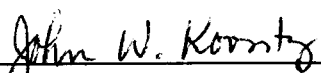


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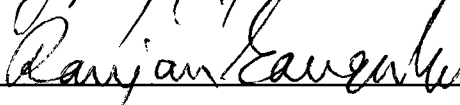
I am submitting herewith a dissertation written by Richard S. Peterson entitled "Functional and *In Vitro* Characterization of an Insulin Responsive Regulatory Region of the Tyrosine Aminotransferase Gene Promoter". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.



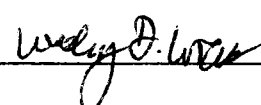
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We have read this dissertation
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Associate Vice Chancellor
and Dean of The Graduate School

Functional and *In Vitro* Characterization of an
Insulin Responsive Regulatory Region of the
Tyrosine Aminotransferase Gene Promoter

A dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Richard S. Peterson

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Abstract

Previous studies from our laboratory have shown that in the KRC-7 rat hepatoma, insulin inhibits tyrosine aminotransferase (TAT) gene expression through a selective decrease in TAT transcription. To further characterize the insulin response similar studies were conducted with the Fao rat hepatoma, which possesses a differentiated phenotype. In agreement with a previous report, it was found that insulin treatment results in an increased rate of basal TAT transcription followed by an increase in hybridizable mRNA^{TAT} in Fao rat hepatoma cells.

To further extend these results, the effect of insulin on dexamethasone- and 8Br-cAMP-mediated induction of TAT gene expression was examined. Although insulin was found to increase basal expression, it was also found to inhibit either dexamethasone- or 8Br-cAMP-mediated increases in mRNA^{TAT}. Insulin has been reported to inhibit either dexamethasone- or cAMP-mediated increases in mRNA^{TAT} in the KRC-7 rat hepatoma. These results suggest that both cell lines may share a common mechanism for the inhibition of enhancer-mediated induction.

In an effort to identify *cis*-element(s) contained in the TAT 5'-flanking sequence which mediate the effect of insulin, a series of 5'-, 3'-, and internal deletion TAT promoter/chloramphenicol acetyltransferase reporter (TAT•CAT) chimeric constructs were transfected into Fao cells for transient expression. These investigations have led to the identification of an insulin-response element(s) (IREs) contained within the proximal promoter sequences located between -351 to -52 bp.

In addition, transient transfection analysis indicates that a liver-specific enhancer activity, located at -3.6 Kb relative to the TAT cap site, is diminished in the Fao clone when compared to that observed in the KRC-7 clone.

Gel shift experiments with fragments of the TAT promoter containing the putative IRE(s) with nuclear extracts from insulin-treated and control Fao cells indicate that specific protein-DNA complexes are formed. No observable differences in protein-DNA complex intensities and/or mobility's resulted with insulin treatment.

This was followed with *in vitro* DNase I footprint analyses of TAT 5'-flanking sequences -351 to -2 bp with nuclear extract from insulin-treated or control cells. Although no insulin-dependent differences could be detected, the investigations have revealed multiple protected regions that suggest a close spacing of several DNA-binding activities. Transcription factor recognition-site assignments, inferred through footprint protection and computer-assisted sequence homologies, suggest important functional roles for liver-enriched DNA-binding proteins.

Since no DNA-binding activities could be correlated with cellular responses to insulin, no insulin response element(s) could be defined using these methods. The results indicate alternative experimental approaches must be employed in an effort to further characterize the molecular mechanism by which insulin directs TAT gene expression in the Fao rat hepatoma.

TABLE OF CONTENTS

CHAPTER	PAGE
I. OVERVIEW.....	1
II. <i>IN VIVO</i> STUDIES - NORTHERN AND RUN-OFF ANALYSES...	17
Introduction	17
Materials And Methods.....	19
Results	25
Discussion	36
III. TRANSIENT TRANSFECTION ANALYSES.....	38
Introduction	38
Materials And Methods.....	56
Results	57
Discussion	71
IV. <i>IN VITRO</i> STUDIES -GEL-SHIFT AND DNASE I FOOTPRINT ANALYSES	75
Introduction	75
Materials And Methods.....	81
Results	84
Discussion	103
V. CONCLUDING REMARKS AND FUTURE EXPERIMENTS	107
LIST OF REFERENCES	115
VITA	121

LIST OF FIGURES

FIGURE	PAGE
I-1. Liver-enriched transcription factors and their consensus DNA recognition sites	4
I-2. DNase I hypersensitive sites and corresponding functional enhancer units of the TAT 5'-flanking sequence	7
I-3. The DNA sequence of the TAT -11 Kb liver-specific enhancer unit	11
II-1. Effect of insulin on mRNA ^{TAT} in Fao cells	26
II-2. Effect of insulin on the transcription of the TAT gene in Fao cells	28
II-3. Effect of insulin on dexamethasone-mediated increases in TAT transcription rate evaluated through nuclear run-off assay in Fao cells	33
III-1. The TAT -3.6 Kb liver-specific enhancer and cAMP response unit	40
III-2. The asymmetric TAT CRE contained in DNA sequence designated BI	44
III-3. Diagram demonstrating the hypothetical redundancy of putative and/or reported HNF-3 and HNF-4 recognition sites in the modular units which together comprise the determinants of TAT expression	47
III-4. The -2.5 Kb TAT glucocorticoid response unit	50
III-5. TAT•CAT and TAT•TK•CAT constructs used in transient transfection studies	58
III-6. Transient transfection fold induction results summary	60

