IDENTIFICATION OF GROWTH HORMONE RESPONSE SEQUENCE IN RAT IGFBP-1 PROMOTER

BY

XINPING LU

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ABSTRACTS

Hepatic transcription of insulin-like growth factor binding protein-1 (IGFBP-1) is enhanced in hypophysectomized (hypox) rat and can be rapidly down regulated by administration of GH. Here we have used DNase-1 protection and transient transfection studies to identify DNA sequences mediating the suppressive effect of GH on the IGFBP-1 promoter. Hepatic nuclear extracts prepared from pituitary intact, hypox and GH-treated hypox rats were analyzed. Three DNase-1 protected regions were identified where there were reproducible differences in the footprint pattern obtained with nuclear extracts from pituitary intact and hypox rats. These regions, F4, F3 and F2, correspond to the nucleotides -235 to -245, -447 to -500 and -748 to -788, respectively. F4 was not responsive to GH whereas the F3 and F2 footprints were acutely responsive to GH, in that a reversion to the normal pattern was apparent within 30 min after administration of GH, at 50 μg/100g body weight to hypox rats. In each case the response after a single injection of GH was transient with a reversion to the hypox pattern apparent after 1h. When F2 region was removed by deletion of fragment -824 to -557 bp, the suppressive effect of GH on the IGFBP-1 promoter CAT activity was lost. Since GH induces hepatic expression of c-fos and c-jun, the effect of AP-1 on the IGFBP-1 promoter was also investigated by co-transfection of the IGFBP-1 promoter CAT plasmid with c-fos or c-jun expression vectors. Co-expression of c-fos suppressed IGFBP-1 CAT activity 4 to 6 fold, while c-jun suppressed activity by less then 2 fold. Co-transfection of c-fos and c-jun together suppressed activity 5 to 7 fold. However, the IGFBP-1 promoter 5' and internal deletion

constructs still retained c-fos suppresive effect. These results indicate that the rat IGFBP-1 promoter GH response element may locate within the region -788 to -748 bp upstream of transcription start site and AP-1 has a suppresive effect on the IGFBP-1 promoter CAT activity.

III. LIST OF ABBREVIATIONS

ALC	acid labile subunit
AP-1	activator protein-1
bp	base pair(s)
BW	body weight
^{0}C	degree centigrade
cAMP	cyclic adenosine monophosphate
CAT	chloramphenicol acetyltransferase
cDNA	complementary deoxyribonucleic acid
DMEM/F-12	Dulbecco's modified Eagle's medium/Ham's F-12 mixture
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
GAS	IFN γ-activated sequence
GH	growth hormone
GHR	growth hormone receptor
GHBP	growth hormone binding protein
h	hour(s)
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
Hypox	hypophysectomized
IFN	interferon

IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IL	interleukin
IRE	insulin response element
kb	kilobase
kda	kilodalton
L	liter
MAP	mitogen-activated protein
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
M	molar
MW	molecular weight
PBS	phosphate buffered saline
PEST	Pro-Glu-Ser-Thr
PKC	protein kinase C
SRE	serum response element
Stat	signal transducer and activator of transcription
SV 40	simian virus 40
µg	microgram
µl	microliter

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V. INTRODUCTION

V. 1. Molecular mechanism of growth hormone action

Growth hormone (GH), a polypeptide hormone, is synthesized and secreted from anterior pituitary cells. Circulating GH is bound to a plasma protein whose structure is virtually identical to the extracellular portion of the GH receptor and is carried to target organs. The most striking and specific effect of GH is the stimulation of linear growth that results from GH action on the epiphyseal cartilage or growth plates of long bones. GH also increases the activity and probably the number of bone modeling units. Visceral organs (liver, kidney, pancreas, intestines), endocrine glands (adrenals, parathyroids, pancreatic islets), skeletal muscle, heart, skin, and connective tissue all undergo hypertrophy and hyperplasia in response to GH. Furthermore, GH has several actions on carbohydrate and lipid metabolism.

V. 1. 1. GH binding, receptor dimerization and activation of Janus kinase and other signal pathways

GH starts its actions by binding to specific receptors localized in cell membranes. The human GH receptor (GHR) is a protein of 620 amino acids consisting of an extracellular hormone binding domain of 246 amino acids, a single 24-amino acid transmembrane region and a long cytoplasmic domain (Leung et al., 1987). In addition to the membrane bound full-length receptor, a soluble GHR is present in blood (Baumann et al., 1986) and other biological fluids (Pastel-Vinay et al., 1991), which is named GH binding protein

(GHBP). GHBP corresponds to the extracellular domain of the membrane receptor and is thus a soluble short form of the GHR (Baumann et al., 1986; Ymer et al., 1985). The human GH molecule has two receptor binding sites. Based on crystallography, size exclusion chromatography, calorimetry, and fluorescence quenching experiments using the solubilized extracellular domain of the GHR, one molecule of GH binds to two GHRs and induces GHR dimerization (Cunningham et al., 1991; de Vos et al., 1992). Receptor dimerization is crucial for GH signal transduction (Duquesnoy et al., 1994; Berg et al., 1993; Savage et al., 1993). One of the most rapid cellular responses to GH is the binding of the tyrosine kinase Janus kinase2 (JAK2) to GHR (Argetsinger et al., 1993; Vander et al., 1994). JAK2 is a member of the Janus family which consists of cytoplasmic tyrosine kinases that currently includes JAK1, JAK2, JAK3, and tyK2 (Firmbach-Kraft et al., 1990; Harpur et al., 1992; Wilks et al., 1991; Witthuhn et al., 1994). JAK2 appears to be the primary JAK family member activated by GH. JAK1 and JAK3 may also be activated by GH and thereby mediate at least some effects of GH. JAK2 is also involved in erythropoietin, prolactin, IL-3, IL-5, GM-CSF and γ-interferon signaling. Activation of JAK2 after GH binding is a very rapid and transient event. JAK2 serves in signal transduction by phosphorylating itself and GHR or other proteins that associate with the receptor (Argetsinger et al., 1993; Foster et al., 1988; Silva et al., 1993).

In addition to JAK2 , some other kinases and substrates have been demonstrated to be tyrosyl phosphorylated in response to GH. The mitogen-activated protein (MAP) kinases are reported to be activated by GH (Anderson et al., 1992; Campbell et al., 1992; Winston et al., 1992; Moller et al.,1992). However, the pathway which mediates the GH activation of MAP kinase has not been defined. It is possible that both the Ras pathway

and the protein kinase C (PKC) route are involved, and other protein kinases, phospholipaseA2, cytoskeletal proteins, and transcription factors have been considered as MAP kinase substrates (Davis et al., 1993).

Some evidence from several studies suggest that PKC may play a role in GH-initiated signal transduction. Diacylglycerol (DAG), a known activator of PKC, is reported to be rapidly and transiently induced by GH (Doglio et al., 1989; Johnson et al., 1990; Rogers et al., 1989; Tollet et al., 1991). However whether GH-dependent PKC activation lies downstream of JAK2 or is a JAK2-independent pathway remains to be determined. Shc, one of the SH2 domain-containing proteins, has been observed to be tyrosyl phosphorylated by GH (Vanderkuur et al., 1995). Upon tyrosine phosphorylation, Shc can serve as a signaling molecule in pathways such as that of the MAP kinase. Another mechanism of signaling for GH could be related to an increase in calcium influx. GH is able to increase the cytosolic free calcium ion concentration in IM-9 cells (Ilondo et al., 1994) and in freshly isolated rat adipocytes (Schwarts et al., 1990; Schwarts et al., 1992), which is independent of protein tyrosine phosphorylation or PKC activation.

V. 1. 2. Nuclear events in response to GH

Recently, a family of transcription factors that serves as a signal transducer and activator of transcription (Stat) was found to participate in GH signal transduction. Stat proteins, originally identified in the IFN-signaling pathway (Darnell et al., 1994), are latent cytoplasmic factors containing SH2 and SH3 domains. Cytoplasmic Stat proteins, upon tyrosyl phosphorylation, often via a JAK kinase-initiated cascade, form complexes with other Stat and/or non-Stat proteins. These Stat protein complexes then translocate into

nucleus and modulate the transcription of target genes by binding to specific DNA regulatory elements. GH has been shown to induce tyrosyl phosphorylation of several Stat (or Stat-related) proteins. Stat1/p91 which was originally identified in IFN signaling, Stat3/APRF (acute-phase response factor) involved in IL-6 and LIF signaling, and/or Stat5/MGF (mammary gland factor) involved in Pri signaling are tyrosyl phosphorylated in GH-treated 3T3-F442A fibroblasts and in liver of hypophysectomized rats treated with GH (Campbell et al., 1995; Gronowski et al., 1994; Gronowski et al., 1995; Meyer et al., 1994). Consistent with their role as transcription factors, Stat proteins are involved in GH-induced DNA-binding complexes for several genes.

Three complexes induced by GH binding to the sis-inducible element (SIE) of the c-fos gene promoter (Campbell et al., 1995; Gronowski et al., 1995; Meyer et al., 1994). These three GH-induced SIE-binding complexes are thought to contain Stat1 and Stat3 homodimers and heterodimers, respectively. In Ba/F3 cells transfected with GHR, GH induces the binding of Stat1-containing complexes to SIE and IFNγ-response region (GRR) (Wang et al., 1995). GH also stimulates the binding of Stat5 to the IFNγ-activated sequence (GAS)-like response element in the serine protease inhibitor 2.1 (Spi 2.1) promoter (Wood et al., 1995; Bergad et al., 1995), insulin promoter (Galsgaard et al., 1996), acid-labile subunit promoter (Ooi et al., 1997) and cytochrome p450-3A10 gene (Subramanian et al., 1995). DNA binding of Stat-containing complexes may represent an important contribution to modulation of gene expression by GH and suggests a direct signal transduction pathway (GHR→ JAK→ Stats→ target gene) by which GH can elicit nuclear events. Activation of Stat proteins is also observed upon ligand binding to many members of the cytokine receptor superfamily (Darnell et al., 1994), suggesting that

activation of Stat proteins is a common mechanism by which cytokines and growth factors regulate gene transcription.

V. 1. 3. Gene response to GH at the transcription level

Several genes have been reported to be acutely induced or suppressed by GH at the transcriptional level (Gronowski et al., 1995). Early response genes, c-fos and c-jun, are rapidly induced by GH (Gronowski et al., 1995; Gurland et al., 1990; Slootweg et al., 1991). C-fos and c-jun proteins are combined to form jun/jun homodimers or c-fos/c-jun heterodimers which are designated as activator protein-1 (AP-1). AP-1 serves as a transcription factor to regulate the expression of many genes. AP-1 is also considered to participate in the regulation of genes that have a long-term response to GH. For example, in 3T3-F442A adipocytes, binding of Fos-containing DNA-binding complexes is required for expression of tissue-specific genes (Spiegelman et al., 1988). Rat hepatic c-fos and c-jun mRNA abundance is increased by GH (Gronowski et al., 1995). In GH-deficient hypox rats the hepatic transcripts of c-fos are almost undetectable. Administration of GH to hypox rats markedly increases the accumulation of hepatic c-fos mRNA (Gronowski et al., 1995). One serum response element (SRE) presented in the c-fos gene promoter was observed to bind to serum response factor (SRF) and TCFs (Treisman, 1992). The SRE has been shown to mediate stimulation of c-fos by GH (Meyer et al., 1993). A rapid and transient induction by GH of binding of SRF-containing complexes to the SRE (Liao et al., 1995) may be related to the regulation of c-fos by GH. MAP kinases, activated by GH and a number of other growth factors, are reported to phosphorylate SRF and TCFs, and therefore stimulate their transcriptional activity (Anderson et al., 1992; Campbell et

al., 1992; Winston et al., 1992; Moller et al., 1992). These observations suggest that another pathway involving GHR→MAP kinase→SRE-associated proteins→Fos may exist. With this pathway the signal of GH is likely to result in nuclear events contributing to the ability of GH to promote cell growth and differentiation.

The transcriptional levels of a liver-specific serine protease inhibitor 2.1 (Spi 2.1) are increased by administration of GH both in vivo and in vitro (Lecam et al., 1987; Yoon et al., 1990). The GH activation of Spi 2.1 gene transcription is mediated via two GH-response elements. One is a GAS-like element, which binds to Stat5 like proteins induced by GH (Sliva et al., 1994; Thomas et al., 1995). Similar elements are also identified in rat insulin and mouse ALS gene promoters and have the ability to interact with Stat5 containing DNA-binding complexes induced by GH (Galsgaard et al., 1996; Ooi et al., 1997). The other element is GA-rich box, which binds to Janus kinase2-dependent, but Stat protein-independent, DNA binding complexes (Legraverend et al., 1996).

Insulin-like growth factor-1 (IGF-1), which mediates some of GH actions, is stimulated by GH predominantly at the gene transcription level (Bichell et al., 1992; Mathews et al., 1986). Although the mechanism is proved difficult to delineate, a DNase hypersensitive site induced by GH has been identified in the second intron of the IGF-1 gene that parallels the pattern of stimulation of IGF-1 transcription by GH. Recently, an Sp1 binding site present in the IGF-1 gene has been observed to be involved in the regulation of IGF-1 gene transcription by metabolism (Zhi et al., 1997). Whether Sp1 participates in mediating the regulation of GH on IGF-1 gene expression remain unknow.

Insulin-like growth factor binding protein-1 (IGFBP-1) is negatively regulated by GH. The regulation of IGFBP-1 by GH is at the transcriptional level. Compared to insulin, GH

has a modest suppressive effect on IGFBP-1 gene transcription under normal circumstances (Kachra et al., 1994). However, in food-deprived rats and hypox rats, hepatic IGFBP-1 mRNA is markedly increased and the injection of GH but not insulin rapidly reduces IGFBP-1 mRNA accumulation (Murphy et al., 1991; Ooi et al., 1990; Senevirante et al., 1990; Hu et al., 1996). Several experiments using cultured hepatocytes also reported that GH administration acutely decreases IGFBP-1 transcription (Gronowski et al., 1995; Kachra et al., 1994; Thissen et al., 1994). Using the rat IGFBP-1 promoter inserted in a chloramphenical transferase (CAT) reporter vector in this laboratory demonstrated that IGFBP-1 promoter activity is suppressed by GH in rat primary hepatocytes. Progressive 5'-deletion and in vitro transfection studies identified that the GH response sequence is located between -930 and -277bp upstream of the transcription start site (Hu et al., 1996). Within this region no GAS-like element, SRE like element or GAGA box was found. It is more likely that the negative regulation of GH on IGFBP-1 gene expression is mediated by different signal transduction pathways.

V. 2. Functions and regulation of IGFBP-1

III.2.1. The functions of IGFBP-1

Insulin-like growth factor binding proteins (IGFBPs) are a group of structurally homologous proteins that specifically bind to IGFs and modulate their biological effects on target tissues. Complementary DNA sequences for eight IGFBPs have been identified (Baxter et al., 1989; Ooi et al., 1990; Ooi et al., 1988; Rosenfeld et al., 1990; Rutanen et al., 1990; Shimasaki et al., 1991; Shimaski et al., 1991; Oh et al., 1997; Synthesis et al., 1997). Most of the circulating plasma IGF-I and IGF-II is associated with IGFBP-3 and an acid-labile subunit (ALS) in a growth hormone-dependent ternary complex which may serve as a relatively static and usually saturated IGF reservoir (Baxter et al., 1986; Blum et al., 1990). Like IGFBP-3, IGFBP-1 is also present in plasma at a concentration sufficient to alter the functions of IGFs. However, IGFBP-1 contains unsaturated serum IGF binding sites (Guler et al., 1989). To date, virtually all of the known functions of IGFBP-1 are related to its ability to specifically bind and modulate the actions of IGFs.

Several experiments in vitro show that IGFBP-1 inhibits the actions of IGF when present in molar excess or in its phosphorylated form. IGFBP-1 inhibits IGF-1 stimulated aminoisobutyric acid uptake (Ritvos et al., 1988), IGF-1 stimulated [^{3}H] thymidine incorporation (Busbywh et al., 1988) and mediated mitogenesis (Franuman et al., 1989; Liu et al., 1991; Angervo et al., 1991). IGF-1 elicits its anabolic effects via binding to its membrane bound receptors. The mechanism of the IGFBP-1 inhibitory effect on IGF-1 is considered to competite the binding of IGF-1 to its receptor by forming a IGF:IGFBP complex (Rutanen et al., 1988; Campbell et al., 1991). Some results demonstrated that

IGFBP-1 also enhances IGF-1 stimulated DNA synthesis in several cell lines (Elgin et al., 1987; Koistinen et al., 1990).

