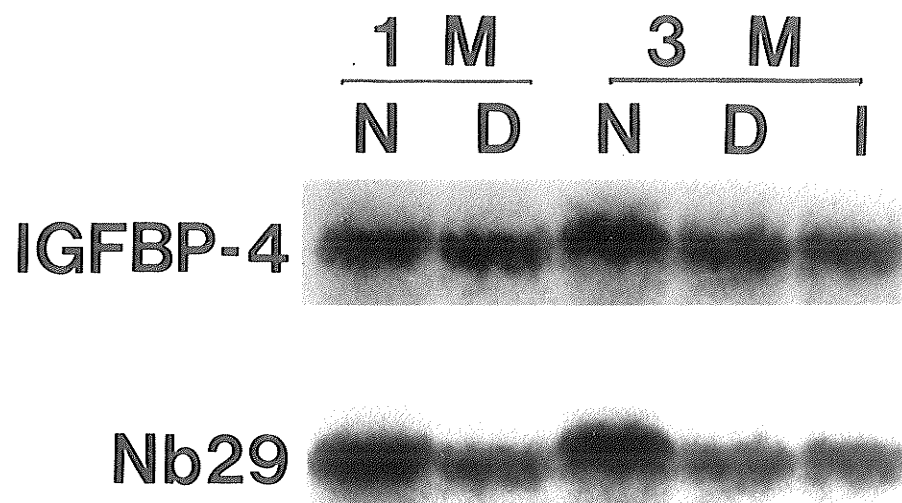
In contrast to the other binding proteins examined, the amount of hepatic IGFBP-4 mRNA was not significantly different in diabetic compared with non-diabetic rats (Fig. 5-12). Intensive treatment with insulin had no effect on the hepatic IGFBP-4 mRNA levels in diabetic rats (Fig. 5-12).

5.4 Discussion

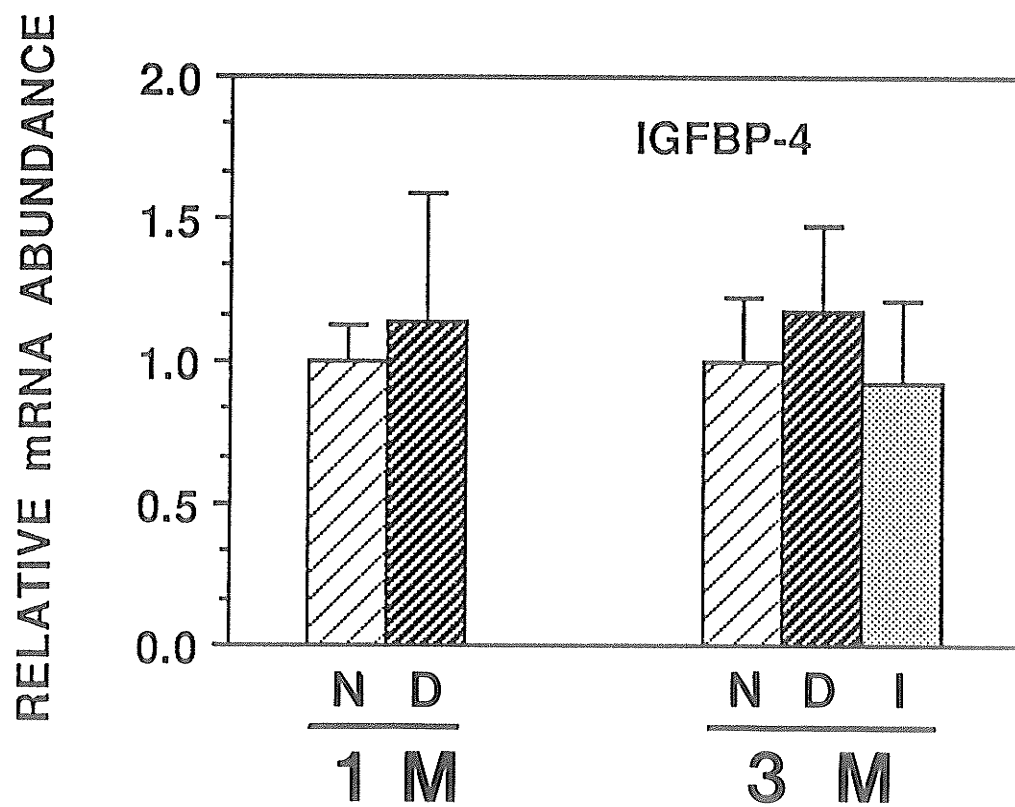
Poor metabolic control in diabetes mellitus is characterized by severe growth retardation and impaired cellular proliferation in many tissues in response to a number of stimuli (Phillips & Young, 1976; Murphy & Lazarus, 1983; Murphy, 1988b). Clearly multiple factors both hormonal and non-hormonal in nature contribute to the impaired growth seen in diabetic animals. It has long been known that circulating IGF activity and IGF-I levels are low in untreated diabetic rats (Phillips & Young, 1976; Maes *et al.*, 1983; 1986). The important role of IGF-I in growth of diabetic rats has been demonstrated in a study from Scheiwiller *et al.* (1986), in which the infusions of recombinant human IGF-I into diabetic rats restored growth toward normal without substantially lowering blood glucose levels. Hepatic IGF-I mRNA levels has been reported to be low in diabetic rats and may contribute largely to the low levels of IGF-I in serum. Since the IGF-I mRNAs are ubiquitously distributed throughout the body, it has been suggested that local expression of IGF-I contributes to functional activity of this peptide in many tissues. Furthermore, the relative contribution of