FIGURE	PAGE
III-7. Transient transfection analysis results summary	62
III-8. The Herpes simplex virus thymidine kinase promoter DNA sequences -105 to -18 bp contained in pBLCAT2 and present in all TAT•TK constructs tested in transient transfection studies	65
IV-1. TAT 5'-flanking sequence -310 to -11 bp	77
IV-2. Representative gel shift of TAT 5'-flanking sequence -208 to -2 bp (pTAT HS206)	85
IV-3. Representative <i>in vitro</i> DNase I footprint of TAT promoter non-coding strand sequence -351 to -2 bp	87
IV-4. <i>In vitro</i> DNase I footprint of TAT promoter non-coding strand sequence -351 to -2 bp	90
IV-5. <i>In vitro</i> DNase I footprint results summary of TAT 5'-flanking sequence -300 to -30 bp	92
IV-6. <i>In vitro</i> DNase I footprint of TAT coding strand sequence -290 to -40 bp with untreated Fao rat hepatoma vs. pig brain nuclear extract protein.....	94
IV-7. <i>In vitro</i> DNase I footprint of TAT coding strand sequence -351 to -2 bp with Fao-derived untreated vs. Fao-derived heat- treated (10 minutes/85°C) vs. pig brain-derived nuclear extract protein	96
IV-8. <i>In vitro</i> DNase I footprint competition of TAT coding strand sequence -351 to -2 bp with 500-fold molar excess competitors.....	99

FIGURE	PAGE
IV-9. <i>In vitro</i> DNase I footprint of TAT 5'-flanking sequence -351 to -2 bp with KRC-7 vs. Fao rat hepatoma derived nuclear extract protein	101

Chapter I

OVERVIEW

Modular Structure of Transcription Factors

Defining the functional role of *cis*-acting promoter elements which coordinately direct appropriate and juxtaposed cell-specific, temporal, and external stimulus-related gene expression has been a longstanding quest of molecular biologists. It has now become apparent that *cis*-acting promoter elements and upstream enhancers do not exist as discrete, high-affinity recognition binding sites but, rather, in assemblies as modular units composed of multiple and cooperative interactions which, individually, possess only moderate specificity (Frankel and Kim, 1991). Modular structure is ideally suited for protein/protein surface contacts which lead to cooperative and/or allosteric interactions.

Several advantages are afforded the eukaryotic cell through the formation of complex macromolecular assemblies. Physically, there will be an overall loss in entropy typically associated with discrete, sequential 'rigid structure' complex formation. Cooperative, multiple interactions assure that the overall specificity of the transcription complex is high, even if some individual interactions are of low specificity. It would be difficult to dissociate or regulate the activity of a transcription complex if every component possessed high affinity for every other component. Furthermore, tight or specific interactions might interfere with the combinatorial use of factors by many promoters. The design of larger, cooperatively interacting complexes composed of individual interactions with only modest specificity may be

necessary for increased flexibility. The increased flexibility allows common and/or cell-type specific factors to be shared by several promoters while also affording a high degree of specificity to a particular combinatorial unit. A structural module paradigm also allows for the evolutionary diversity which is evident by the large number of transcription factors that belong to the leucine zipper and helix-loop-helix classes.

The TAT Gene - an Experimental System for Studies of Mammalian Gene Expression

Due to the large diversity of genes whose expression is restricted to the liver, and the amenability of liver tissue to biochemical experimentation, the cultured rat hepatoma has proven to be a valuable mammalian system in which to study gene expression.

Tyrosine aminotransferase (TAT; EC 2.6.1.5, located on mouse chromosome 8) is a liver-specific enzyme that catalyses the rate-limiting step in tyrosine catabolism. The ease with which TAT can be assayed, coupled with the rapidity and the magnitude of its response to cellular agonists, make TAT particularly favorable for biochemical studies.

TAT expression is subject to complex transcriptional regulation. It is cell-type specific being preferentially expressed in the parenchymal cells of the adult liver (Hargrove and Granner, 1985). Expression is developmentally regulated with basal expression increasing dramatically a few hours after birth (Johnson *et al*, 1988). TAT is also subject to hormonal regulation. Glucocorticoid hormones (Schmid *et al*, 1987) and glucagon (Hashimoto and Schutz, 1984) (acting through cAMP-dependent protein kinase (PK-A)) stimulate TAT transcription while the effect of insulin varies with the cell line

or primary cell culture studied (Lee *et al.*, 1986; Crettaz *et al.*, 1988; Moore and Koontz, 1989).

In addition, TAT gene expression is controlled by two genetically defined regulatory loci. *alf/hsdr-1* (albino locus factor/hepatocyte specific developmental regulatory locus) occupies a region spanning ~315 Kb on mouse chromosome 7 (Ruppert *et al.*, 1992). *alf/hsdr-1* confers the perinatally acquired (newborn) competence for induction of TAT by glucocorticoid hormones (Ruppert *et al.*, 1990). A homozygous deletion of this locus results in the failure of perinatal activation of several liver enzymes, including TAT, phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase, all of which possess roles in gluconeogenesis and are normally inducible by both glucocorticoids and cAMP.

Significantly, the *alf/hsdr-1* deletion mutant phenotype has been found to possess severely depressed mRNAs for the liver-enriched transcription factors hepatocyte nuclear factor (HNF)-1 and HNF-4 and substantially reduced levels of HNF-3 and CCAAT/enhancer binding protein (C/EBP) (Tonjes *et al.*, 1992). Modular *cis*-acting units containing recognition sites for this finite group of liver-enriched nuclear factors have been shown to be essential for appropriate expression of several liver-specific genes (Xanthopoulos *et al.*, 1991) (see Figure I-1) and, most recently, TAT (Nitsch *et al.*, 1993). Taken together, it has been hypothesized that one or more of these factors might be required for hormone-dependent and/or high-level expression of the TAT and PEPCK genes. Indeed, classification of the state of differentiation of hepatoma cell lines includes characterization of both basal and glucocorticoid-inducible expression of TAT (Killary and Fournier, 1984).