In vivo studies have shown that injection of IGFBP-1 into rats induced a small increase in blood glucose concentration (Lewitt et al., 1992). In rats with dexamethasone-induced fetal growth retardation, IGFBP-1 mRNA abundance is increased by 8.5 fold compared to that of sham-injected controls (Pricewa et al., 1990). Studies on transgenic mice that overexpress rat IGFBP-1 have presented a phenotype characterized by modest reduction in birth weight, reduced brain size and fasting hyperglycemia (Rajkamar et al., 1995a; Rajkamar et al., 1995b). These data suggest that in vivo, IGFBP-1 may function to inhibit the action of IGF and this inhibition selectively impairs the development of organs.

V. 2. 2. Molecular characterization of IGFBP-1

Human IGFBP-1 consists of 234 amino acids and has a predicted molecular mass of 25.3 kDa (Brinkman et al., 1988; Julkunen et al., 1988; Luthman et al., 1989). The IGFBP-1 protein can be divided into three regions according to structural characteristics. Region 1 contains the first 79 residues including the N-terminal cysteine cluster (Lee et al., 1988; Brinkman et al., 1991a); Region 2 spans residues 80-144 containing a typical Pro-Glu- Ser- Thr (PEST) domain (Lee et al., 1988), which includes clusters of these four amino acids flanked on each side by a positively charged amino acid. PEST domains are also present in region 2 of rat and bovine IGFBP-1. The presence of PEST regions coupled with the negative charge of the IGFBP-1 protein suggests that IGFBP-1 is rapidly metabolized. Region 3 of IGFBP-1 spans residues 145-234 and includes the C-terminal

cysteine cluster. Both N- and C- terminal regions, and particularly the cysteine residues, contribute determinants required for optimal IGF binding (Brinkman et al., 1991b). Human IGFBP-1 cDNA isolated from several organs are essentially identical (Lee et al., 1988; Brinkman et al., 1988; Julkunen et al., 1988; Luthman et al., 1989; Bremer et al., 199191). The IGFBP-1 transcription start site was identified via primer extension studies using RNA extracted from HepG2 human hepatoma and uterine decidual cells (Cubbage et al., 1989; Tseng et al., 1992). Human IGFBP-1 mRNA is a transcript of around 1.55kb consisting of 165 bp of 5'-untranslated sequence, 777 bp of coding sequence, and at least 612 bp of 3'-untranslated sequence. The 3'-untranslated region contains five ATTTA motifs that are characteristic of mRNA species with a very short half-life (Show et al., 1986). The presence of PEST regions mentioned above in the IGFBP-1 protein and the ATTTA motifs in the IGFBP-1 mRNA may explain the rapid and marked fluctuation of IGFBP-1 levels in vivo.

The human IGFBP-1 transcript is expressed in normal fetal liver, postnatal tissues primarily in secretory endometrium, and in pregnancy decidua and liver. Low levels of expression have been found in Wilm's tumor, but not in normal human fetal or postnatal kidney. In the rat, IGFBP-1 mRNA is also found as a single 1.5 kb transcript with four ATTTA motifs in the 3'-untranslated region (Mohn et al., 1991; Murphy et al., 1990). In contrast to the human, rat kidney expresses easily detectable amounts of IGFBP-1 (Mohn et al., 1991; Murphy et al., 1990; Chin et al., 1992). The expression of rat IGFBP-1 mRNA in other tissue organs are similar to those in human.

The human IGFBP-1 chromosomal gene has been isolated and completely sequenced (Cubbage et al.,1989; Powell et al., 1989; Brinkman et al., 1988; Ehrenborg et al., 1992). The gene is divided into four exons and spans 5.2 kb of chromosomal DNA. The IGFBP-1 gene has been localized to human chromosomal region 7p14-p12 (Alitalo et al., 1989; Ekstrand et al., 1990). IGFBP-1 and -3 genes are contiguously arranged in a tail-to-tail fashion separated by only 20 kb of chromosomal DNA (Ehrenborg et al., 1992).

The region 5' to the human IGFBP-1 mRNA cap site functions as a gene promoter (104, 109). Beginning 28 bp upstream from the cap site is a TATA element, which is important for accurate initiation of transcription in genes transcribed by RNA polymerase II. In addition, a CCAAT upstream promoter element is located 72-68 bp 5' to the cap site. The first 1205 bp 5' to the cap site can direct efficient expression of the reporter genes chloramphenical acetyltransferase and luciferase (Powell et al.,1989; Suwanichkul et al., 1990; Powell et al., 1991). A similar location for the rat IGFBP-1 mRNA cap site was reported and the first 470 bp 5' to this cap site is highly conserved in the human sequence (Unterman et al., 1992). These observations suggest that many of the elements which play an important role in IGFBP-1 gene transcription are located within this 470-bp region. Three sites have been shown to be involved in optimal basal activity of the rat IGFBP-1 promoter in H4-11-E cell. They are the site nt -62/ -50 that binds the hepatic nuclear factor-1 (HNF-1), the site nt -108/ -99 that is a partial insulin response element and the site nt -293/ -286 that is homologous to the putative binding site for transcription factor activator protein-2 (AP-2) (Suh et al., 1996).

V. 2. 3. Regulation of IGFBP-1

Developmental regulation

Serum IGFBP-1 levels in human are elevated in fetal life and decline postnatally (Drop et al., 1984a; Drop et al., 1989; Pancal-Roessler et al., 1989). Fetal serum IGFBP-1 levels appear to rise in the time of midgestation and are about 10-20 fold higher than maternal serum levels (Fant et al., 1988). An elevated IGFBP-1 level in amniotic fluid in midgestation was also reported (Baxter et al., 1987). Fetal and amniotic IGFBP-1 levels fall in late gestation. After birth, serum IGFBP-1 levels decline progressively until puberty and remain at low levels constantly in adulthood (Giudice et al., 1992). Similar variations in hepatic IGFBP-1 mRNA abundance following development stages were also demonstrated in human and rats (Brinkman et al., 1988; Murphy et al., 1990; Ooi et al., 1990).

Metabolic and hormonal regulation

Human IGFBP-1 levels in plasma are reported to have rapid daily fluctuations ranging as much as 10-fold or higher in normal, healthy individuals (Baxter et al., 1987; Cotterrill et al., 1988). The fall in IGFBP-1 levels occurs immediately after a meal, while increases occur during fasting (Yeoh et al., 1988). IGFBP-1 levels also appear to be abnormally regulated in some disease states. Elevated IGFBP-1 levels were reported after prolonged exercise (Suikkari et al., 1989), in children with chronic renal failure (Lee et al., 1989) and in insulin-dependent diabetes mellitus (Batch et al., 1981; Crosby et al., 1992). Low plasma levels of IGFBP-1 are observed in individuals suffering from obesity, polycystic

ovary syndrome (Suikkari et al., 1989; Pekonen et al., 1989; Conway et al., 1990; Lwashita et al., 1990) and acromegaly (Hall et al., 1988; Holly et al., 1991).

To date, IGFBP-1 is regulated by several hormones at the gene transcription level. Insulin is the dominant inhibitory agent in the regulation of human and rat IGFBP-1 gene expression. In vitro studies demonstrated that the transcription of IGFBP-1 is inhibited by insulin administration in rat H35 hepatoma cells (Powell et al., 1991), rat primary hepatocytes (Villafuerte et al., 1991), human HepG2 cells (Orlowski et al., 1991) and human fetal liver explants (Lewitt et al., 1989). Insulin also inhibits IGFBP-1 transcription in vivo. Increased hepatic IGFBP-1 mRNA abundance was reported in streptozotocin induced diabetic rats and insulin infusion was found to decrease hepatic and kidney IGFBP-1 gene transcription (Unterman et al., 1990; Ooi et al., 1992). An insulin response element has been identified in human and rat IGFBP-1 gene promoters, which is located in the rat promoter region -135/ -92bp upstream of the transcription start site (Robertson et al., 1994; Goswami et al., 1994; Suh et al., 1994). This sequences is conserved in the human IGFBP-1 promoter (Suwanichkul et al., 1993) and is homologous to the insulin response element of the phosphoenolpyruvate carboxykinase gene, which is also rapidly inhibited by insulin (O'Brien et al., 1990; O'Brien et al., 1991a; O'Brien et al., 1991).

Glucocorticoids have been shown to increase circulating levels of IGFBP-1 in experimental animals and human subjects (Luo et al., 1990; Conover et al., 1993). Glucocorticoids directly stimulate hepatocellular expression of IGFBP-1 at the level of transcription (Unterman et al., 1991; Orlowski et al., 1990). Four cis-elements in the rat IGFBP-1 promoter were shown to be involved in mediating the effects of glucocorticoids

on the regulation of IGFBP-1 gene transcription. One is a potential glucocorticoid response element (GRE) which is located between nt -91 and -77 bp. The other three sites: hepatic nuclear factor-1(HNF-1) binding site (-62/ -50), a partial insulin response element (-108/ -99) and site -253/ -236 are also involved in mediating dexamethasone stimulatory effect under some, but not all, circumstances. Those cis-elements in rat IGFBP-1 promoter cooperate, in varying combinations to allow optimal dexamethasone stimulated promoter activity (Suh et al., 1996). Insulin inhibits the basal and dexamethasone stimulated IGFBP-1 promoter activities.

Several other hormones, such as glucagon, IGF-1 and progesterone, are also involved in the regulation of IGFBP-1. In vitro studies, glucagon was found to have a stimulatory effect on hepatocyte IGFBP-1 transcription (Lewitt et al., 1989; Kachra et al., 1994). In vivo studies, different results have been reported. Some investigators found that different glucagon concentrations in plasma had no consistent effect on IGFBP-1 levels (Jensen et al., 1991). Others demonstrated that a single subcutaneous dose of glucagon administration to fasting, GH-deficient adults resulted in a small, but significant increase in IGFBP-1 levels (Hilding et al., 1992).

V. 2. 4. Regulation of IGFBP-1 by GH

IGFBP-1 has been found to be negatively regulated by GH at the transcriptional level. Elevated hepatic IGFBP-1 mRNA abundance was observed in GH-deficient hypox rats (Senevirante et al., 1990) while low levels were observed in acromegaly (Hall et al.,1988; Holly et al., 1991). Several laboratories have reported that IGFBP-1 expression is

decreased after GH treatment in rat primary hepatocytes (Senevivante et al., 1992; Kachra et al., 1991; Kachra et al., 1994; Thissen et al., 1994). Compared with the effect of insulin, GH has a modest inhibitory effect on IGFBP-1 gene transcription under normal circumstances. In fasting rats and hypox rats, in which hepatic IGFBP-1 transcription was up-regulated, injection of GH, but not insulin suppressed IGFBP-1 transcription (Murphy et al., 1991; Hu et al., 1996). However, the GH signal transduction pathway for IGFBP-1 gene regulation is still not clear. Previous studies in this laboratory have localized the GH response element(s) of the rat IGFBP-1 promoter to the region -930 to -270bp upstream of the transcription start site (Hu et al., 1996). But the GH response cis-element(s) has/have not yet been identified. In this project, DNase-1 protection assay and deletion mutation analysis were used to identify the GH response cis-element(s) in the rat IGFBP-1 promoter.

VI.Materials and Methods

VI. 1. Materials

Hypox or sham-operated pituitary intact male Sprague-Dawley rats of approximately 100g body weight (BW) were obtained from Charles River Canada (Quebec, Canada). Animals were provided with standard laboratory chow and water and observed for changes in body weight for at least 4 weeks before the beginning of the experimental protocol. Human pituitary GH (3 U/mg) was kindly provided by Dr. I. Worsley (University of Manitoba, Manitoba, Canada). GH (1μg, 10μg, 50μg or 100μg/100 BW) or equivalent volume of saline was administrated i.p. to groups of hypox rats. The animals were sacrificed 0.5, 1, 3 and 12 h later. The livers were rapidly removed and used for preparation of nuclear extracts. All animal experiments was performed in accordance with protocols approved by the animal care committee of the faculty of Medicine. University of Manitoba

Radioisotope ^{32}P-labeled nucleotides were obtained from ICN Biochemical Canada Ltd. (Mississauga, Ontario) and Amersham Canada Ltd (Oakville, Ontario). [^{3}H] acetyl Co-enzyme A was obtained from NEN products (Boston, USA). Restriction enzymes were purchased from Pharmacia (Canada) Inc. (Baie d'Urfe, Quebec). Poly(dI-dC).Poly(dI-dC), collagen, collagenase and transferrin were purchased from Sigma Chemical Co. (St louis, USA). The protein assay dye reagent was bought from Bio-Rad laboratories (CA, USA). Ketalean was obtained from MTC Pharmaceuticals (Ontario).

Rompun was obtained from MILES Canada Inc. (Ontario). Cell microsieves were bought from BioDesign Inc. (New York, USA). Rat IGFBP-1 promoter luciferase plasmids containing native IRE or mutations of IRE construct were kindly sent by Dr. Unterman from Chicago.

VI.2. Nuclear extracts preparation

Nuclear extracts were prepared from rat liver as previously described (Gorski et al.,1986). All steps were performed at 4°C or on ice. Phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT) were added to the buffers just prior to use. Animals were sacrificed by decapitation. Fresh rat livers were quickly removed and transferred to a container with 50 ml of PBS buffer. The livers were cut to small pieces and than transferred to a motor driven 30 ml Teflon-glass homogenizer. The volume was brought up to 20 ml with homogenization buffer (10 mM HEPES-KOH, pH 7.6, 25 mM KCL, 0.15 mM Spermine, 0.5 mM spermidine, 1mM EDTA, 2M Sucrose, 1 mM DTT, 0.1 mM PMSF, 10% glycerol). The minced livers were homogenized by approximately 10 up-down strokes. After that, the homogenate was diluted to 60 ml with the homogenization buffer. Two 30 ml homogenate aliquots were layered over two 12 ml cushions of the same homogenization buffer in two ultracentrifuge tubes and centrifuged at 33,000 rpm for 45 min at 4°C in a Ti 60 Wortmen rotor. The combined nuclear pellets were resuspended in 20 ml of low-salt buffer (20 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCL_2$, 20 mM KCL, 0.2 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 25% Glycerol) and centrifuged at 4000 rpm for 10 min at 4°C in a PR-7000 rotor. Packed nuclei were

resuspended in 1 ml of the same low-salt buffer. While the suspend nuclei was vortexed, high-salt buffer (20 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 1.6 M KCl, 0.2 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 25% glycerol) was added in a dropwise fashion to a final concentration of KCl of approximately 380 mM. Nuclear proteins were extracted from the nuclei by continually gentle mixing for 1 h. Nuclei were precipitated by centrifugation at 13,000 rpm in a microcentrifuge for 30 min at 4^0C. The supernatant was transferred to a dialysis bag and dialysed against 500 ml of dialysis buffer (20 mM HEPES-KOH, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 20% glycerol) for 2 h. At the end of dialysis a precipitation was formed and that was removed by centrifugation at 13,000 rpm for 30 min. Nuclear extracts were aliquoted and quickly frozen on dry ice and then stored at -70^0 C. Protein concentration was determined using the Bio-Rad protein assay.

VI. 3. Probe labeling

5'-protruding DNA fragments or synthesized double strand oligonucleotides were end-labeled with ^{32}P as previously described (Sambrook et al.,1989). Plasmid DNA was cut by appropriate restriction enzymes which produce a 5' protruding end. The radionucleotides [α-^{32}P]dCTP or [α-^{32}P]dATP were incorporated onto the 3'- end by M-MLV reverse transcriptase at 37^0C for 1h. The radionucleotide [γ-^{32}P] ATP was also used in some cases by incorporating it into the 5'-end by T4 polynucleotide kinase at 37^0C for 30 minutes. Radioisotope end-labeled DNA fragments were obtained by digestion with a second restriction enzyme and isolation of the appropriate fregment by agarose gel

electrophoresis. The radioactivity of probes were measured using a Beckman scintillation counter.

VI. 4.DNase-1 footprinting

The DNase-1 footprint assay was performed according to the protocol as described (Allegro et al., 1990) with some modifications. Nuclear extracts (10-20 μg) were incubated on ice for 20 min in a volume of 20 μl containing 10 mm HEPES-K, pH 7.9, 1 μg of Poly(dI-dC).Poly(dI-dC), 50 mM KCl, 0.1 mM EDTA, 0.25 mM dithiothreitol, 0.1 mM phenylmethysulfonyl fluoride and 10,000 cpm of DNA probe. Tubes were placed at room temperature for 2 min. Two microliter of DNase-1 (2-20 ng) solution with 25 mM CaCl2 was introduced and incubated for 70 seconds. The digestion reaction was terminated by adding 180 μl of a stop solution (0.2 M NaCl, 20 mM EDTA, 1% SDS, 50 μg/ml yeast total RNA). The reaction mixture was extracted with an equal volume of phenol-chloroform (1:1). DNA was precipitated with 0.3 M sodium acetate and 70% ethanol. After washing with 70% ethanol, packed DNA was dried in a speedvac for 15 min. The pellets were dissolved in 4 μl of formamide buffer, boiled for 3 min and quickly quenched on ice. The probes were separated by electrophoresis though a 7% polyacrylamide-7 M urea gel at 30 W for 2 h. Gels were dried at 80°C under vacuum for 2 h and subjected to autoradiography at -70°C with intensifying screens.