Fig. 5-12. The effects of spontaneous diabetes and insulin treatment on hepatic IGFBP-4 mRNA abundance. (A) A representative Northern blot of total RNA (80 μ g per lane) extracted from non-diabetic (N), diabetic (D) and diabetic rats treated with intensive insulin for 3 weeks (I). Rats were killed 1 month (1 M) or 3 months (3 M) after the onset of diabetes. The pattern of hybridization obtained with a rat IGFBP-4 antisense oligonucleotide probe is shown in the upper autoradiogram. As a control for gel loading, the pattern of hybridization obtained with Nb29 cDNA is shown in the lower autoradiogram. (B) The mean $\pm$ SEM. IGFBP-4 mRNA levels for six rats per group were quantitated by densitometry, normalized for the loading control, and expressed relative to those from the age-matched non-diabetic rats.

A.



B.



circulating and tissue-derived IGF-I to the available IGF-I in any given tissue may vary under different experimental conditions.

The present study was undertaken to determine whether STZ-induced diabetic rats have reduced IGF-I gene expression in multiple tissues and if so, whether such reduction is related to the duration of their diabetes. The relative abundance of IGF-I mRNA in 7 different tissues at various stages after induction of diabetes with STZ was examined. In each of the 7 tissues, IGF-I mRNA was reduced and the level of reduction was of similar magnitude in most tissues. It had been previously reported that IGF-I mRNA is reduced in the liver, kidney, and lung of diabetic rats (Goldstein *et al.*, 1988; Fagin *et al.*, 1989b). My observations confirm their reports and also suggest that the reduction in IGF-I expression is a generalized phenomenon seen in most, if not all, tissues. Furthermore, I showed that IGF-I mRNA levels decreased more dramatically in most tissues when the duration of diabetes was increased. At present, there is no data to directly address the question as to whether the decreased IGF-I mRNA levels are caused by the decrease in synthesis or stability of IGF-I transcripts. Whether the low insulin alone or the alteration of metabolic status in diabetes is responsible for the decreased IGF-I mRNA levels has not been clearly clarified yet. Insulin has been shown to increase IGF-I mRNA levels in cultured hepatocytes (Johnson *et al.*, 1989), suggesting that low insulin in diabetes may contribute the reduced levels of IGF-I mRNA. The diminished

hepatic and renal IGF-I gene expression was not completely reversed by 1 week of insulin treatment in our study. These findings are in contrast with the report of Fagin *et al.* (1989b), who demonstrated that insulin treatment reversed the diminished IGF-I expression seen in liver and kidney of STZ-induced diabetic rats. However, in their study insulin treatment started early after the onset of diabetes whereas in my study insulin-treatment was initiated after 1 month of untreated diabetes. It is possible that 1 week insulin treatment was insufficient to reverse established changes in chronically diabetic rats.