Figure I-1. Liver-enriched transcription factors and their consensus DNA recognition sites.

C/EBP, DBP, and LAP are members of the leucine zipper dimerization family. HNF-4 is a member of the steroid hormone super-family. The DNA recognition sites are aligned to demonstrate a similarity in core motif (the double-underlined DNA bases).

<u>Cloned Liver-Enriched DNA-Binding Activities</u>		
<u>Factor</u>	<u>Lineage</u>	<u>Consensus Recognition Site (5'→3')</u>
C/EBP	leucine zipper dimerization family Landschulz <i>et al.</i> (1988)	TTGC GCAA or T(T/G)TGG(T/A)(A/T)(A/T)
DBP	Mueller <i>et al.</i> (1990)	"
LAP	leucine zipper dimerization family Descombes <i>et al.</i> (1990)	"
HNF-1	POU-homeobox gene family Baumheter <i>et al.</i> (1990)	(G/A)GTTAATN ATTAAC(C/A)
HNF-3	<i>Drosophila</i> homeotic fork head-related Lai <i>et al.</i> (1990)	ATTATTGACTTAGCTT
HNF-4	Sladek <i>et al.</i> (1990)	TGNAC(C/T)TTG
<u>Uncloned Liver-Enriched DNA-Binding Activities</u>		
HNF-5	Grange <i>et al.</i> (1991)	T(G/A)TTTG(C/T)
Tf-LF1	Mendelzon <i>et al.</i> (1990)	TGGCGCAATCTCTTTGACCT
Tf-LF2	"	GCTTTGAAAT TG ATTCAAAG
<u>Identified Insulin-Response Elements</u>		
GAPDH IRE-ABP	Alexander-Bridges (1992)	(C/T)CTTTG(A/T)
PEPCK IRE	O'Brien <i>et al.</i> (1991)	TGGTGTTTTGACAA

Figure I-1.

Recently, the gene encoding fumarylacetoacetate hydrolase (FAH), an enzyme of tyrosine metabolism whose absence is the primary defect in a fatal liver disease of infants, has been shown to be positioned within the *alf/hsdr-1* locus (Ruppert *et al.*, 1992). Taken together, it may be suggested that FAH, alone, is responsible for the *alf/hsdr-1* phenotype, although it has not been demonstrated whether the 315 Kb deletion contains other candidate gene(s) and/or critical flanking sequence of a linked gene(s) required for the full *alf/hsdr-1* phenotype.

The second regulatory locus, *tissue specific extinguisher* (*Tse-1*, located on mouse chromosome 11), is a dominant negative regulator of transcription in somatic cell hybrids. *Tse-1* has been found to encode a distinct regulatory subunit of PK-A (R 1- α) (Boshart *et al.*, 1991). *Tse-1* targets within the TAT 5'-flanking sequence include the TAT CRE (located within sequences -3641 to -3655 bp) and the proximal promoter region (flanking sequences -351 to +1 bp) (Nitsch *et al.*, 1990). Expression of R 1- α correlates directly with *Tse-1*-mediated extinction of TAT gene expression (Boshart *et al.*, 1991).

Three Liver-specific Enhancer Units Comprise the Determinants of TAT Expression in vivo.

Mapping of DNase I hypersensitive sites in TAT gene chromatin of TAT-expressing and non-expressing cells has offered a means to locate potential *cis*-regulatory elements important in the control of TAT gene expression (Nitsch *et al.*, 1990). Analyses of putative transcriptional control element-CAT fusion genes in TAT-expressing and non-expressing cell lines has allowed the assignment of functional roles for several regions of the TAT 5'-flanking sequence (see Figure I-2).

Figure I-2. DNase I hypersensitive sites and corresponding functional enhancer units of the TAT 5'-flanking sequence.

Vertical arrows designate DNase I hypersensitive sites present in TAT 5'-flanking sequence in TAT-expressing cells. The open arrow designates the TAT gene start site (+1). Boxes with various pattern fills represent DNA *cis*-element recognition sites for DNA-binding proteins. DNA-binding proteins are identified below each *cis*-element recognition site. See the accompanying text for further details. Taken from Nitsch *et al.* (1993).

The cell-specific differentiated phenotype can be extinguished experimentally by fusing cells with dissimilar phenotypes forming somatic cell hybrids (e.g., hepatoma/mouse embryo fibroblasts). The basis of this dominant negative-regulation leading to extinction has recently been established for the TAT gene by G. Shutz's laboratory (Nitsch *et al.*, 1993). Comparative DNase I hypersensitive site examination of a TAT-expressing hepatoma vs. that observed in somatic cell hybrids or fibroblasts together with transient transfection analyses of identified functionally important enhancer regions clearly demonstrates that extinction of TAT expression is a consequence of the absence of transcriptional activation rather than a mechanism consisting of active repression.

The primary contributing component of extinction is the lack of expression of the liver-enriched transcriptional activators HNF-4 (and HNF-1), C/EBP, and HNF-3 β/γ . Note the similarity between those liver-enriched transcription factors which serve as the principal determinants of TAT expression *in vivo* and those liver-enriched transcription factors whose absence is associated with the *alf/hsdr-1* phenotype (Figure I-2 and Table IV-1).