VI. 5. Gel mobility shift assay

Gel mobility shift assays were performed as described (Galsgaard et al., 1996) with some modifications. Two double-strand oligonucleotides were used as probes: footprint region F1 oligonucliotide: top strand 5'- AGTTTTCCTTGCAATATAAA AGAATGAA-3'; bottom strand' 5'-TTCATTCTTTTATATTGCAAGGAA-3', and footprint region F2 oligonucliotide: top strand 5'-TAGGAGTTATTCATAGACCG GG-3'; bottom strand 5'-CCCGGTCTATGAATAACT-3'. The probes were radiolabeled with [α-^{32}P]dATP or dCTP in a 'fill-in' reaction using M-MLV reverse transcriptase. 10 μg of hepatic nuclear extracts were incubated with 10,000 cpm probe in a volume of 15 μl containing 20 mM HEPES, pH 7.9, 50 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 0.1mM phenylmethylsulfonyl fluoride, 10% glycerol for 30 min at room temperature. Free and bound probes were separated on a 5% polyacrylamide gel containing 2% glycerol and 0.25×TBE (25 mM Tris/HCl, 25 mM boric acid, 0.25 mM EDTA; pH 7.9). The gel was preelectrophoresed for 90 minutes at 4^{0}C, 10V/cm with 0.25×TBE buffer. After samples were loaded, electrophoresis was continued for 2-3 h. The gel was dried at 80^{0}C under vacuum for 2 h and exposed to x-ray film at -70^{0}C with intensifying screens.

VI. 6. Isolation and preparation of rat hepatocytes

Male Sprague-Dawley rats of approximately 300g BW were anesthetized with an i.p. injection of a mixture of Ketalean (130 μg/100g BW) and Rompun (50 μg/100g BW). The livers were perfused via the portal vein to the right atrium with Swim's S-77 medium supplemented with penicillin/streptomycin (100 u/ml and 100 μg/ml, respectively) as described (Bissell et al., 1980) with modifications. A single-pass (nonrecirculation)

perfusion was performed. Approximately 200 ml of Swim's S-77 medium with 0.5 mM EDTA was first perfused through the liver to wash away blood. Collagenase (40 mg) dissolved in 100ml of the same medium supplemented with 5 mM calcium chloride was then perfused into the liver for 10 to 15 minutes. During liver perfusion the temperature of the medium was maintained at 37°C and the pH was kept between 7.4-7.6. Collagenase digested liver was carefully removed from the ligaments, washed with the same Swim's S-77 medium then transferred to a clean container containing 50 ml DMEM/ F-12 cell culture medium. Hepatocytes were separated gently and isolated by filtration through a cellmicrosieve. The cell suspension was centrifuged at 1000 rpm with a PR-7000 rotor for 1 minute at 4°C. The supernatant was discarded by aspiration. Cells were washed twice with basal DMEM/F-12 medium (without serum or hormones) and finally resuspended in DMEM/ F-12 medium with 10% fetal calf serum (FCS).

Collagen was dissolved in a dilute acidic acid solution to a final concentration of 0.8 mg/ml. twenty-five µl of this solution was spread over the surface of a 60 mm cell culture dish and dried at room temperature. Hepatocytes were plated at a density of 4×10^6/dish in DMEM-Ham's F-12 medium with 10% FCS. Nonadherent cells were removed after a 1 h incubation, and medium containing 3% FCS, 1 mg/ml bovine serum albumin (BSA), and 0.1 mg/ml transferrin was added. On the following day, the medium was replaced with fresh medium and DNA transfection experiments were performed.

VI.7. Plasmids and transfection

The IGFBP-1/ CAT plasmid was constructed by ligating a Pst1-SfaN1 fragment of the rat IGFBP-1 gene, encompassing the sequence -1627 to +148, into a pCAT-Basic vector (Promega Corp., Madison, WI). In the plasmid Δ-824/-557-CAT, a fragment -824 to -557bp upstream of the transcription start site was deleted from the 1627-CAT construct, in the plasmid Δ-557/-320-CAT , a fragment -557 to -320bp was deleted and in the plasmid Δ-277/-81-CAT , a fragment -277 to -81 bp was deleted from the 1627-CAT plasmid. A c-fos expression vector was constructed by inserting human c-fos cDNA into a pBK28 plasmid, which uses the FBJ murine sarcoma virus LTR as its promoter. A c-jun expression vector was constructed by inserting human c-jun cDNA into pUC18 plasmid, which uses RSVLTR as its promoter. All the plasmids were prepared by a large-scale preparation and purified by cesium chloride gradient ultracentrifugation.

Hepatocytes were plated as described above. On the second day, fresh DMEM/F-12 medium containing 3% FCS, 1 mg/ml BSA and 0.1 mg/ml transferrin were replaced in a volume of 2 ml per dish. A total of 3.5 μg plasmid DNA mixed with 240 mM $CaCl_2$ in a volume of 83 μl was slowly dropped into a same volume of 2×HBS solution (50 mM HEPES, pH 7.1, 280 mM NaCl, 1.5 mM Na_2HPO_4), while bubbling air was introduced into the 2×HBS solution through a pipette. At the end of mixing, a slightly opaque solution appeared. The reaction was then incubated at room temperature for 30 minutes. A total of 150 μl of the mixture was added dropwise into the cell culture dish. After 6 h of incubation, medium was replaced by 1 ml of basal DMEM/ F-12 medium containig 10% of glycerol for 1 minute. Cells were washed twice with PBS buffer and then cultured in 4 ml of DMEM/F-12 medium containing 3% FBS, 1mg/ml of BSA and 0.1mg/ml of transferin.

Dexamethasone (10^{-7} M) and human GH (0.5 μg/ml) were administered immediately after glycerol shock. Hepatocytes were harvested for a CAT assay 30 h later after transfection.

VI.8. CAT Assay

Transfected hepatocytes were rinsed twice with PBS and scraped off the plates with 1 ml of trypsin/EDTA solution. Cells were collected by centrifugation for 2 minutes at a microcentrafuge and resuspended in 250 μl cell lysis buffer (100 mM Tris-HCl pH 7.8, 0.1% Triton X-100). Cells were lysed for 15 min on ice and the cell debris was precipitated by centrafugation for 15 min at 4^0 C. The protein concentration of the supernatant was measured. Cell extracts containing 100-200 μg of protein were transferred to a new tube and brought to a volume of 0.2 ml with cell lysis buffer. Endogenous acetylase was heat-inactivated at 65^0C for 10 min. After cooling to room temperature, cell extracts were transferred into a scintillation vial and mixed with 75 ml of [^{3}H] acetyl CoA solution containing 3.3 mM chloramphenicol, 0.1 M Tris-HCL pH 7.8, 2.5 nCi/ml [^{3}H] acetyl CoA. Scintillant (3 ml) was added on the top of the mixture and CAT activity was quantitated using a phase separation liquid scintillation assay in a Beckman scintillation counter.

VI. 9. Statistical analysis

Statistical analyses were performed with the Student's t test to determine the significance of the differences between control groups and GH or insulin treated groups. Values are means ± SEM. The definition of statistical significance was $p < 0.05$.

VII. RESULTS

VII. 1. DNase-1 protection analysis of IGFBP-1 promoter region -930 to -270 bp upstream of the transcription start site.

As mentioned before, rat IGFBP-1 is regulated by GH at the transcriptional level. IGFBP-1 transcripts were suppressed by administration of GH in primary hepatocytes (Kachra et al.,1994; Thissen et al., 1994), and IGFBP-1 mRNA abundance and transcription rates were up-regulated in hypox rats and markedly decreased by a single injection of GH (Gronowski et al., 1996; Murphy et al.,1990). Previous studies in this laboratory, using the rat IGFBP-1 promoter inserted into a CAT reporter plasmid, have localized the GH response element(s) to the promoter region -930 to -270 bp (Hu et al., 1996). Therefore, DNA-protein interactions were analyzed within this region by DNase-1 protection assay. As we know, regulation of gene transcription is mediated by specific DNA binding proteins. These proteins, after stimulated, bind to or are released from the corresponding cis-elements located in gene or gene promoter region and enhance or suppress gene transcription. Since hypox rats show a marked increase in hepatic IGFBP-1 transcription and mRNA abundance and these abnormalities can be corrected by single injection of GH, these rats were used as the animal model in this experiment. Hepatic nuclear proteins were extracted from pituitary intact sham-operated, hypox and GH treated hypox rats. DNA-protein interactions were compared among these three groups. Within promoter region -930 to -270 bp, fragment -824 to -557 bp and -557 to -270 bp were end-labeled with radioisotope and mixed with nuclear proteins prepared from these

different groups of rats. DNase-1 protection assay revealed that two regions, F2 (-788/-748 bp) (Fig.1) and F3 (-500/-447bp) (Fig.2) had different footprint patterns between sham-operated rats and hypox rats. Administration of GH to hypox rats rapidly reversed these two footprint patterns to the normal pattern. These data indicate that these two regions may be involved in the GH signal transduction pathway. Besides these two regions, a third region F1, (-670/-630 bp) was apparent with the nuclear proteins extracted from GH treated hypox rats, but identical pattern was observed in sham-operated rats and hypox rats.

VII. 2. DNase-1 protection analysis of fragment -277 to -81 bp

Previous studies in this laboratory have found that fragment -277 to -81 bp has different nuclear protein binding abilities between sham-operated rats and hypox rats by using electrophoretic mobility analysis (Hu et al., 1996). To further investigate the interaction of nuclear proteins within this region, DNase-1 protection assay was performed. As shown in Fig3, within the region -245 to -235bp, a footprint F4 was identified. The footprinting pattern was different between sham-operated rats and hypox rats, but GH had no effect on it. That is, the footprinting pattern was identical between hypox rats and GH treated hypox rats. Another footprinted region, F5 (-277/-258 bp) was only apparent with the nuclear proteins extracted from GH treated hypox rats. There was no DNA-protein interaction observed when nuclear proteins extracted from sham-operated rats and hypox rats were used.

VII. 3. Time course of GH effects on footprinting pattern of IGFBP-1 promoter

Some evidence suggests that the regulation of IGFBP-1 gene transcription by GH is rapid and transient. Therefore the time course of GH effects was investigated by DNase-1 protection analysis with the fragment -824 to -557 bp, -557 to -277 bp and -277 to -81 bp. Human pituitary GH (50 μg/100g BW) was administered i.p. to hypox rats. Animals were sacrificed 0.5 h, 1 h, 3 h and 12 h after GH administration. The livers were rapidly removed and used for nuclear extracts preparation. End-labeled DNA fragments were footprinted with these nuclear proteins. Within fragment -824 to -557 bp, as mentioned above, two footprinted regions F1 and F2 were observed to have responses to GH. The pattern in the F1 region was identical between sham-operated rats and hypox rats. However, a different pattern was induced within 0.5 h of GH administration and persisted for 3 h. This changed pattern switched back to the hypox pattern by 12 h of GH treatment. Footprint F2, in which the footprinting pattern was different between sham-operated rats and hypox rats, revealed an acute response to GH, in that a rapid conversion of the footprinting pattern to the normal pattern was apparent within 0.5 h of injection of GH to hypox rats. This response was rapid and transient with reversion to the hypox pattern within 1 h (Fig 1). Within fragment -557 to -277 bp, one footprint region F3 was observed. As with F2, different footprinting patterns were observed between sham-operated rats and hypox rats, conversion to the normal pattern was induced within 0.5 h of GH administration and this GH reponse was also rapid and transient with reversion to the hypox pattern in 1 h (Fig 2). In fragment -277 to -81 bp, two footprints, F4 and F5, were

found. F4 had no response to GH, while within the F5 region, no footprint was observed with the nuclear extracts from sham-operated rats and hypox rats, but a footprint was induced by GH administration 1 h later and that persisted for 12 h (Fig 3A).

VII. 4. Dose response of GH effect on footprints in IGFBP-1 promoter

Varying amounts of GH; 1μg, 10 μg, 50 μg and 100 μg/100g BW, were injected i.p. into hypox rats. Animals were sacrificed 0.5 h and 3 h after GH administration. Nuclear extracts were prepared and incubated with radioisotope end-labeled fragments -824 to -557 bp, -557 to 277 bp. The change in F1 was induced by GH at a dose of 10 μg/100g BW whereas a higher dose of 50 μg/100g BW is required to normalize the footprinting pattern of F2 (Fig 1C). The change in F3 induced by GH required a dose of 50 μg/100g BW (Fig 2B).

VII. 5. Internal deletion constructs from the -1627bp IGFBP-1 CAT plasmid.

Five footprinted regions were observed in the IGFBP-1 promoter. Four of these were modulated by GH administration. In order to identify and confirm which footprinted region was involved in GH signal transduction, internal fragment deletions were performed. The restriction enzyme map of the IGFBP-1 promoter CAT plasmid and the IGFBP-1 promoter region -1627/+147 bp that was used in this experiment is shown in Fig 4. Three fragment deletion constructs are presented in Fig 5. The procedures used for deletion construction are described in Fig 6 and Fig 7.

VII. 6. The effects of GH on CAT activities of deleted mutants

To determine which footprinted region is functionally involved in the GH signal transduction pathway, the three fragment deleted constructs were transiently transfected into isolated hepatocytes. CAT activity was measured. As shown in Fig 8, GH suppressed the 1627bp IGFBP-1 promoter CAT activity to around 30% or 20% of the control group. Construct C (-557 to -320 bp deleted) and construct D (-277 to -81 bp deleted) retained GH responsiveness and demonstrated GH suppresive effects around 17%, 16% of the contral group, respectively. Construct B (-824 to -557 bp deleted) lost its responsiveness to GH, in that the CAT activity of GH treated group was the same as that of the non-GH treated group. These results indicate that the GH response element(s) is likely located within the promoter region -824 to -557 bp.

VII. 7. Effects of in vivo GH administration on the electrophoretic mobility of the oligonucleotides corresponding to the F1 and F2 footprints.

To further investigate the DNA-protein interaction, two double strand oligonucleotides were synthesized according to the sequences of footprint region F1 and F2. The sequences of these two oligonecleotides were described in Materials and Methods. Electrophoretic mobility shift assays were performed. As shown in Fig 9, radioisotope end-labeled double strand oligonucleotide F1(o) forms one DNA/protein complex with the nuclear extracts from sham-operated rats and hypox rats. Administration of GH to hypox

rats resulted in another DNA/protein complex within 30 minutes as indicated by the arrow. Radioisotope end-labeled double strand oligonucleotide F2(o) forms four DNA/protein complexes with the nuclear extracts from sham-operated rats. However, only two retarted bands were apparent with the nuclear extracts from hypox rats, as shown in Fig 10. The hypox retardation pattern was reverted to the normal pattern within 30 min after GH administration. This response to GH was transient with a reversion to the hypox retardation pattern by 1 h after GH injection.

VII. 8. The relationship of GH and insulin in the regulation of IGFBP-1 gene transcription

As mentioned before, both insulin and GH have suppressive effects on IGFBP-1 gene transcription. Under normal circumstances, insulin is the dominant factor in IGFBP-1 gene regulation. However, in some situations insulin loses its inhibitory effect and GH becomes the major hormone to suppress IGFBP-1 gene transcription. It is postulated that some relationship between GH and insulin signal transduction may exist. Here two constructs were used , one is the rat IGFBP-1/Luciferase vector containing the SauI/HgaI fragment of the IGFBP-1 promoter which extents -360 bp upstream of the transcription start site. This fragment contains the native IGFBP-1 IRE with its two insulin response sequences (AACGCAAAACAAACTTATTTTGAACA) and it is inserted immediately upstream to the luciferase gene in the pGL2 vector from Promega. The second one contains mutations in both halves of the IRE (AACGCAccgacgcCcaggccTGAACA) and does not respond to insulin. Both constructs were transiently transfected into hepatocytes isolated from

pituitary intact rats. GH and insulin were administered 6 h before harvesting the cells for the measurement of luciferase activities. As shown in Fig 11, with the normal IRE construct, insulin suppressed IGFBP-1 luciferase activity to about 50% of the control group, whereas GH had no effect. With the mutant construct, there was no response to either GH or insulin.