Studies in human subjects have demonstrated that circulating levels of IGFBP-1 in patients with insulin deficiency are elevated and rapidly normalized with insulin administration (Brismar *et al.*, 1988). In glucose clamping studies, exogenous insulin caused a dose-dependent decrease in the serum IGFBP-1 levels and these effects were independent of the glucose levels (Suikkari *et al.*, 1988; 1989). These observations indicate that insulin plays a role in regulation of serum IGFBP-1 levels. In this study, I demonstrated that expression of IGFBP-1 gene is enhanced in both the liver and kidney of STZ-induced diabetic rats. These data are consistent with the human studies and support the report of Unterman *et al.* (1989), that increased amounts of the IGFBP-1 are present in serum from STZ-induced diabetic rats. Furthermore, my findings indicate that the change of IGFBP-1 in diabetic rats occurs at the mRNA levels. When I completed this studies and

submitted to a journal for publication, a report from Ooi *et al.* (1990) appeared. They showed that hepatic IGFBP-1 mRNA levels are dramatically increased in STZ-induced diabetic rats, and this result confirmed our data. In my studies, treatment with insulin for 1 week significantly decreased the IGFBP-1 mRNA levels in kidney from diabetic rats. The direct effects of insulin on IGFBP-1 production has been demonstrated in an *in vitro* system. Studies with explants of human fetal liver (Lewitt & Baxter, 1989), human decidual cells (Thraikill *et al.*, 1990), human hepatoma cells (Conover & Lee, 1990a; Singh *et al.*, 1990), and rat H4IIE hepatoma cells (Unterman *et al.*, 1991) showed that insulin can directly reduce the production of IGFBP-1. It was recently demonstrated by Powell *et al.* (1991) that the effect of insulin on IGFBP-1 occurred at the level of IGFBP-1 gene transcription. A region spanning from 103 to 529 base pairs 5' to the IGFBP-1 mRNA cap site in human IGFBP-1 gene was proved to be necessary to demonstrate the inhibitory effect of insulin in CAT assay (Powell *et al.*, 1991). A 5' flanking region of rat IGFBP-1 genomic DNA was cloned recently in our laboratory, and the sequencing of this DNA is under way at present. Further studies will be needed to look whether an "insulin-response element" as reported in the promoter/enhancer region of other insulin sensitive genes (Forest *et al.*, 1990; Klemm, *et al.*, 1990; Nasrin *et al.*, 1990; O'Brien *et al.*, 1990) is present in this 5' flanking region.

In these studies, the inverse correlation between hepatic IGF-

I mRNA and glucose concentrations did not achieve statistical significance, however, in a more detailed study reported by Goldstein et al. (1988), where graded doses of STZ were administered, a significant inverse correlation between hepatic IGF-I mRNA and serum glucose was observed. While there was no significant correlation between hepatic IGFBP-1 and IGF-I mRNAs, a highly significant correlation was observed between hepatic IGFBP-1 mRNA levels and serum glucose concentrations. It has been reported that lower glucose in the medium and lower intracellular glucose stimulate the production of IGFBP-1 in human fetal liver explants *in vitro* (Lewitt & Baxter, 1989; 1990). The strong correlation between hepatic IGFBP-1 mRNA levels and serum glucose concentrations suggests that, in addition to the stimulating effect of low insulin on IGFBP-1 gene expression, low intracellular glucose levels in diabetic rats, which had high serum glucose levels, may enhance the IGFBP-I gene expression as well.

In addition to the studies of STZ-induced diabetic rats, I investigated the expression of IGFBPs in spontaneously diabetic rats because this model of diabetes may be more relevant to the human diabetes and the expression of IGFBPs in spontaneously diabetic rats has not been reported. The present study is the first report in which the levels of IGFBP-1, -2, -3, and -4 mRNAs have been simultaneously examined in a spontaneously diabetic animal model. Also, I studied a group of diabetic rats which received intensive insulin treatment in order to maintain near-

normal glycaemic control.