A second component of TAT expression extinction is the loss of *in vivo* binding and, hence, activity of ubiquitous factors (e.g., CREB, glucocorticoid receptor) that, together with the prerequisite liver-enriched transcriptional activators (see above), physically interact to create the critical regulatory enhancer units at -11 kb (the principal determinant of TAT expression *in vivo*), -3.6 kb (the liver-specific cAMP responsive unit), and -2.5 kb (the liver-specific glucocorticoid responsive unit) (Nitsch *et al.*, 1993).

Uncertainties Associated With Assigning In Vivo Identity to DNA-binding Activities

TAT flanking sequence -10548 to -10483 bp (the -11 Kb enhancer - see Figure I-3) possesses a principal role in directing TAT expression (Nitsch *et al.*, 1993). The 65 bp unit, comprising the -11 Kb enhancer, was cloned in front of a HSV TK promoter/CAT reporter construct (pBLCAT2) and transiently transfected into hepatoma and fibroblast cells. Normalized CAT activity was 90 pM·min·mg vs. 3 pM·min·mg respectively.

Paradoxically, *in vitro* DNase I footprinting has revealed two distinct DNA-binding activities, both of which are present in extracts from hepatoma cells and fibroblasts, although no protein binding was observed in fibroblasts when analyzed through *in vivo* genomic footprinting (Nitsch *et al.*, 1993).

Initial efforts by Schutz's laboratory to identify the two DNA-binding activities included identifying DNA sequences within the 65 bp unit that possess homology to previously characterized DNA recognition sites (Nitsch *et al.*, 1993). Gelshift studies confirmed the identity of a putative HNF-3 site. Competition with a 100-fold molar excess of the well characterized HNF-3 binding site from the transthyretin gene was complete. In addition, retarded complexes were selectively depleted with antisera specific for HNF-3. Furthermore, the concatamerized putative TAT HNF-3 site was capable of driving hepatocyte-specific CAT reporter expression.

Interestingly, the second DNA-binding activity, -11/2 A (contained within region -11/2) (see Figure I-3), results in identical complex mobility-patterns and intensities in gel-shift analyses when using nuclear extract derived from either hepatoma cells or fibroblasts. Examination of the -11/2 A motif reveals a sequence that is both 100% homologous (10/10) to the ubiquitous transcription factor, NF-Y, and 90% homologous (9/10) to the liver-enriched

Figure I-3. DNA sequence of the TAT -11 Kb liver-specific enhancer unit.

-11/2 and -11/2 A are subset sequences of the enhancer unit that were used for gel shift and transfection studies by Nitsch *et al.* (1993). Boxed DNA sequences denote sequence similarity and/or experimentally validated identity to a consensus DNA recognition site for a particular DNA-binding protein. See the accompanying text and Nitsch *et al.* (1993) for information on individual DNA-binding protein identities.

transcription factor, C/EBP. Although the identity of this DNA-binding activity has yet to be determined, the transiently transfected 33 bp -11/2 fragment (TAT flanking sequence -10548 to -10515 bp) cloned into pBLCAT2 is active in hepatoma cells only (115 pM·min·mg in hepatoma cells vs. 10 pM·min·mg in fibroblasts).

Taken together, it appears that the *in vivo* -11/2 DNA-binding activity in hepatoma cells is either distinct from that present in fibroblasts or, alternatively, an additional component subsequent to the DNA-binding event facilitates reporter expression selectively in hepatoma cells.

Reported Insulin-Responsive Promoter regions

Recently, insulin-response DNA elements (IREs) have been described for the genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Nasrin *et al.*, 1990), phosphoenolpyruvate carboxykinase (PEPCK) (O'Brien *et al.*, 1991), and α -amylase (Keller *et al.*, 1990).

Using transient transfection of GAPDH/CAT fusion genes, M. Alexander-Bridges's group demonstrated that the stimulatory effect of insulin is mediated through *cis*-acting sequences located between -488 and +21 bp (Nasrin *et al.*, 1990). The minimal sequence contacted by the GAPDH IRE-A DNA-binding protein (IRE-ABP), as determined by methylation interference studies, includes an unusually large domain (18 nts), 5'-CTTTCCCGCCTCTCAGCC-3'.

The same group has recently cloned a protein (IRE-ABP) that binds a sequence partially contained within the extreme 3'-boundary of the IRE-A element (the single-underlined sequence), 5'-CTCAGCCTTTGAAAG-3' (Alexander-Bridges, 1992). IRE-ABP is a member of the HMG (High Mobility

Group) class of transcriptional regulators and is 67% identical within its HMG box domain to the candidate gene for testis-determining factor, SRY. Both IRE-ABP and SRY share binding specificity for the IRE-A sequence which contains a highly conserved core motif 5'-(T/C)CTTTG(A/T)-3' (the above double-underlined sequence). Functionally, the diverse members of the HMG family regulate expression of genes that initiate and maintain the differentiated phenotype.

Transient transfection studies by D. Granner's group have shown that a 22-mer oligo containing PEPCK sequences -416 to -402 bp (5'-TGGTGTTTTGACAAC-3') is sufficient to mediate the insulin-response (58% inhibition) when four of the 22-mer oligos are concatamerized and cloned into a linker-scanning derivative of pBLCAT2 at a position immediately adjacent to the TFIID site (at -32 bp) (O'Brien *et al.*, 1991). This result may be interpreted as follows. The PEPCK-IRE is position- and orientation-independent and the mechanism of action involves active repression. Alternatively, the 22-mer oligo is recognized by at least two DNA-binding activities. Gel-shift studies provide support for the latter interpretation. First, there is extremely limited migration of the protein-bound complex when compared to the free probe. In addition, there appear to be at least two distinct, specific complexes present, both of which are competed with cold competitor probe.