VII. 9. The effect of activator protein-1(AP-1) on IGFBP-1 promoter CAT activity.

AP-1 is a Jun/Jun homodimer or Fos/Jun heterodimer and functions as a transcription factor. Several studies have demonstrated that as an early-response gene, c-fos can be stimulated by administration of GH in NIH 3T3F442A cells (Gurland et al., 1990; Sumantran et al., 1992), and c-fos mRNA abundance is increased by GH treatment in mouse osteoblasts in primary cell culture (Slootweg et al., 1991) and within rat central nervous system (Minami et al., 1992). The levels of hepatic c-fos mRNA in hypox rats is undetectable, but can be acutely increased by a single injection of GH within 30 minutes and this GH effect is transient with c-fos mRNA declining to baseline levels in 1 hour (Gronowski et al., 1994). The transcription level of c-jun is also increased by GH treatment in several cell lines and hypox rats, although the induction of c-jun transcripts is consistently less than that of c-fos. Therefore the AP-1 complex was hypothesized to be involved in the GH signal transduction pathway for regulation of IGFBP-1 gene transcription. In this experiment a c-fos expression vector which uses the sarcoma virus LTR as promoter was co-transfected with the 1627 IGFBP-1 promoter CAT plasmid into isolated primary cultured hepatocytes. As shown in Fig 12(A), varying amounts of c-fos

expression plasmid from 5 ng to 1 μg were used in the transfection studies. Overproduction of c-fos suppressed IGFBP-1 CAT activity significantly. The inhibitory effect was observed even at a dose of 5 ng of c-fos expression vector. The effect of c-fos on transfection efficiency was assessed by co-transfection with the pCH 110 β-galactosidase plasmid. Over production of c-fos had no effect on β-galactosidase activity. C-jun and c-fos/c-jun were also co-transfected into hepatocytes with the IGFBP-1 CAT promoter vector. As shown in Fig 12(B), co-transfection of c-fos suppressed IGFBP-1 CAT activity 4 to 6 fold, while c-jun suppressed CAT activity less than 2 fold. Co-transfection with c-fos and c-jun together suppressed CAT activity 5 to 7 fold.

VII. 10. Localization of AP-1 site

Previous studies have confirmed that the AP-1 binding consensus sequence is TGAGTCA. In order to identify the AP-1 binding site in IGFBP-1 promoter, 5' deletion and internal fragment deletion constructs of the IGFBP-1 promoter were co-transfected with the c-fos expression vector. As shown in the right panel of Fig 13, Three plasmids were used: IGFBP-1 promoter -1627/CAT, IGFBP-1 promoter -277/CAT and a 277 to -81 bp deletion construct. The left panel demonstrates that c-fos inhibitory effect was retained in the IGFBP-1 promoter -277/CAT construct. With further deletion of fragment -277 to -81bp from the 1627bp IGFBP-1 plasmid, the CAT activity was still suppressed by overexpression of the c-fos gene.

VIII. DISCUSSION

To date, several genes have been found to be regulated by GH at the transcriptional level. Some GH response elements including Sis-inducible element (SIE) (Compbell et al., 1995), serum response element (SRE) (Meyer et al., 1993), IFNγ-response region (GRR) (Wang et al., 1995), IFNγ-activated sequence (GAS)-like response element (GLE-1) (Wood et al., 1995) and a GAGA box (Legraverrend et al., 1996) have been identified. The GH signal transduction pathway (GHR→JAK2→Stats→target gene) is involved in the transcriptional regulation of c-fos, Spi 2.1, insulin, and ALS genes, and all of those genes are positively regulated by GH. IGFBP-1 is negatively regulated by GH. Previous studies in this laboratory have localized GH response element(s) of IGFBP-1 promoter to the region -930 to -277 bp upstream of the transcription start site. However, compared with the above identified GH response elements, no homologous sequence was found within this region.

IGFBP-1 appears to have rapid dynamic regulation in human plasma, with levels varying more then 10-fold within a few hours (Baxter et al., 1989; Hall et al., 1991; Holly et al., 1991). Under normal circumstances, insulin is the dominant inhibitory agent in IGFBP-1 gene regulation and GH only modestly affects IGFBP-1 gene expression. However, in fasting rats and hypox rats, hepatic IGFBP-1 mRNA abundance was markedly increased, and the injection of GH, not insulin rapidly reduced IGFBP-1 mRNA to normal levels (Murphy et al.,1991; Hu et al., 1996). It seems that normal and fasting or hypox rats have different regulation mechanisms for IGFBP-1 gene transcription, and these differences are related to the effect of GH. Therefore, hypox rats were chosen as the animal model in this study.

The rat IGFBP-1 promoter GH response element(s) seems to be located within the region -788 to -748 bp (F2) and -500 to -447 bp (F3) upstream of transcription start site. The footprint pattern in these two regions were different between pituitary intact rats and hypox rats, and injection of GH to hypox rats rapidly reverted the footprint pattern to the normal pattern. The effect of GH was transient. In each case a reversion to the hypox pattern was apparent 1 h after GH administration. The IGFBP-1 promoter region -245 to -235 bp (F4) also has different nuclear protein binding activates between pituitary intact rats and hypox rats, but these DNA-protein interactions are GH-independent, since the footprint pattern of the hypox rats didn't change after administration of GH. Suh et al reported that this region is conditionally required for dexamethasone stimulation (Suh et al.,1996). The synthesis of glucocorcorticoid is stimulated by adrenocorticotropic hormone (ACTH), which is also synthesized and released from pituitary. Therefore, the ACTH→glucocorticord axis is also impaired in hypox rats. The different footprint patterns between pituitary intact rats and hypox rats in site F4 may be glucocorticoid related.

Within the IGFBP-1 promoter region -670 to -630 bp (F1) a DNA-protein interaction was induced by administration of GH to hypox rats, but the pattern in region F1 was identical between pituitary intact and hypox rats. According to the evidence that GH-deficient hypox rats and pituitary intact rats have marked differences in the IGFBP-1 gene expression, nuclear protein interaction in region F1 is not likely to be important in this GH response. In the promoter region -277 to -258 bp (F5), one footprint was induced by GH administration. In this region no footprint was observed in pituitary intact and hypox rats. Previous study have found that this region is a cAMP responsive element (Suwanichkul et al., 1993) that confers at least

part of the stimulatory effect of cAMP on the human IGFBP-1 promoter. The data demonstrated here indicate that multiple pathways may be involved in GH signal transduction.

GH response element(s) in the rat IGFBP-1 promoter is more likely located within the region -824 to -557 bp. A mutant plasmid with deletion of fragment -824 to -557 bp from the original IGFBP-1 promoter CAT vector resulted in total loss of the GH response. However, mutant plasmids containing deletion of fragments -557 to -320 bp or -277 to -81 bp retained GH responsiveness. Two DNA-protein interaction sites were observed within this region, F1 and F2. Comparing the sequence of F1 and F2 with other identified specific protein binding sequences, we found that the sequence GCAAT within the F1 region is the same as the core sequence of IGF-1 gene footprinting region III and the sequence TTATTC within F2 is the same as the core sequence of IGF-1 gene footprinting region V (Pao et al.,1995). Using in vitro transcription assays, Pao et al found that templates lacking region III or V no longer demonstrated differences in transcriptional activity with nuclear extracts from streptozotocin-induced diabetic and normal rats. Using gel mobility shift analysis, they further observed that nuclear protein binding to region III and V was reduced with extracts from diabetic animals, typically 30-50% of that of normal rats (Pao et al., 1995). IGFBP-1 modulates the actions of IGF-1 by competition with IGF-1 receptor binding. Circulating IGFBP-1 and IGF-1 are both mainly produced from liver and are regulated by GH at the gene transcriptional level. IGF-1 gene expression is increased while IGFBP-1 is suppressed by GH administration (Bichell et al., 1992; Gronowski et al.,1995). IGF-1 is also considered to mediate most of the physiological functions of GH. Since these close relationships of regulation and function between IGF-1 and

IGFBP-1, the similarity in footprint sequences indicates that the regulation of IGFBP-1 and IGF-1 by GH may be via a same pathway but may result in different effects.

The DNA-protein interactions in the IGFBP-1 promoter F1 and F2 regions were GH-affected. An oligonucleotide with a sequence corresponding to region F1 formed one retarded band with the nuclear extracts prepared from pituitary intact and hypox rats. After GH administration, an extra retarded band was apparent. This corresponds to the data obtained from footprint analysis, in that injection of GH to hypox rats resulted in a changed pattern in the F1 region. The F2 region oligonucleotide formed four retarded bands with the nuclear extracts from pituitary intact rats and only two bands with the hypox rat nuclear extracts. This seems hard to explain in terms of the footprint pattern in the F2 region, in that a strong protein interaction was found. However, these data indicate that there are marked difference in DNA-protein interaction in the F2 region between hypox and pituitary intact rats. The data from the F2 oligonucleotide gel shift assay further indicate that some of the nuclear protein interactions were GH-dependent. Administration of GH to hypox rats reverted the gel retardation pattern to the normal pattern within 30 min. The effect of GH was transient. A reversion to the hypox retardation pattern was apparent with the nuclear extracts from hypox rats within 1 h after GH treatment. These data indicate that some of the nuclear protein interactions in the F2 region are GH dependent and that the effect of GH is rapid and transient.

As mentioned before, six nucleotides in the IGFBP-1 promoter footprint region F2 are the same as that of the IGF-1 gene footprint region V. Zhu et al reported that an oligonucleotide corresponding to the IGF-1 gene footprint region V exhibited a strong gel shift with the nuclear transcription factor Sp1, and Sp1 antibody led to a supershifted band. Mutations in

region V abolished Sp1 binding in gel shift analyses and also led to greatly reduced transcriptional activity (Zhu et al., 1997). Sp1 protein is a nuclear transciption factor, so whether Sp1 participates in the regulation of IGFBP-1 gene transcription by GH through promoter region F2 needs to be further studied.

The kinetics of the GH effects on the IGFBP-1 promoter footprinting pattern were performed using nuclear proteins extracted from hypox rats treated with GH at different times. In the F1 region, the change in DNA-protein interaction was induced within 30 minutes after GH injection, and this altered nuclear protein binding activity persisted for 3 h. In the F2 region, DNA-protein interactions were also modulated within 30 minutes after GH administration. However, this GH response was transient, with the nuclear protein interaction reverting to the hypox pattern within 1h after GH treatment. These results confirm previous observations which showed that IGFBP-1 gene transcription, as measured by nuclear run-on assay, was reduced within 30 min of a single GH injection (Seneviratne et al., 1990). Gronowski also reported that the abundance of IGFBP-1 mRNA was diminished to 50% of baseline within 30 min after in vivo GH treatment (Gronowski et al.,1995). These results indicate that the effect of GH on IGFBP-1 gene regulation is rapid and transient.

Previous experiments have demonstrated that hepatic expression of the IGFBP-1 and circulating IGFBP-1 concentration are reversibly regulated by insulin. In both the human and the rat IGFBP-1 gene, a negative insulin response element (IRE) has been identified. In the human IGFBP-1 promoter IRE is located between -120 to -96 bp 5' of the mRNA cap site and is 100% conserved in the rat IGFBP-1 promoter (Goswami et al.,1994). In vitro transcription studies performed previously in this laboratory demonstrated that both GH and insulin had

suppressive effects on IGFBP-1 promoter CAT activity in hepatocytes isolated from pituitary intact rats. In contrast, insulin had no effect on promoter CAT activity in hepatocytes isolated from hypox rats whereas GH resulted in comparable suppression of CAT activity in hepatocytes from both hypox and pituitary intact rats (Hu et al.,1996). These observations suggest that the suppressive effect of insulin on IGFBP-1 transcription seems to be GH-dependent or at least these two hormones have some interaction in regulating IGFBP-1 gene expression. In this experiment, using hepatocytes from pituitary intact rats, the results showed that with the normal IRE construct, IGFBP-1 promoter luciferase activity was suppressed by insulin to about 50% of the control group, but GH had no effect. The mutant construct had no response to either insulin or GH. As mentioned before that the GH reponse element(s) may be located within the region -824 to -557 bp, further investigations on the interactions of GH and insulin using longer promoter region with mutant IRE or/and hepatocytes from hypox rats should be performed.

The data from co-transfection of c-fos and c-jun expression vectors with the IGFBP-1 reporter plasmid indicate that AP-1 has a suppressive effect on IGFBP-1 promoter activity. Previous experiments demonstrated that the nuclear transcripts of c-fos, one of the components of AP-1, is rapidly induced by GH administration within 15 minutes (Gronowski et al., 1995). Multiple GH reponse cis-elements in the c-fos promoter have been identified (Chen et al.,1995). Gronowski et al reported that cycloheximide (CHX), a protein synthesis inhibitor, suppressed GH-stimulated AP-1 DNA binding activity, but did not alter the hormone-activated JAK-Stat pathway or block GH-inducible expression of the IGF-1 and Spi 2.1 genes. In contrast, IGFBP-1 mRNA was superinduced by CHX treatment. These observations suggest

that the synthesis of a new protein may play a role in mediating the inhibitory effect of GH on IGFBP-1 mRNA accumulation. Using in vitro transfection studies, we found that overproduction of c-fos or c-jun proteins markedly suppressed the IGFBP-1 promoter CAT activity. Most investigators considered that the IGFBP-1 promoter region -308 to -302 bp upstream of transcription start site is AP-1 binding site, in which the sequence is TGAGTTG. Five neucleotides in that region are identical to the AP-1 consensus sequence TGAGTCA (Suh et al., 1996). But the data from cotransfection studies showed that construct -277CAT still conferred the c-fos inhibitory effect. DNase-1 protection analysis of fragment -557 to -277 bp did not reveal a protected region in the site of -308 to -302 bp. Furthermore, Suh et al reported that mutation of the sequence from -308 to -302 bp did not affect IGFBP-1 promoter activity (Suh et al.,1996). This evidence indicates that AP-1 has a negative effect on IGFBP-1 gene transcription, but whether AP-1 mediates the suppressive effect of GH on IGFBP-1 is still not clear.

In summary, the observations in this study demonstrate that the sequence from -788 to -748 bp and from -670 to -630 bp may represent the GH response cis-elements in the rat IGFBP-1 promoter.

VII. TABLES

Table 1. Sequence of footprints

	Region	Sequence
F1	-670/-630	5'-TTCTGAAGTTTTTCCTTGCAATAT
		AAAAAGAATGAAAAAAACAA
F2	-788/748	5'-CTGGGGACATAGGAGTTATTCA
		TAGACCGGGGGGGGGGGG
F3	-500/-447	5'-AGGTATAACTGTTTCTTTTGGAATGGGG
		TGAACAATGAATTTCTTCACTTGATG
F4	-245/-235	5'-CTCCCCCATC
F5	-277/-257	5'-CCTTTTGATTTCTCCCTGAAC

Table 2. Response to GH

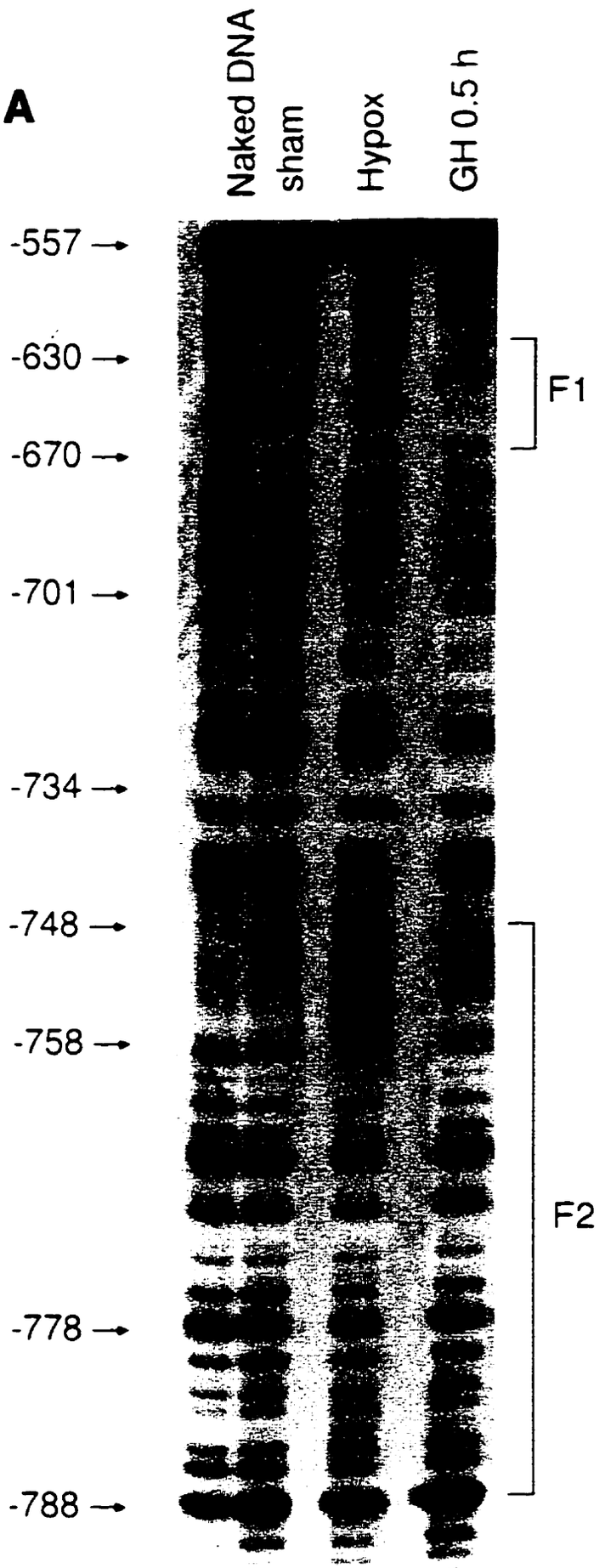
Footprint Region	**Difference Between Pituitary Intact and Hypox Rats**	**GH Response**
F1	No	Yes
F2	Yes	Yes
F3	Yes	Yes
F4	Yes	No
F5	No	Yes