One month after the onset of diabetes, the hepatic IGFBP-1 mRNA level was significantly increased in BB/W rats. No further increase in hepatic IGFBP-1 mRNA was observed at 3 months after the onset of diabetes in BB/W rats. This contrasts with the changes in hepatic IGFBP-1 mRNA level seen in STZ-induced diabetic rats, in which a more marked and progressive increase (nine-fold) in IGFBP-1 mRNA levels was seen with increasing duration of diabetes. This difference between the STZ-induced diabetic rats and BB/W rats may, in part, reflect the fact that the BB/W rats were treated with a small amount of insulin. In addition, no significant increase in serum IGFBP-1 was detected by immunoblotting and the 27-30 KDa binding proteins detected by ligand blotting were reduced in BB/W diabetic rats. These results are in contrast to those reported by Unterman *et al.* (1989) who demonstrated a significant increase in IGFBP-1 in the serum of STZ-induced diabetic rats by both Western ligand blot and immunoblotting techniques. However, in their study, the rats received large doses of STZ and were markedly hyperglycaemic. The small amount of insulin used in our BB/W diabetic rats on a daily basis may have been sufficient to prevent the very marked increase in serum IGFBP-1. It is possible that a five- to six-fold increase in hepatic IGFBP-1 mRNA level in BB/W diabetic rats is not great enough to produce significantly increased IGFBP-1 levels in the serum. Alternatively, some modulation of translation and post-translation processes in BB/W

diabetic rats may affect the hepatic production of IGFBP-1. Enhanced clearance of IGFBP-1 from the circulation of diabetic rats cannot be excluded as a possible mechanism for the discordance between hepatic IGFBP-1 mRNA and serum IGFBP-1 levels. Finally, the ligand blot and immunoblotting technique may not be sufficiently sensitive or precise enough to detect small changes in IGFBP-1. In this regard, it should be noted that a significant decrease in the intensity of the 27-30 KDa bands identified by ligand blotting was observed in BB/W diabetic rats. At least three binding proteins of approximately this size have been identified: IGFBP-1, -2, and degraded IGFBP-3 which lacks the COOH terminus (Zapf et al., 1988; Yang, et al., 1989). Presumably a decrease in the amount of other IGFBPs of this size may mask modest increases in the concentration of IGFBP-1 seen in autoradiograms of Western-ligand blotting. Treatment of BB/W diabetic rats with intensive insulin for 3 weeks completely normalized the hepatic IGFBP-1 mRNA levels, demonstrating the reversible nature of the increase in hepatic IGFBP-1 mRNA. This is similar to the result seen in STZ-induced diabetic rats.

A dramatic increase in hepatic IGFBP-2 mRNA levels (14- to 32-fold) was seen in BB/W diabetic rats. Treatment of diabetic rats with insulin for 3 weeks decreased the levels of IGFBP-2 mRNA. An increase in IGFBP-2 mRNA was observed by other investigators in STZ-induced diabetic rats (Boni-Schnetzler et al., 1989; Ooi et al., 1990). In one study, treatment with insulin for 7 days

reduced IGFBP-2 mRNA to control levels, whereas insulin treatment failed to decrease IGFBP-2 mRNA in the other reported study. The data in spontaneously diabetic rats in this thesis demonstrated that intensive insulin treatment can reduce the hepatic IGFBP-2 mRNA levels in diabetic rats. The direct inhibitory effect of insulin on IGFBP-2 expression has been demonstrated in rat cultured hepatocytes (Boni-Schnetzler *et al.*, 1990). The enhanced hepatic IGFBP-2 gene expression may be caused directly by the low levels of insulin in BB/W diabetic rats.