Also, it should be noted that a region of the PEPCK-IRE sequence is similar to the IRE-ABP and SRY core recognition motif (the double-underlined bases). Interestingly, all of the consensus recognition sites for the liver-enriched transcription factors (with the exception of HNF-1) contain a region with DNA sequence similarity to the core recognition motif of the HMG class of transcriptional regulators, 5'-(C/T)CTTTG(A/T)-3' and see Figure I-1.

The transgenic approach has been used by M. Meisler's group to study insulin regulation of pancreatic amylase gene expression. Hybrid genes containing sequences from the amylase promoter ligated to the insulin-unresponsive elastase promoter, which was then coupled to a CAT reporter, has allowed the delineation of a 30 bp fragment (-167 to -137 bp) as the insulin-dependent element (IDE) (Keller *et al.*, 1990). Contained within the IDE is a pancreas-specific factor (PTF1) recognition site (-158 to -123 bp). A portion of the IDE/PTF1 sequence 5'-ACTTTTAG-3' (-144 to -136 bp) is homologous to the HMG family core recognition motif.

Rationale and Objective of the Proposed Work

An increased understanding of the molecular mechanism of insulin-mediated regulation of gene expression in higher eukaryotes includes, as an integral component, the identification of insulin-dependent *cis*-acting elements within the promoters of insulin-responsive genes. Once identified, insulin-responsive *cis*-acting elements can be used in a Southwestern screening approach to isolate, purify, and ultimately clone genes encoding the corresponding insulin-responsive *trans*-acting protein factor(s).

We have therefore initiated a study of the regulation of expression of the TAT gene in the Fao rat hepatoma. The TAT gene was chosen because of the large amount of available background information, both on the expression of the gene in the adult liver *in vivo* and in cultured rat hepatoma clones derived from the Reuber H35 minimal-deviation rat hepatoma.

The aim of the present study is to functionally identify relevant IRE(s) contained in TAT 5'-flanking sequence whose deletion results in the loss of appropriate insulin responsiveness of the TAT gene.

Once identified we then sought to determine, through *in vitro* DNase I footprinting, if alterations in IRE DNA-binding activities were detectable. The results would ultimately lead to an increased understanding of insulin action leading to changes in gene expression.

Chapter II

IN VIVO STUDIES

NORTHERN BLOT AND TRANSCRIPTIONAL RUN-OFF ANALYSES

Introduction

Insulin regulation of TAT expression has been examined in a number of different cell systems. These systems have included various developmental stages of the animal liver (Lee *et al.*, 1986), both adult and fetal primary hepatocytes (Schmid *et al.*, 1987; Cake *et al.*, 1989), and several hepatoma clones; four of which have been derived from the same parental clone, the Reuber H35 minimal-deviation rat hepatoma (Crettaz *et al.*, 1988; Schubart *et al.*, 1986; Messina *et al.*, 1992; Moore and Koontz, 1989).

In the adult rat liver insulin enhances the expression of TAT through a rapid and transient increase in its transcription rate leading to an increase in mRNA^{TAT}, and consequently, increased enzyme activity (Johnson *et al.*, 1988). A similar result has been reported for the cultured rat hepatoma cell line, Fao (Crettaz *et al.*, 1988).

In addition to the above studies, other labs have reported different and, in some instances, conflicting responses of the TAT gene to insulin. Thus, insulin had no observable effect on mRNA^{TAT} in isolated adult hepatocytes (Schmid *et al.*, 1987). Insulin alone had no effect on basal mRNA^{TAT} levels in the FTO-2B rat hepatoma cell line, but antagonized increases that resulted from treatment with the cAMP analog, CPT-cAMP (Schubart *et al.*, 1986). Recently, it has been reported that in the H4IIE rat hepatoma insulin mediates a biphasic action on TAT gene expression (Messina *et al.*, 1992). In the well-differentiated

rat hepatoma cell line, H4IIE (H4), insulin was found to rapidly inhibit TAT transcription while increasing TAT activity. Transcription rates returned to control values after 4 hours of insulin exposure. Long-term exposure to insulin resulted in a maximum 3-fold increase in TAT transcription by 12 hours which decreased gradually thereafter.

We have previously reported that insulin rapidly inhibits expression of the TAT gene in KRC-7 cells, a variant of the Reuber H35 rat hepatoma (Moore and Koontz, 1989). This inhibition is observed as a decrease in the rate of transcription accompanied by a decrease in the level of the TAT mRNA. In addition, insulin was found to inhibit both dexamethasone and cAMP-mediated enhanced transcription (Moore and Koontz, 1989).

The conflicting insulin responses observed in the KRC-7 and H4 hepatoma cell lines versus that which have been reported in the adult rat liver and Fao cells may be attributed to a variety of parameters. Cell culture conditions including maintenance medium and hormonal or serum supplements may influence the cellular phenotype. Also, manipulations such as the length of time between serum withdrawal and treatment addition or state of cell confluency may affect experimental results.

Alternatively, the cell types may represent different developmental phenotypes in which insulin has been reported to stimulate TAT expression, e.g., the adult rat liver and neonatal rat liver 72 and 92 hours postpartum (Lee *et al.*, 1986; Johnson *et al.*, 1988), or suppress expression of the TAT gene, e.g., the fetal rat liver, isolated fetal hepatocytes, and the neonatal rat liver 20-48 hours postpartum (Johnson *et al.*, 1988; Cake *et al.*, 1989; Cake *et al.*, 1986).