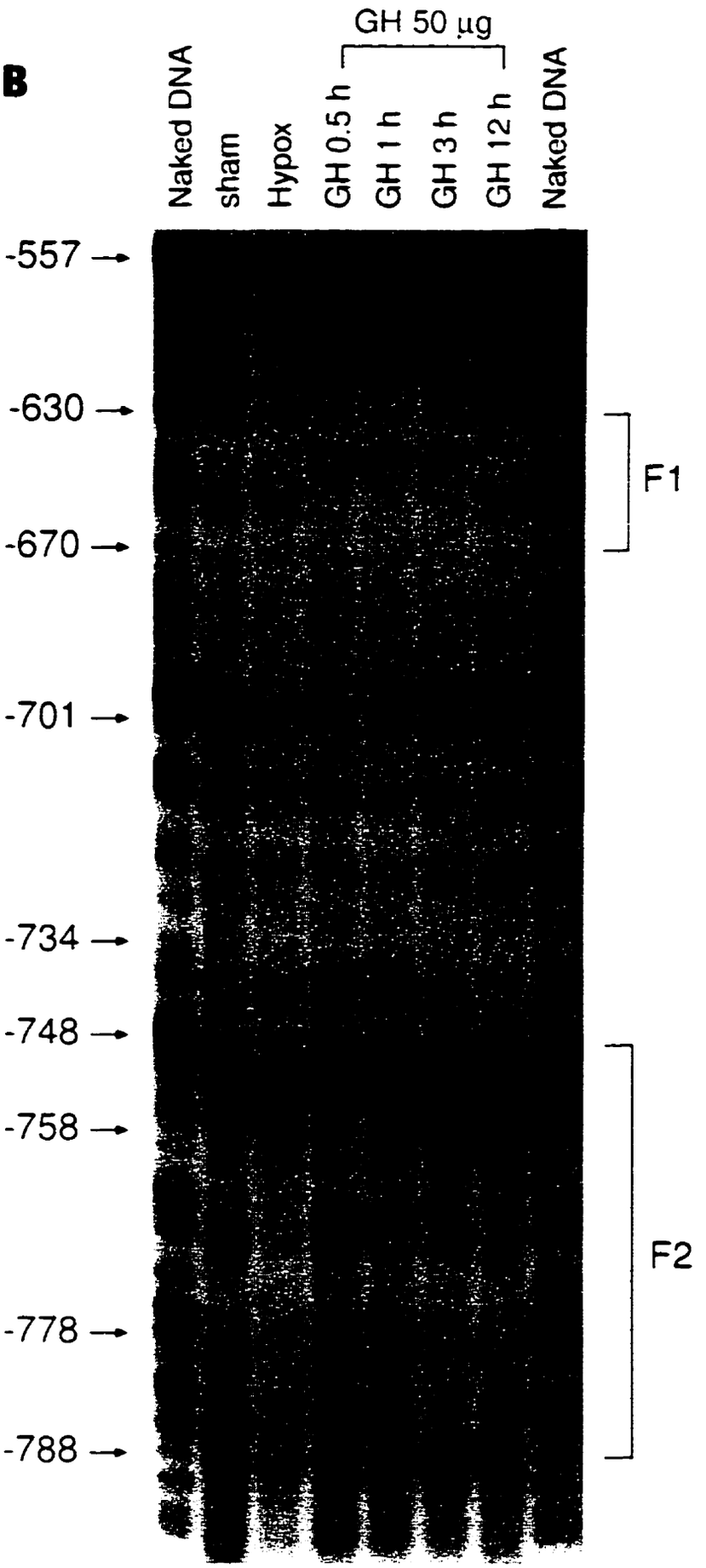
X. FIGURES

Figure 1. DNase-1 protection analysis of IGFBP-1 promoter region -824 to -557 bp

Two livers were pooled to prepare nuclear extracts in each group. Labeled DNA fragments (10,000cpm) were incubated with DNase-1 alone or with both nuclear extracts and DNase-1. Nucleotide positions, 5' to the IGFBP-1 promoter transcription start site are marked to the left of each panel. The autoradiograph was exposed overnight at -70°C with intensifying screens.

Footprints of coding (**panel A**) and non-coding strands (**Panel B and C**) of fragment -824 to -557 bp are presented. Two regions, F1 (-670 to -630 bp) and F2 (-788 to -758 bp), are noted, The pattern in region F1 is identical between nuclear extracts from sham-operated rats and hypox rats. However, a change in the pattern is apparent with the nuclear extracts from GH treated hypox rats. This change is induced within 0.5 h of GH administration at a dose of 10 mg/100g BW and persists for 3 h, but 12 h later the pattern reverts to the hypox pattern. The footprint pattern in region F2 is different between sham-operated rats and hypox rats and an acute response to GH is apparent, in that a reversion to the normal pattern is also induced within 0.5 h of administration of GH at a dose of 50 mg/100 BW to hypox rats. This effect of GH on F2 is transient, since the footprint pattern reverts to the hypox pattern within 1 h after GH treatment.


A
Naked DNA
sham
Hypox
GH 0.5 h
-557
-630
-670
-701
-734
-748
-758
-778
-788
F1
F2


B
GH 50 μg
Naked DNA
sham
Hypox
GH 0.5 h
GH 1 h
GH 3 h
GH 12 h
Naked DNA
-557
-630
-670
-701
-734
-748
-758
-778
-788
F1
F2

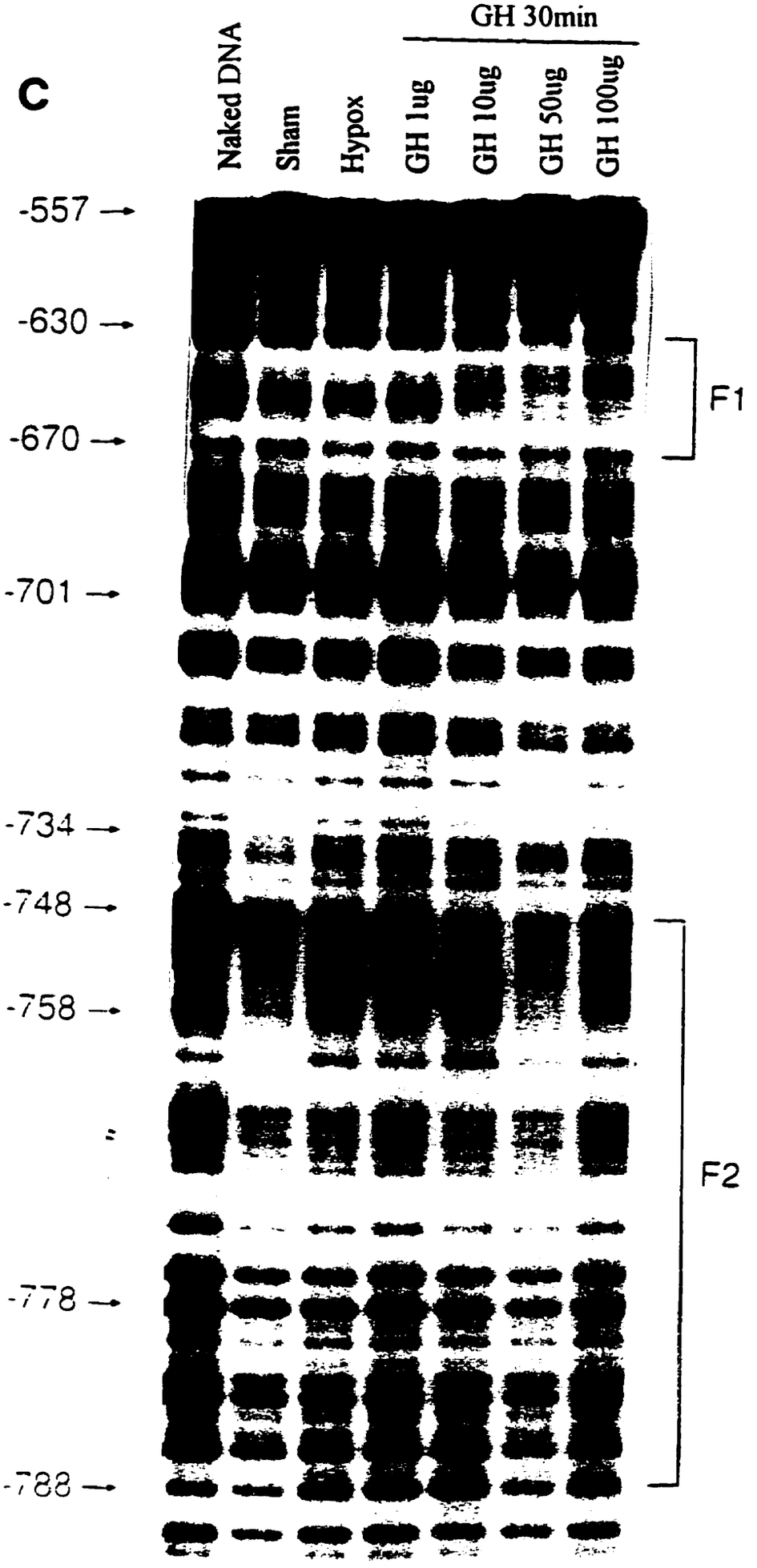
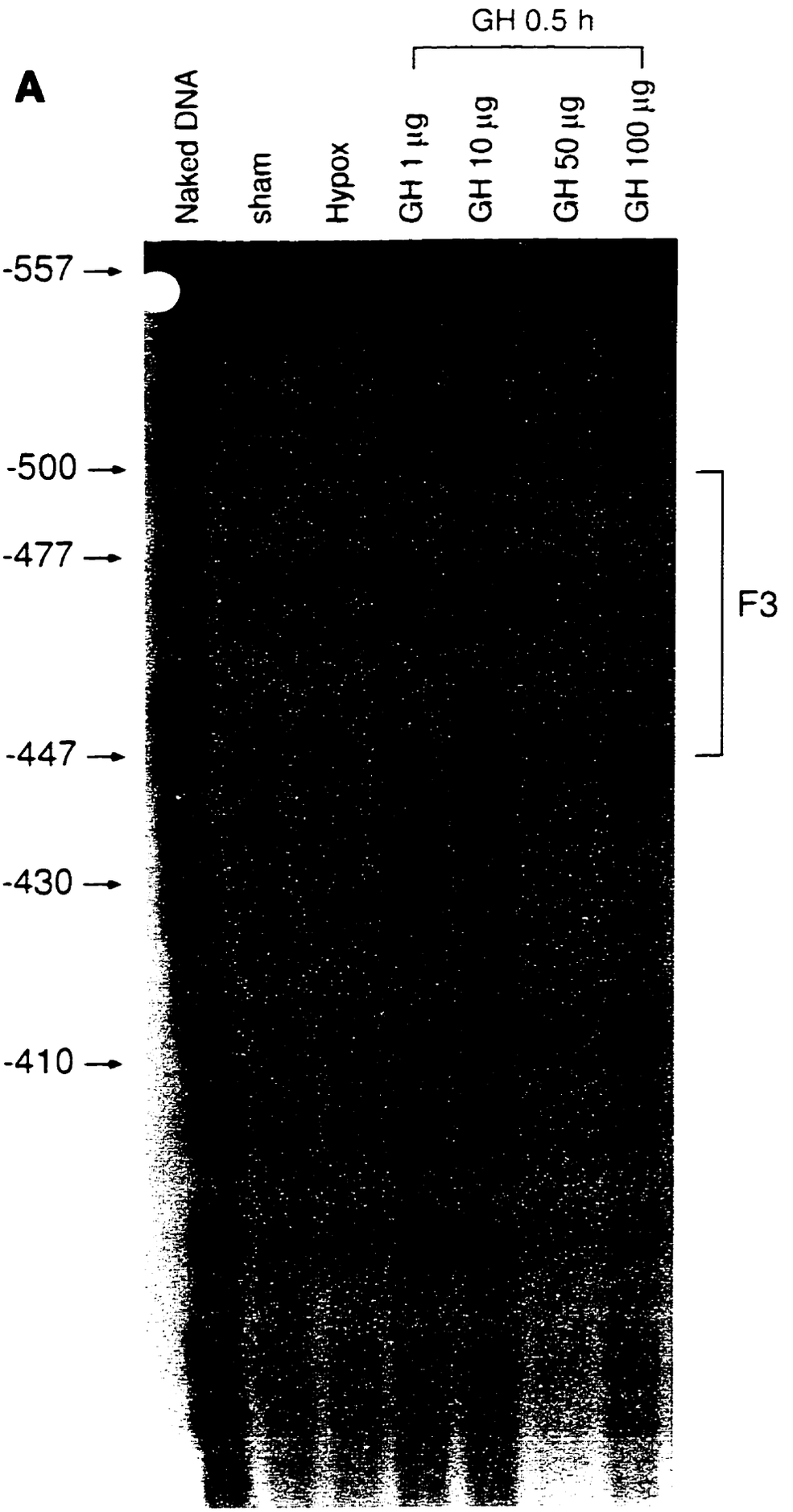

C
Naked DNA
Sham
Hypox
GH 30min
GH 1ug
GH 10ug
GH 50ug
GH 100ug
-557
-630
-670
-701
-734
-748
-758
-778
-788
F1
F2

Figure 2. DNase -1 protection analysis of IGFBP-1 promoter region -557 to -277 bp

Two livers were pooled to prepare nuclear extracts in each group. End-labeled DNA fragments (10,000cpm) were incubated with DNase-1 alone or with both nuclear extracts and DNase-1. Nucleotide positions, 5' to the IGFBP-1 gene transcription start site are marked to the left of each panel. The autoradiograph was exposed overnight at -80° C with intensifying screens.

Footprints of coding (**Panel A**) and non-coding strands (**Panel B**) of fragment -557 to -277 bp are presented. One footprinted region, F3 (-477 to -437 bp), is noted. The footprint pattern in F3 is different between sham-operated rats and hypox rats and an acute response to GH is apparent, in that a reversion to the normal pattern is induced by GH administration within 0.5 h at a dose of 50 μg/100g BW.