Decreased serum IGFBP-3 has been reported in diabetic patients and STZ-induced diabetic rats (Baxter & Martin, 1986; Zapf *et al.*, 1989). Consistent with these reports, the ligand blot experiments in this study demonstrated that serum IGFBP-3 is reduced in diabetic BB/W rats. In pregnancy, serum IGFBP-3 was found to be elevated by radioimmunoassay (Baxter & Martin, 1986), but decreased when assayed by Western-ligand blotting (Davenport *et al.*, 1990; Guidice *et al.*, 1990; Hossenlopp *et al.*, 1990). Reduced serum IGFBP-3 levels measured by Western-ligand blotting in pregnancy is believed to be related to the serum protease produced or increased during pregnancy which changes the IGFBP-3 affinity for IGF radioligands (Davenport *et al.*, 1990; Guidice *et al.*, 1990; Hossenlopp *et al.*, 1990; Gargosky *et al.*, 1991; Suikkari & Baxter, 1991). Therefore, decreased serum IGFBP-3 levels detected by Western-ligand blotting do not correctly reflect the real levels of IGFBP-3 in pregnancy serum. No similar protease activity was found

in diabetic rat serum, since co-incubation of diabetic rat serum with normal rat serum did not reduce the abundance of IGFBP-3 in normal rat serum detected by Western-ligand blotting (data not shown) whereas pregnancy serum did. Therefore, decreased IGFBP-3 levels in diabetic serum detected by Western-ligand blotting were due to the reduced levels of IGFBP-3 rather than protease in serum of diabetic rats. Furthermore, the decreased hepatic IGFBP-3 mRNA level in these diabetic rats was demonstrated in this study. As to the best of my knowledge, this is a first report showing that decreased production of IGFBP-3 in diabetic rats occurs at mRNA level. Since IGFBP-3 has been shown to be an IGF-I-dependent protein (Clemmons *et al.*, 1989; Zapf *et al.*, 1989), the low levels of IGF-I in diabetic rats may be responsible for the reduced expression of IGFBP-3. Although intensive insulin treatment reduced the elevated IGFBP-1 and -2 mRNA levels in BB/W diabetic rats, the level of IGFBP-3 mRNA was not restored by treatment for 3 weeks with insulin. Graubert *et al.* (1991) have reported a similar sluggishness in restoring serum IGFBP-3 levels with insulin treatment of STZ-induced diabetic rats.

Rat IGFBP-4 cDNA has been recently cloned from a rat liver cDNA library. Also it has been shown that IGFBP-4 mRNA is highly expressed in liver. A single 2.6 Kb IGFBP-4 mRNA was detected by an IGFBP-4 oligonucleotide probe in our Northern analysis of liver RNA. However, no significant change in hepatic IGFBP-4 mRNA levels was found in BB/W diabetic rats compared with control rats.

In this study, the amount of laboratory chow that was consumed by diabetic and control rats was not systematically determined. It has been reported that STZ-induced diabetic rats have extreme hyperphagia. The effects of nutrition on IGF-I and IGFBPs have been well studied. In fasted rats, expression of IGF-I and IGFBP-3 is decreased, whereas expression of IGFBP-1 and -2 is increased. Refeeding results in an increase in IGF-I and IGFBP-3 expression, and a decrease in IGFBP-1, and -2 expression (Emler & Schalch, 1987; Blum & Ranke, 1990b; Ooi *et al.*, 1990; Murphy *et al.*, 1991). Hyperphagia observed in STZ-induced diabetic rats may have similar effects as refeeding, although it has not been reported. The observation that diabetes decreased IGF-I and IGFBP-3 expression and increased IGFBP-1 and -2 expression, suggests that hyperphagia in diabetic rats is unlikely a cause of these changes.

The functional role of IGFBPs remains controversial as I discussed in the previous chapter. In general, IGFBPs restrict the capillary permeability of IGFs and would therefore inhibit the access of IGFs to receptors and presumably prolong the half-life of the IGFs. The effects of IGFBPs on IGF action in local tissues have not been directly demonstrated, and the available information of the functions of IGFBPs comes from the *in vitro* studies. Most studies documented the inhibitory effects of IGFBP-1 on IGF action *in vitro*. IGFBP-2 was reported to be able to inhibit IGF receptor binding and mitogenic actions of IGF (Knauer & Smith, 1980; Han *et al.*, 1988; Feyen *et al.*, 1991). IGFBP-3 have been shown to have

both inhibitory and stimulating effects on IGF-I action *in vitro* depending on experimental conditions. In addition, IGFBP-3 has been demonstrated to prolong half-life of IGF-I in circulation (Zapf *et al.*, 1986; Cascieri *et al.*, 1988b). IGFBP-4 has been shown to inhibit basal, IGF-I-stimulated and serum-stimulated bone cell proliferation (Mohan *et al.*, 1989).