In an effort to distinguish which of the above is responsible for the differential insulin response, we initiated a series of studies with the Fao clone

similar to those previously performed with the KRC-7 clone (Moore and Koontz, 1989). A cell culture maintenance and experimental manipulation regime identical to that employed with the KRC-7 cells was maintained. In agreement with a previous report (Crettaz *et al.*, 1988), it was found that insulin treatment results in a rapid and transient increased rate of TAT transcription followed by an increase in hybridizable mRNA^{TAT} in the Fao rat hepatoma.

To further extend our understanding of insulin action in the Fao cell line, the effect of insulin or 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on dexamethasone or cAMP-mediated TAT induction was examined. When added simultaneously with either 8Br-cAMP or dexamethasone, insulin was found to significantly inhibit the increased TAT mRNA accumulation observed with either agonist alone. TPA significantly inhibited cAMP-mediated increases in mRNA^{TAT} while having no appreciable effect on dexamethasone-mediated increases.

Further examination indicates that insulin antagonizes dexamethasone-mediated increases in mRNA^{TAT} through a dominant inhibitory action on the dexamethasone-mediated induction of TAT transcription rate. This observation is consistent with results obtained with the KRC-7 and H4 hepatoma cell lines suggesting a dominant role for insulin in hormone-mediated expression of the TAT gene.

Materials and Methods

Materials.

Porcine insulin (lot # 7RH87D) was obtained from Elanco Products Co., Indianapolis, IN. Cell culture medium, Dulbecco's modified Eagle's medium (DMEM), was from Gibco, Grand Island, NY. Calf serum and fetal bovine

serum were products of M.A. Bioproducts, Walkersville, Md. Fe-supplemented calf serum was obtained from Hyclone Laboratories, Inc., Logan, Utah. Cell culture dishes were from Corning Glass Works, Corning, NY or Costar, Cambridge, MA. guanidine iso-thiocyanate was purchased from Research Organics, Cleveland, Ohio, agarose from FMC Corp., Rockland ME, cesium chloride from Bethesda Research Laboratories (BRL), Bethesda, Md., formamide from Fisher Scientific, Fairlawn, NJ, [α - ^{32} P]dCTP and [α - ^{32} P]UTP from ICN Radiochemicals, Irvine, CA. All other chemicals were of analytical grade and were obtained from Sigma Chemicals or Baxter Scientific.

Preparation and Handling of Compounds Evaluated

Insulin was prepared as a 1 mg/ml stock in 0.1 M HCL, quantitated at OD 276 where an absorbance of 1.05 = 1 mg/ml insulin, and stored at 4°C. Immediately prior to treatment, a 1 μ M (100x) stock solution was prepared in 1.0 mM HCL. Final treatment concentration was 10^{-8} M unless otherwise indicated. Dexamethasone was prepared as a 100 μ M solution in serum-free DMEM medium and stored in 1 ml aliquots at -20°C. The final concentration used in induction experiments was 1 μ M. 8Br-cAMP was prepared as a 20 mM stock solution in serum-free DMEM medium and stored at -20°C. The final concentration used for induction was 500 μ M (71). 12-O-tetradecanoyl-phorbol-13-acetate (TPA) was prepared as a 1.6 mM stock solution in 95% ethanol. The maximal final treatment concentration with TPA was 1.6 μ M. In these experiments, control dishes received 0.095% ethanol.

Cell Culture.

Fao is a well-differentiated subclone derived from the Reuber H35 rat hepatoma (Deshatrette and Weiss, 1974). Fao cells were obtained from Dr. Ronald Kahn and Dr. Mary Weiss. Cells were maintained by weekly serial passage in DMEM supplemented with either 5% fetal bovine serum and 5% calf serum or 10% Fe-supplemented calf serum. The cells were maintained at 37°C in a humidified atmosphere of 90% air, 10% CO₂. For preparation of total RNA, cells were cultured on 100 mm dishes. Upon reaching ~70 % confluency serum-containing medium was removed by aspiration and replaced with serum-free medium for approximately 20 hr prior to addition of the various compounds.

Total RNA Isolation.

Initially, total cellular RNA was prepared by the guanidinium thiocyanate-cesium chloride method of Chirgwin *et al.*, (1979). Cells were washed 3 x with ice-cold PBS (0.9% NaCl, 0.2% KCl, 20 mM K₂HPO₄, pH 7.2) and dissolved in 4 M guanidinium thiocyanate. The suspension was layered over a 5.7 M cesium chloride cushion and centrifuged for 18 hr at 36,000 rpm at 20°C. The resulting RNA pellet was dissolved in 0.1% diethylpyrocarbonate- (DEPC) treated water, precipitated, and dissolved in DEPC-H₂O for storage at -20 C.

For most recent experiments, total RNA isolations were performed by the recently reported method of Xie and Rothblum (1991). This rapid method results in higher total RNA recoveries of equivalent quality with fewer manipulations.

After washing with PBS, the cells were lysed, in plate, with 1.6 ml solution A [water-saturated phenol (0.04% w/w 8-hydroxyquinoline):Solution B (4 M guanidinium thiocyanate, 25 mM NaCitrate (pH 7.0)):2 M NaOAc (pH 4.0)] in the ratio 1:1:0.1 amended with 720 μ l β -mercaptoethanol/100 ml Solution B, and transferred to 2 ml capacity microfuge tubes. 160 μ l chloroform:isoamyl (24:1) is added, tubes are vortexed, incubated on ice 15-30 minutes and centrifuged at 12000g for 20 minutes at 4°C. The aqueous layer is added to microfuge tubes containing 800 μ l isopropanol, mixed, and incubated at for 1 hr at -20°C. Total RNA is sedimented by centrifugation at 12000 g for 20 minutes at 4°C. The RNA pellet was washed two times with 70% EtOH, dried, and resuspended in DEPC-H₂O. RNA was quantitated by measuring OD 260 of the solution using an absorbance of 1.00 equal to 40 μ g/ml RNA.