A
GH 0.5 h
Naked DNA
sham
Hypox
GH 1 μg
GH 10 μg
GH 50 μg
GH 100 μg
-557
-500
-477
-447
-430
-410
F3

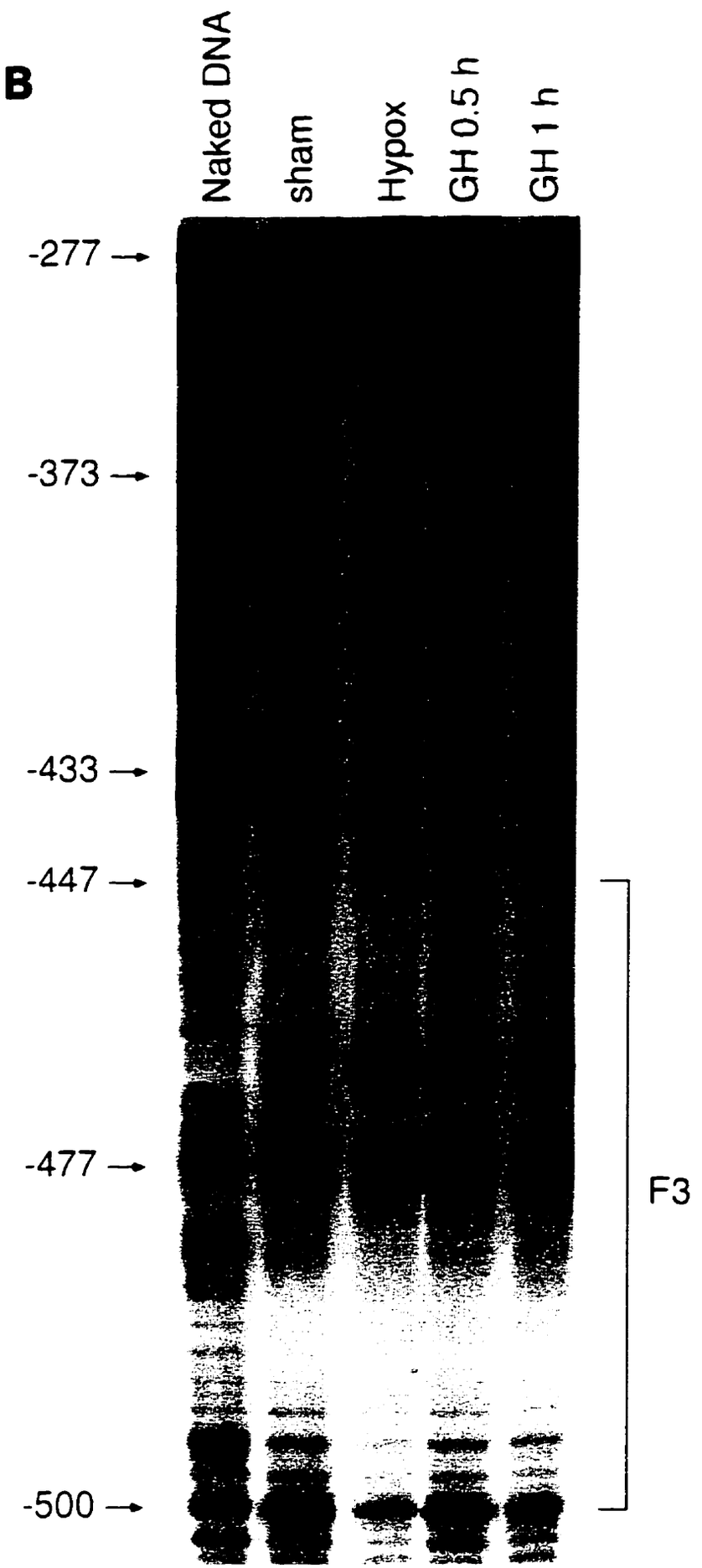
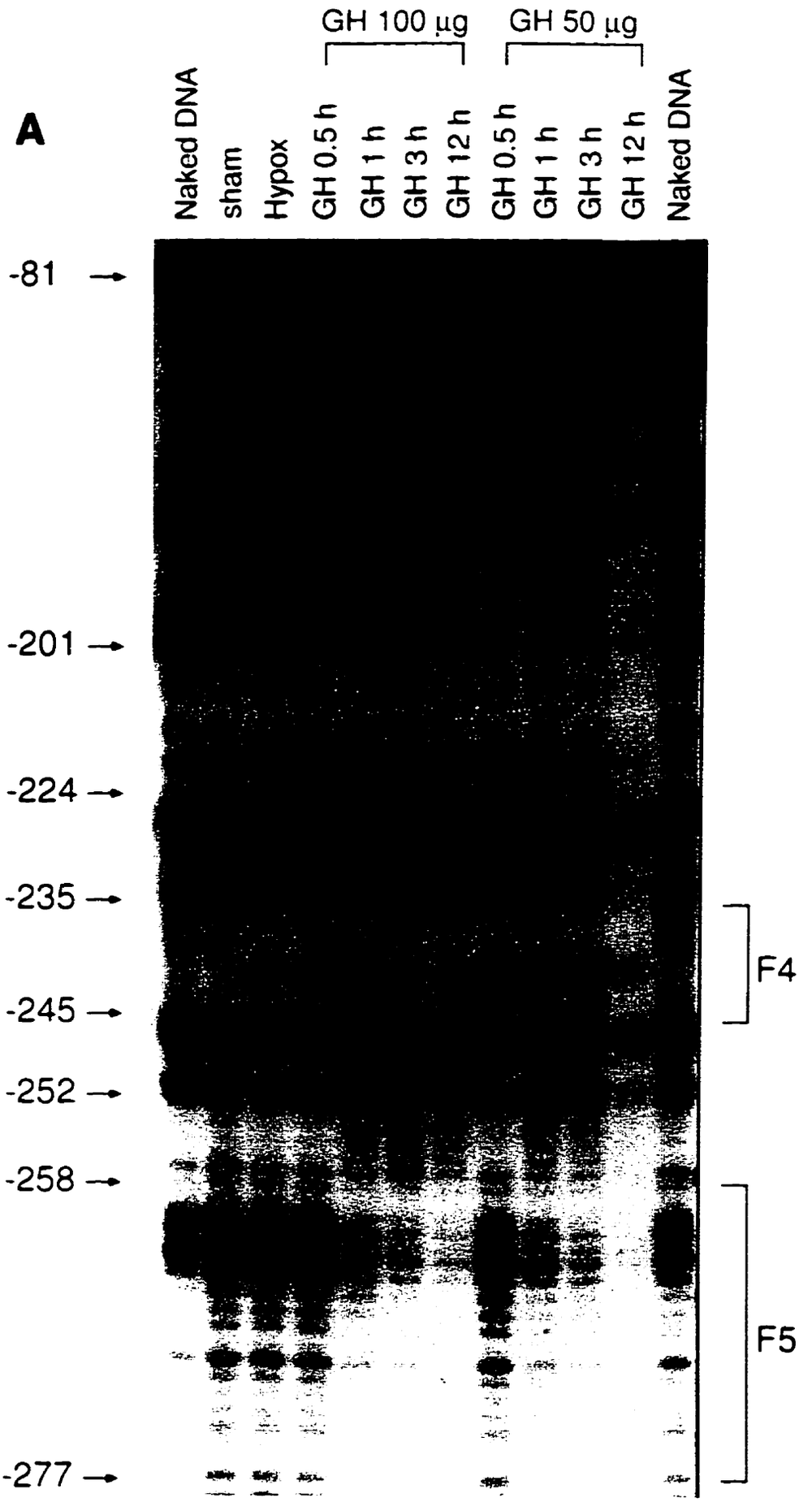
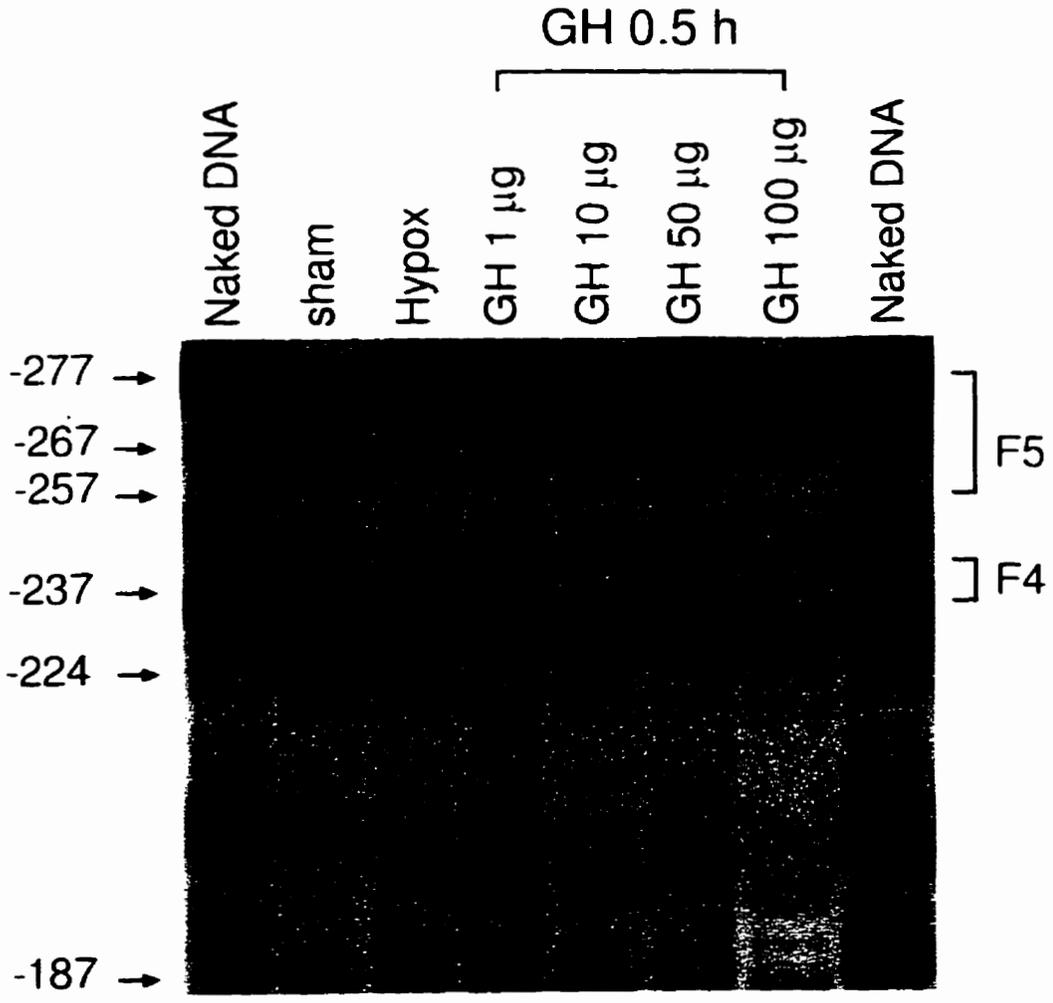

B
Naked DNA
sham
Hypox
GH 0.5 h
GH 1 h
-277
-373
-433
-447
-477
-500
F3

Figure 3. DNase-1 protection analysis of IGFBP-1 promoter region -277 to -81 bp

Footprint of the coding strand (**Panel A**) of the fragment -277 to 81 bp. Two footprint regions, F4 (-245 to -235 bp) and F5 (-277 to -258 bp), are apparent. The footprint pattern of region F4 is different between sham-operated rats and hypox rats but is not affected by administration of GH to hypox rats, in that the footprinting pattern is identical between hypox rats and hypox rats treated with GH. Region F5 show a footprint only in nuclear extracts from GH treated hypox rats. The effect of GH on F5 is apparent by 1 h of GH treatment and persists for 12 h. There is no footprint observed in region F5 with the nuclear extracts from sham-operated rats and hypox rats. **Panel B** is the footprint of the non-coding strand of the fragment -277 to -81 bp. The sequence from -277 to -237 bp, within which footprnting region F4 and F5 were located, was not separated clearly on the top of the gel.


A
GH 100 µg
GH 50 µg
Naked DNA
sham
Hypox
GH 0.5 h
GH 1 h
GH 3 h
GH 12 h
GH 0.5 h
GH 1 h
GH 3 h
GH 12 h
Naked DNA
-81
-201
-224
-235
-245
-252
-258
-277
F4
F5

B


GH 0.5 h
Naked DNA
sham
Hypox
GH 1 μg
GH 10 μg
GH 50 μg
GH 100 μg
Naked DNA
-277
-267
-257
-237
-224
-187
F5
F4

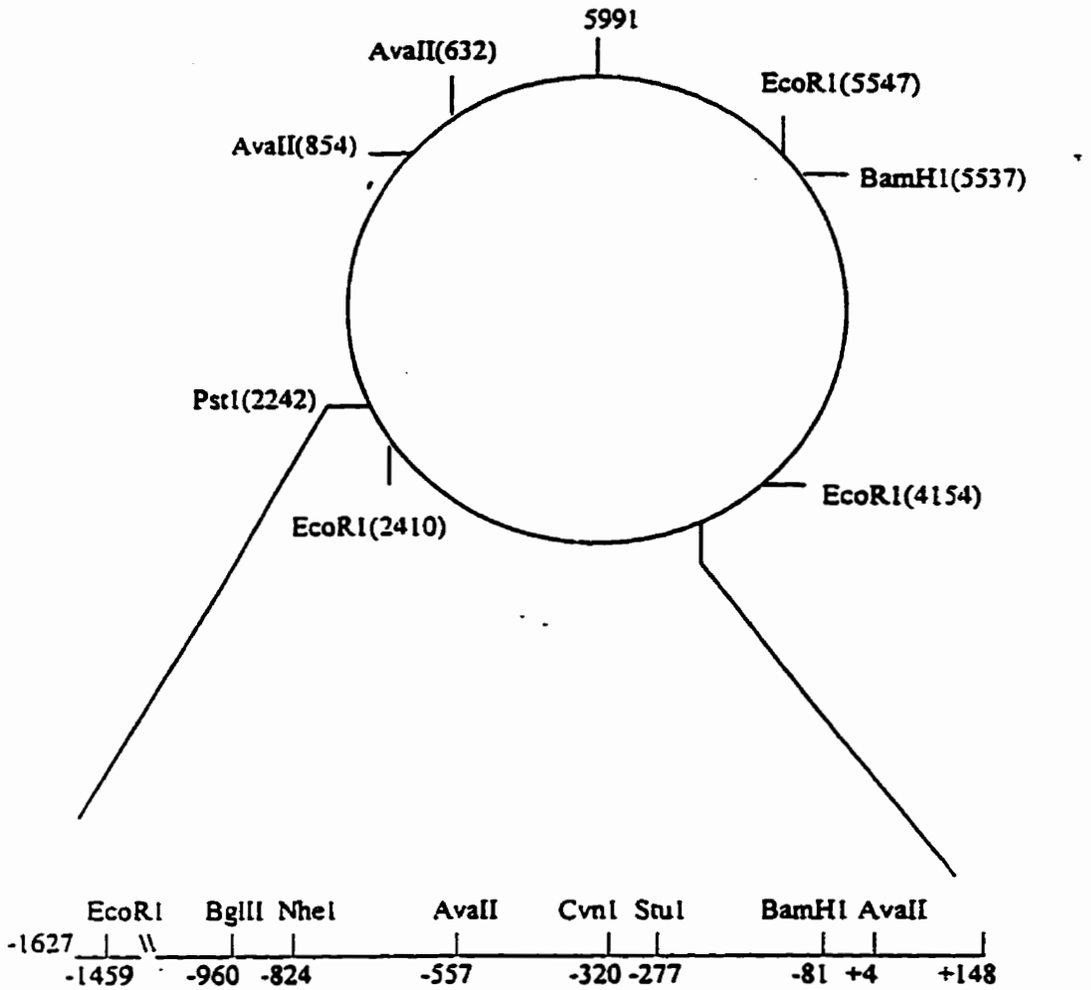


Fig 4. Restriction enzyme map of rat IGFBP-1 promoter -1627 CAT plasmid and IGFBP-1 promoter fragment -1627 to +148 bp.

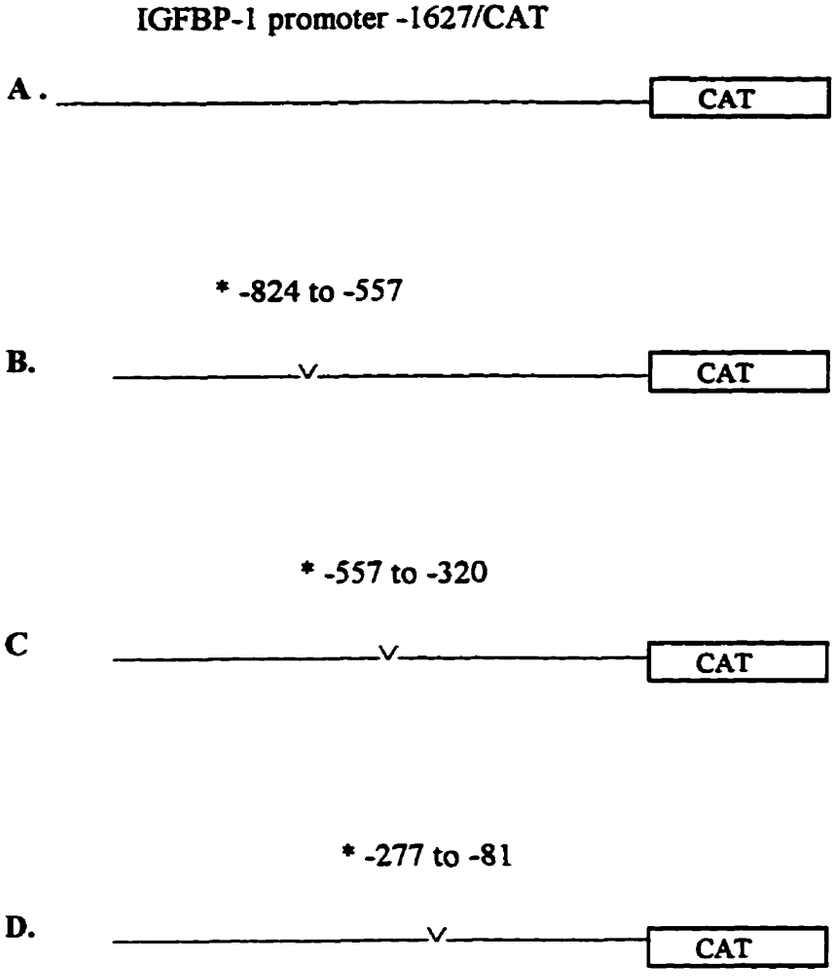
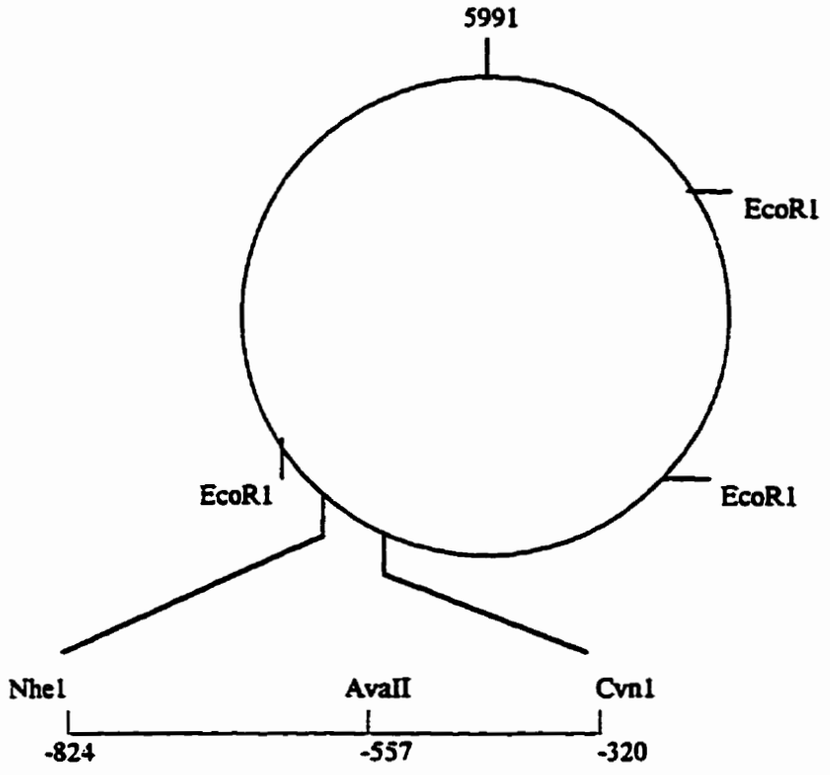


Fig 5. Three deletion constructs B, C and D.