In this study, I conclude that: 1) there is a general defect in IGF-I gene expression in diabetic rats; 2) expression of IGFBP-1, and -2 genes is elevated in diabetic rats, and insulin treatment can inhibit these elevated gene expression; 3) IGFBP-3 gene expression is diminished and a decrease in serum IGFBP-3 concentration is the major change in circulating IGFBPs in spontaneously diabetic rats. The growth retardation seen in diabetic rats may be explained in part by changes in IGFBP expression in addition to the general defect of expression of IGF-I gene. If the overall function of IGFBP-1 and -2 is to inhibit IGF-I actions, while IGFBP-3 prolongs half-life of IGF-I and enhances IGF-I actions, then the changes in gene expression of these binding proteins in diabetic rats would serve to diminish the activities of circulating and tissue IGF. If the function of IGFBP-3 is to inhibit the action of IGF-I, the reduced gene expression of IGFBP-3 in diabetic rats may serve to compensate the low biological activities of IGF-I. Further studies are required to investigate the exact function of each of these binding proteins in growth of diabetic rats.

Chapter 6: Conclusions and future directions:

The findings of this thesis are consistent with the hypothesis that the perturbation of both IGF-I and IGFBP expressions may contribute to the growth retardation seen in GH deficiency, glucocorticoid excess and diabetes. From the observations in this thesis, I conclude that:

1. IGFBP-1 expression is inversely regulated by GH at the transcriptional level. The overexpression of IGFBP-1 in GH deficiency may inhibit the actions of already reduced IGF-I, and contribute to the impaired growth observed in this disorder.

2. Glucocorticoids inhibit IGF-I expression and stimulate IGFBP-1, -3 expression. Reduced tissue expression of IGF-I together with decreased bioavailability of IGF-I caused by enhanced production of IGFBP-1 and -3 may contribute to the growth retardation seen in glucocorticoid excess.

3. The defect of IGF-I expression is present in many tissues of diabetic rats. Expression of IGFBP-1 and -2 is elevated while expression of IGFBP-3 is diminished in diabetic rats. The changes in expression of IGFBPs may decrease IGF-I activities. This together with inhibition of IGF-I expression may be responsible for the impaired growth observed in diabetic rats.

The information obtained from the studies described in this thesis is useful for further elucidating the molecular mechanisms involved in regulation of IGF-I and IGFBPs in GH deficiency, glucocorticoid excess and diabetes. The understanding of these mechanisms may lead to new therapeutic approaches, with the hope of improved growth in children with these disorders.

Future studies would be a series of experiments *in vitro* to address the question whether the effects of GH, DXM or insulin on expression of IGF-I and IGFBPs seen in our *in vivo* studies are direct or indirect. Cultured hepatocyte would be a good system for these studies. In the cases where the direct effects of hormones can be demonstrated *in vitro*, I would like to further determine the level at which the hormones act to regulate mRNA levels by using nuclear run-on and mRNA stability analysis. If the regulation of the transcription rate of the IGF-I or IGFBP genes can be proven, I would like to localize the hormone-response sequences in the 5' flanking region of the genes.

Recently, I have cloned a potential 5' flanking sequence of the rat IGF-I gene, which contains at least 15 Kb sequence upstream of the transcriptional start sites. Also, a 5' flanking sequence of rat IGFBP-1 gene has been cloned in our laboratory. These two sequences provide fundamental tools for studying the hormone-response elements. Experiments involving deletions and mutations of 5' flanking sequences may reveal hormone response sequences in

these genes. The information obtained could allow for DNA footprinting studies which would lead to the determination of potential trans-acting factors that interact specifically with the genes. The results of these studies would provide some insight into the molecular mechanisms by which the IGF-I and IGFBP genes are regulated.

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