RNA Fractionation and Hybridization.

Fifteen micrograms of total RNA was separated by electrophoresis in a 1.1% agarose gel containing 6% formaldehyde and capillary blotted onto nitrocellulose paper or GeneScreenTM using 20X SSC (175.3 g NaCl/liter, 88.2 g NaCitrate/liter, pH 7.2) as the transfer buffer (Thomas, 1980). Visualization was achieved by staining with ethidium bromide, destaining and irradiation by short-wavelength ultraviolet. The nitrocellulose blots were visually examined for RNA transfer, 28 and 18S ribosomal bands were delineated for future reference, the blots were air-dried and baked in a vacuum oven at 80°C for 1-1.5 hours or UV-irradiated to ensure irreversible RNA binding.

The blots were incubated overnight in a rotary oven at 42°C in a prehybridization solution containing 50% formamide, 10X Denhardt's (0.2%

ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA Pentax fraction V), 5X SSPE (0.1875 M NaCl, 6.425 mM NaH₂PO₄, 1.375 mM EDTA, pH 7.4), 100 ug/ml denatured salmon sperm DNA, and 0.1% SDS. The full-length cDNA probe, pTAT 123 (Grange *et al.*, 1985), was nick translated, using a nick translation kit obtained from Promega Biotechnology, to a specific activity of 2.5×10^7 - 10^8 cpm/ μ g with [α -³²P]dCTP. The denatured labeled probe was then added to the prehybridization solution and allowed to hybridize for an additional 16 hours. The blot was then put through a series of washes of increasing stringency to eliminate any nonspecific binding of the probe. Washes 1 and 2 were with 2x SSC and 0.1% SDS for 30 min at room temperature. Washes 3 and 4, if necessary, were with 0.1 X SSC and 0.1% SDS for 30 min at 55°C (Amasino *et al.*, 1986). The damp blot was prepared for autoradiography by being wrapped in plastic wrap, sandwiched between Kodak XRP film and a Cronex intensifying screen, and stored in a cassette at -70°C. The film was developed and analyzed as described below. If necessary the blot was put back on film and stored for a different length of time to attain greater or lesser exposure.

The relative TAT mRNA levels were quantitated by scanning densitometry of autoradiographs, basal mRNA levels being arbitrarily set at 1 unit. An LKB model 2222-010 Ultrosan XL laser densitometer was used for analysis. To ensure a linear relationship between densitometry values and quantities of TAT mRNA, varying amounts (ranging from 2-16 μ g) of total RNA from cells treated with known inducers of mRNA^{TAT} were analyzed by Northern dotblot analysis. Multiple exposures of each autoradiograph were performed to ensure linearity of response. Exposure times were chosen such that RNA input directly correlated with signal intensity.

Measurement of transcription in isolated nuclei.

To measure the elongation of transcripts in control or treated cells, nuclear run-off assays were performed as described previously (Moore and Koontz, 1989). Briefly, nuclei were isolated by the method of Granner *et al.* (1983) and transcription was measured according to the method of McKnight and Palmiter (1979). Following run-off transcription, the RNA was isolated by the method of Groudine *et al.* (1981). Plasmids were denatured according to McKnight and Palmiter (1979) and spotted onto GeneScreen (New England Nuclear, Boston, MA) using a dot-blot apparatus (Bio-Rad, Richmond, CA). The blot was UV irradiated and then prehybridized at 43°C in 0.25 M NaH₂PO₄ (pH 7.2), 7% SDS, 1 mM EDTA, 50% formamide, 0.25 M NaCl, and 100 µg/ml salmon sperm DNA (Amasino, 1986). After prehybridization, a normalized input of isolated RNA ($1-2 \times 10^7$ cpm) was added and hybridized for 65 hr at 43°C. The filters were then washed two times with 2X SSC/0.1% SDS at 55°C for 20 min, followed by two washes with 25 mM NaH₂PO₄ (pH 7.2), 1 mM EDTA, and 0.1% SDS at 50°C for 20 min. The filters were then washed in 2X SSC/10 µg/ml RNase A for 30 min at 37°C, followed by 25 mM NaH₂PO₄ (pH 7.2), 1 mM EDTA, and 1.0% SDS at 50°C for 20 min. The filters were then autoradiographed followed by counting of individual cut-out spots to determine counts hybridized. Nascent TAT transcript was detected using the genomic sub-clones pTAT EH 0.95, pTAT EH 2.45, and pTAT EE 1.05 which together represent 40% of the total 11 Kb heteronuclear TAT transcript before splicing to form the mature 2.4 Kb TAT transcript (Hashimoto *et al.*, 1984). Plasmid pGEM-3Z (Promega Corp., Madison, WI) served to control for non-specific hybridization. The relative transcriptional rate was calculated in reference to vehicle-treated controls for each experiment.

Results

Effect of insulin on mRNA^{TAT} in Fao cells.