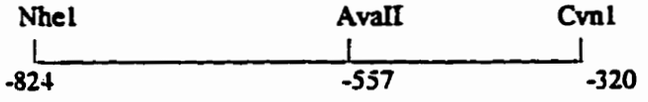
Plasmid A is the basic IGFBP-1 promoter CAT construct used in these experiments. Construct B, C and D are deletion mutants in which fragment -824 to -557 bp, -557 to -320 bp, and -277 to -81 bp were deleted, respectively.

Fig 6. Procedure used for construction of -824 to -557 bp deletion mutant and -557 to -320 bp deletion mutant.

The IGFBP-1 promoter -1627 bp CAT plasmid was digested with restriction enzyme NheI (-824 bp) and CvnI (-320 bp). The fragment corresponding to nucleotides -824 to -320 bp and the remaining linear vector were separated and isolated by electrophoresis in a 0.8% agarose gel. Fragment -824 to -320 bp was further digested with enzyme AvaII (-557 bp). Two fragments, -824 to -557 bp and -557 to -320 bp, were produced. After separation and isolation by electrophoresis in 2% agarose, these two fragments were religated to the above deleted vector. After transformation and preparation of plasmid DNA, positive constructs were selected by digestion of plasmid DNA with restriction enzymes EcoR1 and Bgl1 1/Stu1.



↓ Digest with Nhe1/Cvn1 and separate on agarose gel.



↓ Digest with AvaII and separate on agarose gel.

↓

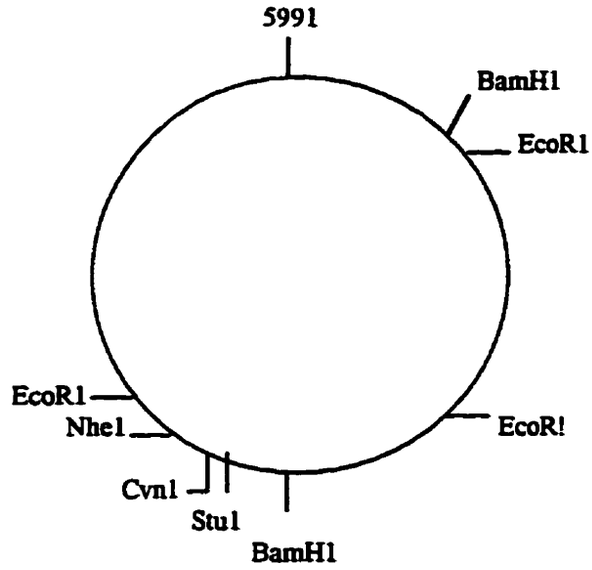
These two fragments were inserted back separately into the linear vector, in which fragment -824 to -320 bp had been deleted.

Construct B was developed by ligation of fragment -557/-320 with the linear vector.

Construct C was developed by ligation of fragment -824/-557 with the linear vector.

Fig 7. Procedure used for construction of -277 to -81 bp deletion mutant.

Since there are two BamH1 cutting sites in the IGFBP-1 promoter -1627 CAT plasmid, to delete fragment -277 (Stu1) to -81(BamH1)bp, this vector was first partially digested with the enzyme BamH1. Linear plasmids were isolated and then digested with Stu1. The linear vector, in which the 196 bp of fragment -277 to -81 was deleted, was separated and isolated by electrophoresis in a 0.8% agarose gel. After ligation, transformation and preparation of plasmid DNA, positive constructs were selected by the enzyme EcoR1 or Bgl11/BamH1.



↓

Plasmid DNA was partially digested with BamH1 and linear vectors were isolated by electrophoresis in a 0.8% agarose gel.

↓

Linear vectors were digested by Stu1. The linear vector, in which 196 bp of fragment -277(Stu1) to -81(BamH) bp was deleted, was separated and isolated in a 0.8% agarose gel.

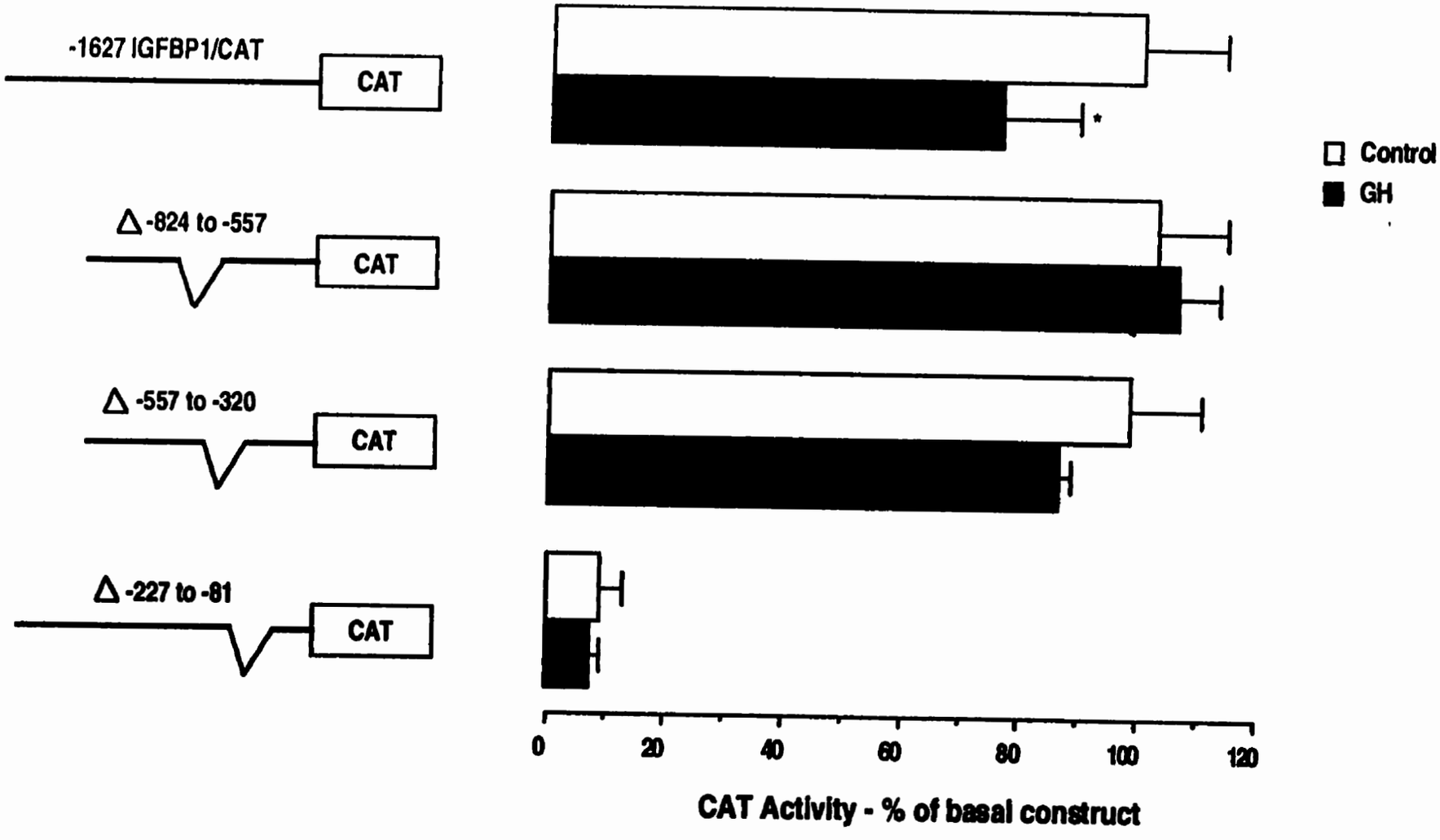
↓

This fragment deleted linear vector was religated.

Fig 8. Localization of GH response element(s) in the IGFBP-1 promoter by deletion analysis.

Panel A. Three internal deletion constructs are presented where internal fragments were deleted from the IGFBP-1 promoter CAT plasmid containing 1627 bp of the 5' flanking DNA of the IGFBP-1 gene.

Panel B. 3.5 μg of each plasmid was transiently transfected into hepatocytes isolated from normal rats. Glycerol shock was performed 6 hours post-transfection, then dexamethasone and human pituitary GH, at a final concentration of 10^{-7} M and 0.5 μg/ml respectively, were added to the culture medium. CAT activity was measured 30 hours after transfection as described in Materials and Methods. The data represent the mean ± SEM relative to CAT activity for separate hepatocyte cultures from 4-6 animals for each of the plasmids as indicated. The suppressive effect of GH on IGFBP-1 promoter CAT activity was lost when the fragment -824 to -557 bp was deleted. * indicates $P < 0.05$ for the difference from the basal untreated hepatocyte culture.


-1627 IGFBP1/CAT
CAT
Δ -824 to -557
CAT
Δ -557 to -320
CAT
Δ -227 to -81
CAT
*
Control
GH
0
20
40
60
80
100
120
CAT Activity - % of basal construct

A

B

Figure 9. Effects of in vivo GH administration on the electrophoretic mobility of a double strand oligonucleotide corresponding to the sequence of region F1.

Hepatic nuclear extracts from pituitary intact, sham-operated rats, hypox rats and hypox rats treated with GH 30 and 60 minutes prior to death, were incubated with the radiolabeled oligonucleotide probe and the DNA-protein complexes were resolved by native polyacrylamide gel electrophoresis. An arrow indicates the retarded band induced by administration of GH to hypox rats.

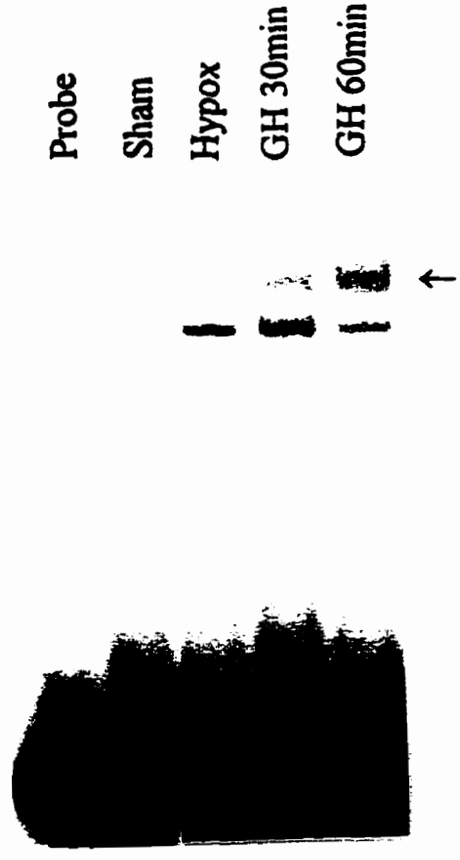


Figure 10. Effect of in vivo GH administration on the electrophoretic mobility of a double strand oligonucleotide corresponding to the sequence of region F2.

Hepatic nuclear extracts from pituitary intact, sham-operated rats, hypox rats and hypox rats treated with GH 30 and 60 minutes prior to death were incubated with the radiolabeled oligonucleotide probe and the DNA-protein complexes were resolved by native polyacrylamide gel electrophoresis. Arrows indicate the retarded band induced by administration of GH to hypox rats.

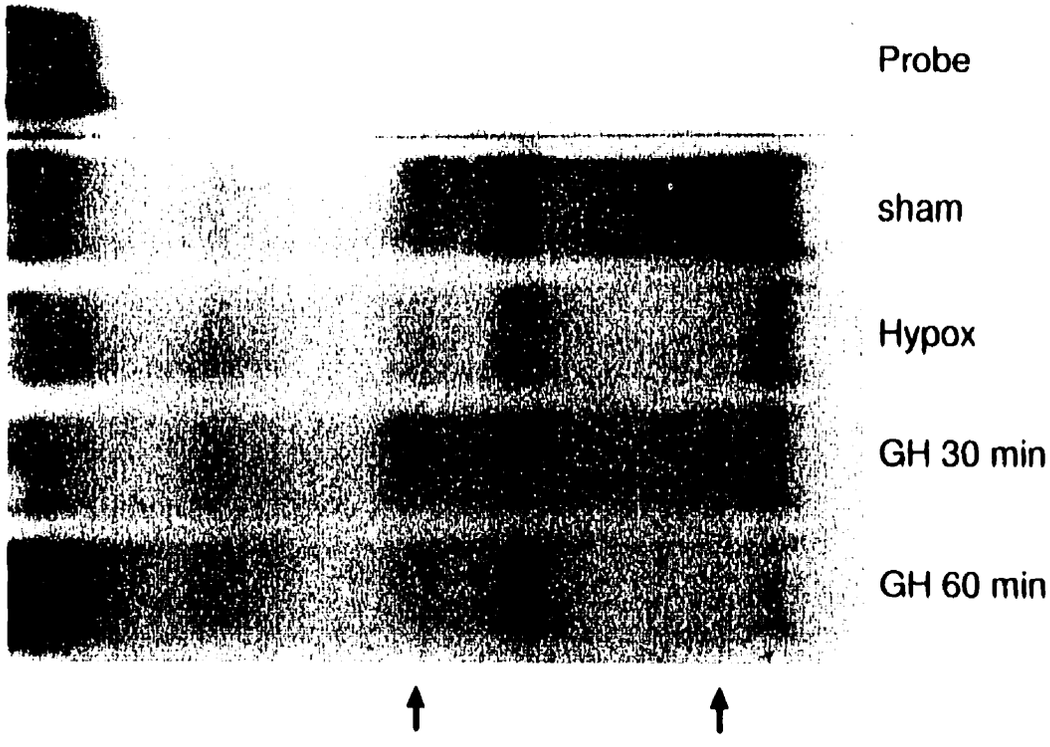

Probe
sham
Hypox
GH 30 min
GH 60 min

Figure 11. The relationship between GH and insulin on IGFBP-1 promoter luciferase activity

Two plasmids were used in this experiment. The IGFBP-1/Luciferase vector contains the SauI/HgaI fragment of the rat IGFBP-1 promoter which extends -360 bp upstream from the transcription initiation site. This fragment contains the native IGFBP-1 IRE with its two insulin response sequences (AACGCAAAAACAAACTTATTTTGAACA) and it is inserted immediatly upstream from the luciferase gene in the pGL2 vector from Promega. The other one is a mutant vector which contains mutations of both halves of the IRE insulin response sequences (AACGCAccgacgcCcaggccTGAACA) and it dose not respond to insulin. Both plasmids were transiently transfected into primary hepatocytes isolated from pituitary intact rats. Glycerol shock was performed 6 h after transfection. GH (0.5 μg/ml) and insulin (10^{-8} M) were administrated 6 h before harvesting the cells for a luciferase activity assay. The data represent the mean ± SEM relative luciferase activity for separate hepatocyte cultures from 5 animals. The level of luciferase activity is expressed as a percentage of the level obtained with -360 IGFBP-1/luciferase plasmid alone. * indicates $P < 0.05$ for the difference from the basal untreated hepatocyte cultures.

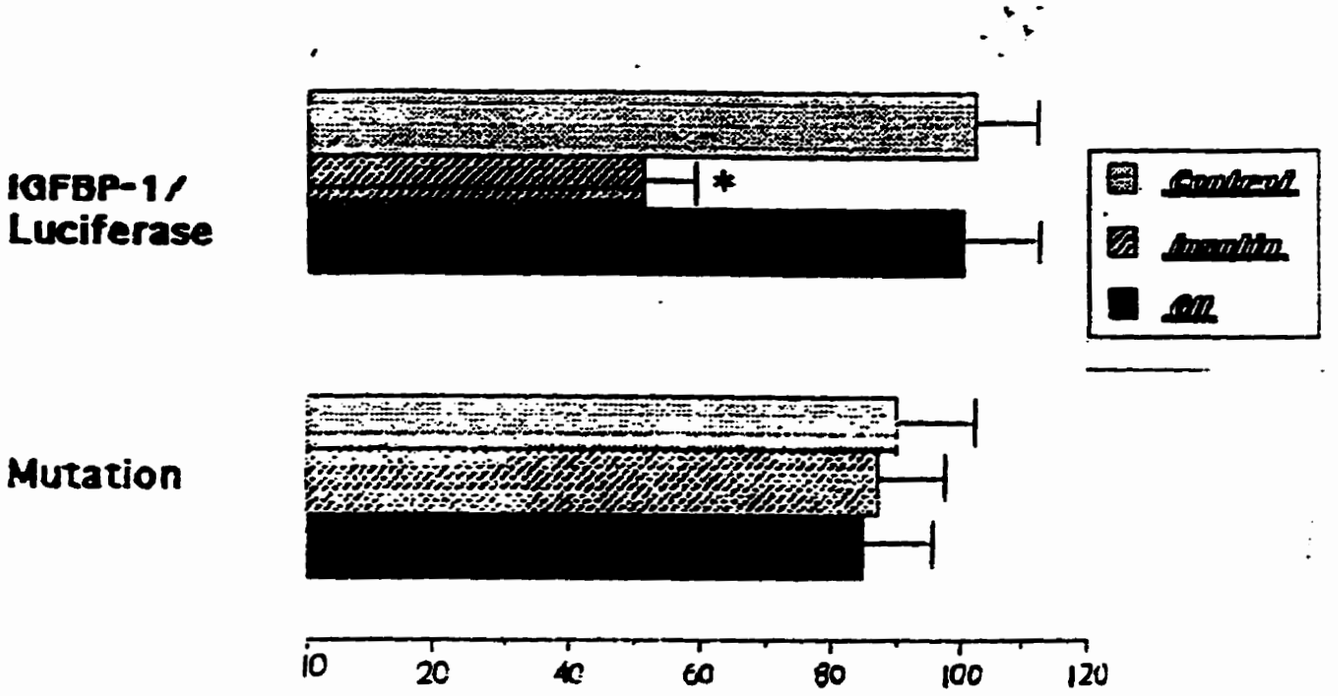
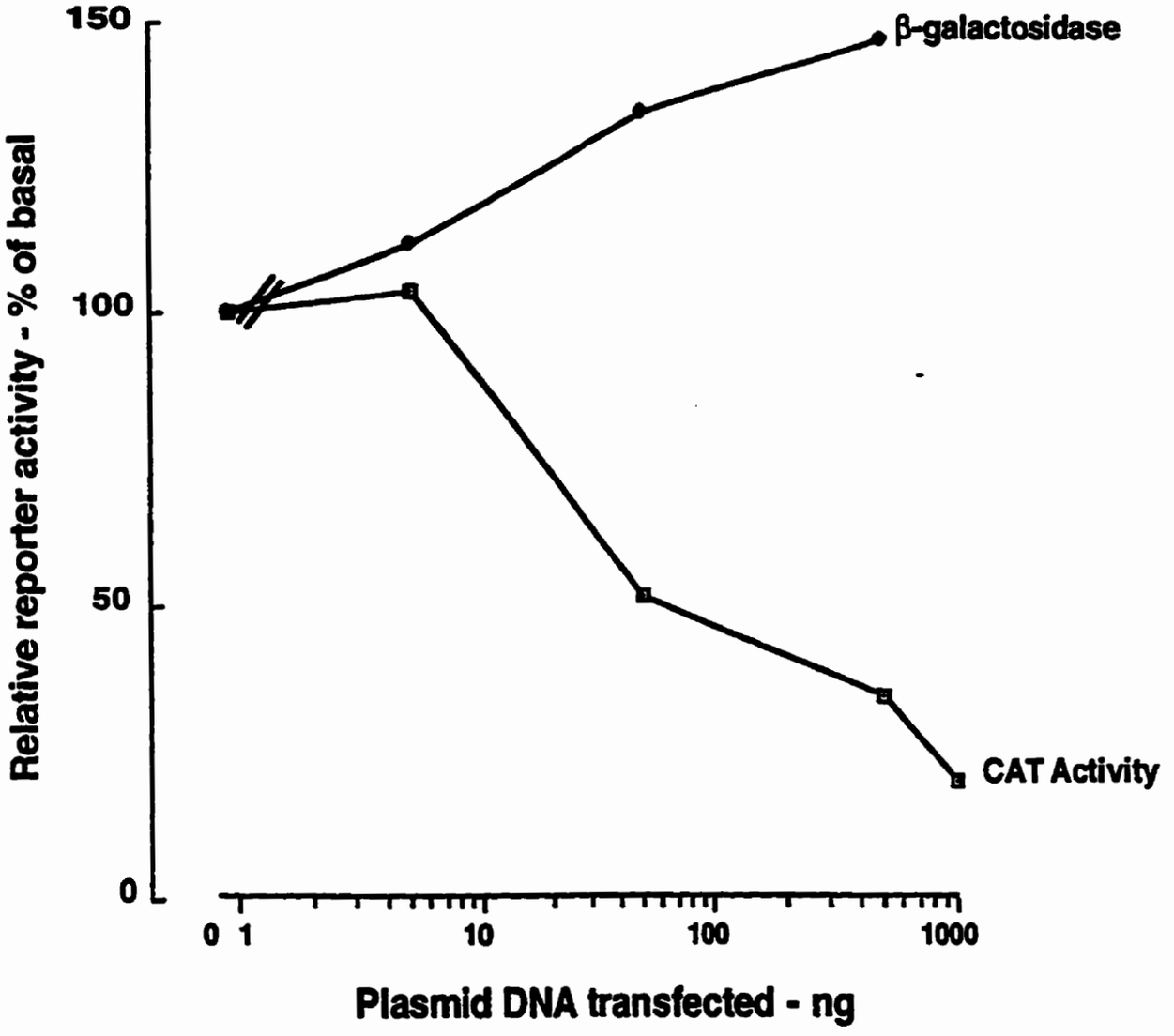

IGFBP-1/
Luciferase
Mutation
*
Control
Insulin
IGF
10
20
40
60
80
100
120
Luciferase activity - % of basal construct

Figure 12. The effects of AP-1 on IGFBP-1 promoter activity

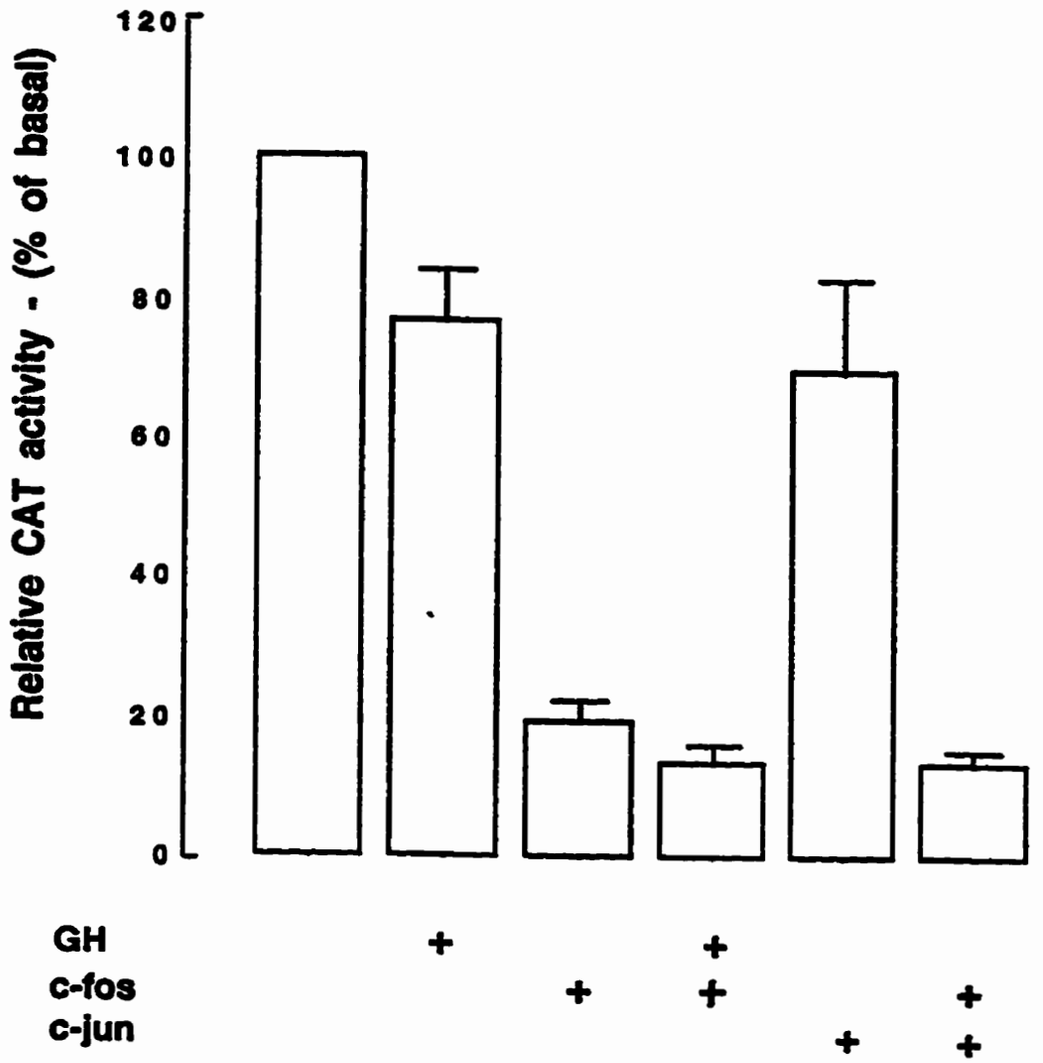
Primary hepatocytes were isolated from pituitary intact rats. The IGFBP-1 /CAT reporter plasmid was co-transfected with the c-fos or c-jun expression vectors.

Panel A. Hepatocytes were transfected with varying amounts of c-fos expression vector together with either the -1627 IGFBP-1/CAT plasmid or the pCH110 β-galactosidase plasmid. CAT activity and β-galactosidase activity were measured and expressed as a percentage of the basal levels observed in cultures not transfected with the c-fos expression vector. Co-expression of c-fos had no effect on β-galactosidase activity but suppressed CAT activity.

Panel B. The -1627 IGFBP-1/CAT reporter plasmid was transfected either alone or together with 0.5 μg of c-fos, c-jun or c-fos and c-jun expression vector together. The data represent the mean ± SEM relative to CAT activity for separate hepatocyte cultures from 3-7 animals. The level of CAT activity is expressed as a percentage of the level obtained with IGFBP-1/CAT plasmid alone. For comparison, the effect of the addition of GH is also shown. * indicates $P < 0.05$ for the difference from basal levels with the transfection of IGFBP-1 CAT plasmid alone.


150
100
50
0
Relative reporter activity - % of basal
β-galactosidase
CAT Activity
0 1
10
100
1000
Plasmid DNA transfected - ng

A

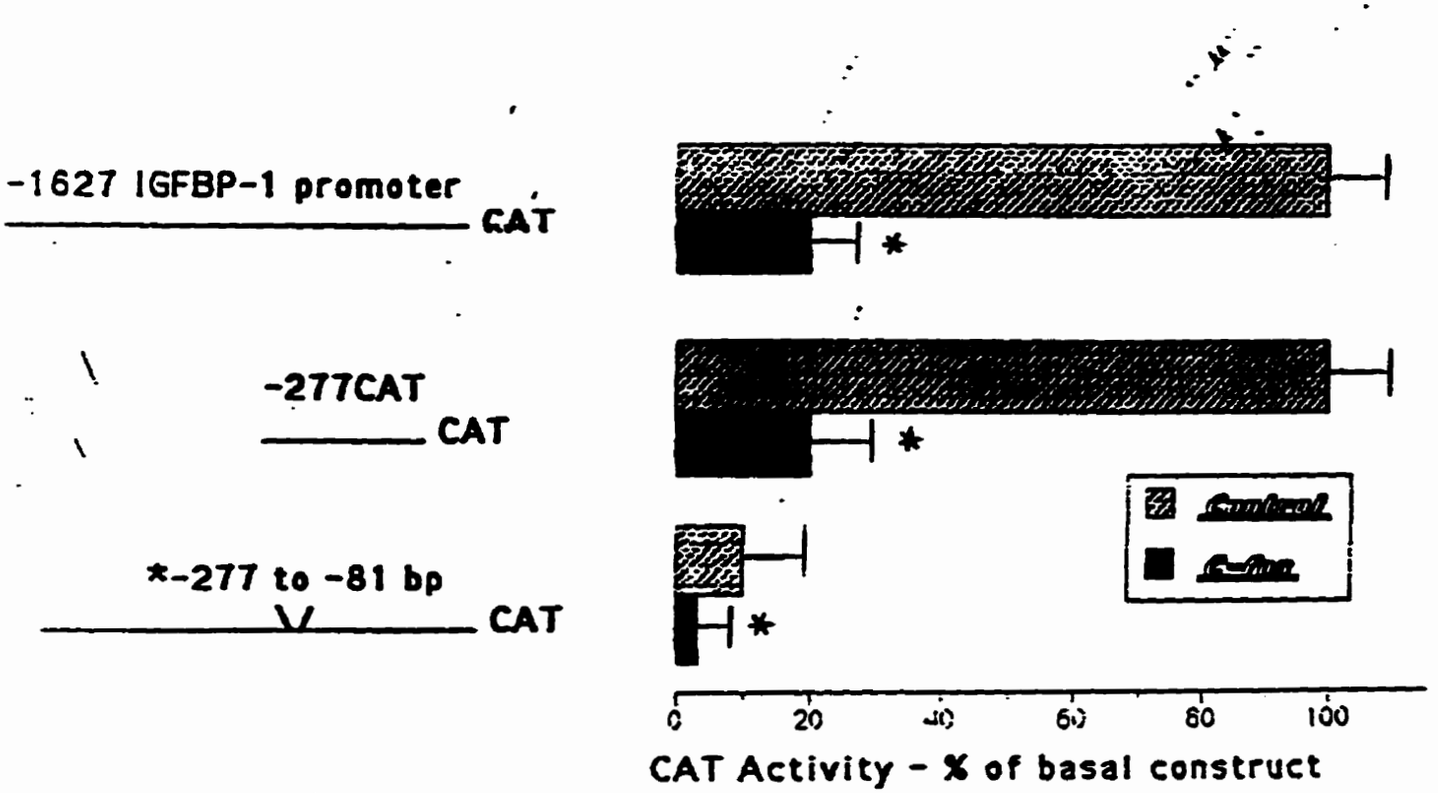

Relative CAT activity - (% of basal)
120
100
80
60
40
20
0
GH
c-fos
c-jun
+
+
+
+
+
+
+
+

B

Figure 13. Localization of the AP-1 binding site on IGFBP-1 promoter

Panel A: Two deletion constructs are presented. Construct B contains IGFBP-1 5'-flanking region -277/+184 bp inserted in CAT reporter plasmid. Construct C contains IGFBP-1 5'-flanking region -1627/+184 bp inserted in CAT reporter plasmid with the fragment -277 to -81 has been deleted.

Panel B: 3 μg of each construct plasmid and 0.5 μg of c-fos expression vector were co-transfected into hepatocytes isolated from normal rats. Glyceral shock were performed 6 hours post-transfection. CAT activity were measured 30 hours after transfection. The data represent the mean ± SEM relative CAT activity for separate hepatocyte cultures from 4-8 animals for each of the plasmid as indicated. * indicates $P < 0.05$ for the difference from the IGFBP-1 CAT plasmid transfection alone hepatocyte cultures.


-1627 IGFBP-1 promoter
CAT
-277CAT
CAT
*-277 to -81 bp
CAT
Control
Cells
0
20
40
60
80
100
CAT Activity - % of basal construct

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