Preliminary characterization of the insulin effect on TAT gene expression in Fao cells included Northern blot analysis of mRNA^{TAT} following insulin treatment. Consistent with previously published results (Crettaz *et al.*, 1988), it was found that insulin rapidly increases the abundance of the mRNA^{TAT} in a time and dose-dependent manner (Figure II-1.). The insulin effect on TAT mRNA is rapid (Figure II-1a.), achieving half-maximal levels by 30 minutes. A maximum 3-fold increase in mRNA^{TAT} abundance is achieved by 2 hours and maintained up to 4 hours after insulin treatment. This is followed by a steady decline back to basal levels. The dose-response results (Figure II-1b.) demonstrate that the insulin effect is mediated at physiological concentrations of insulin and is consistent with the effect being mediated specifically through the high-affinity insulin receptor.

Effect of insulin on TAT transcription rates.

The effect of insulin on TAT gene expression was further investigated by measuring the relative rate of transcription in isolated nuclei through the 'nuclear run-off' assay. *In vitro* transcription by isolated nuclei was performed with [α -³²P]UTP as a radioactive precursor. As shown in Figure II-2 insulin acutely, and transiently, increases the relative rate of TAT transcription with half-maximal increases obtained within 15 minutes of insulin exposure. Maximum induction of ~2.5-fold is achieved within 60 minutes. Within 120 minutes of insulin exposure the relative transcription rate is declining progressively towards that of control values.

Figure II-1. Effect of insulin on mRNA^{TAT} in Fao cells.

a.) Time course of the effects of insulin on TAT mRNA.

Fao cells were treated with 10^{-8} M insulin for the indicated times. Each point represents the mean of 4 separate experiments except for the 30 minute time point ($n = 2$), plus or minus one standard error of the mean.

(b.) Insulin Dose Response. Total RNA was harvested 6 hours after treatment of the cells with the indicated concentration of insulin.

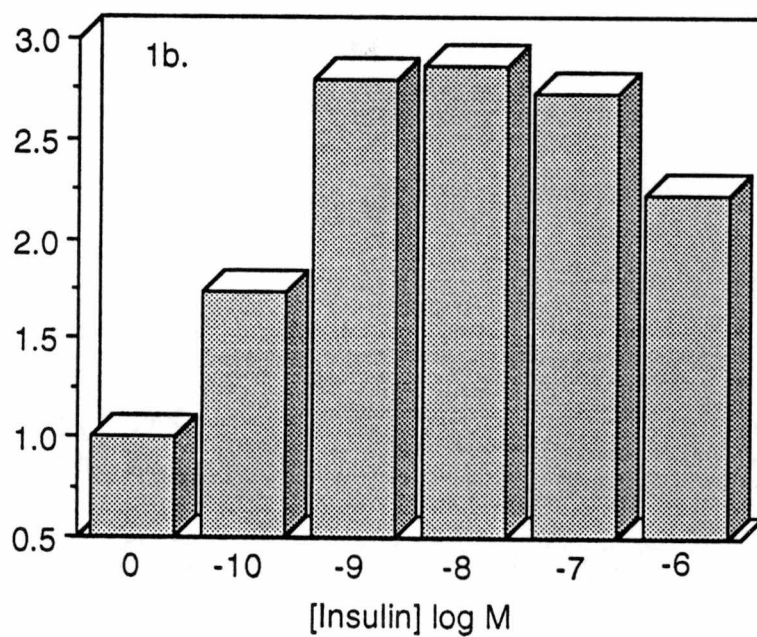
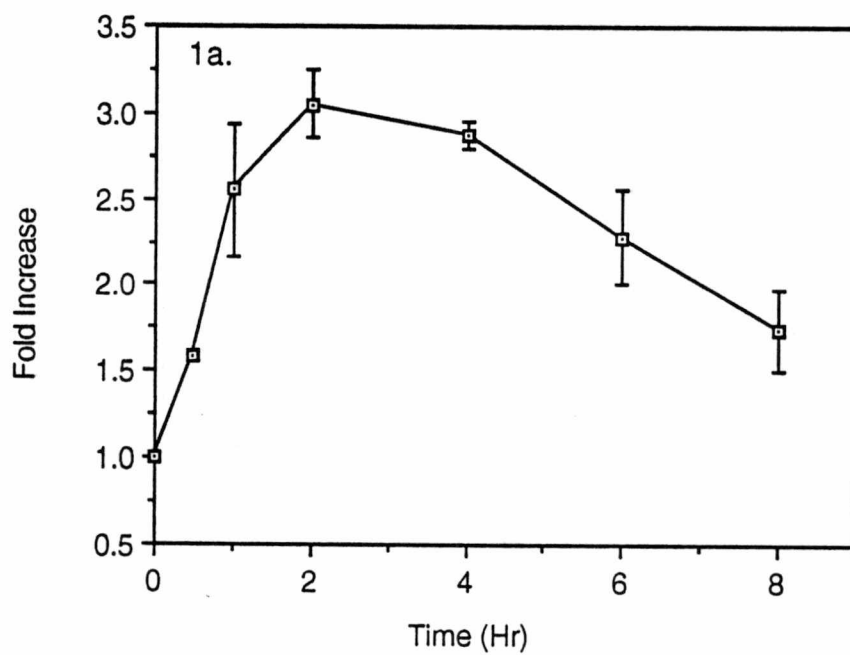


Figure II.1.

Figure II-2. Effect of insulin on the transcription of the TAT gene in Fao cells.

Cells were treated with 10^{-8} M insulin, harvested at the indicated times, and assayed for TAT transcription as described in Materials and Methods. Replicates (n) for each time point is: 15 and 30 minutes, 5; 60 minutes, 4; 90 and 120 minutes, 3. Data are presented as the average plus or minus one standard error of the mean.

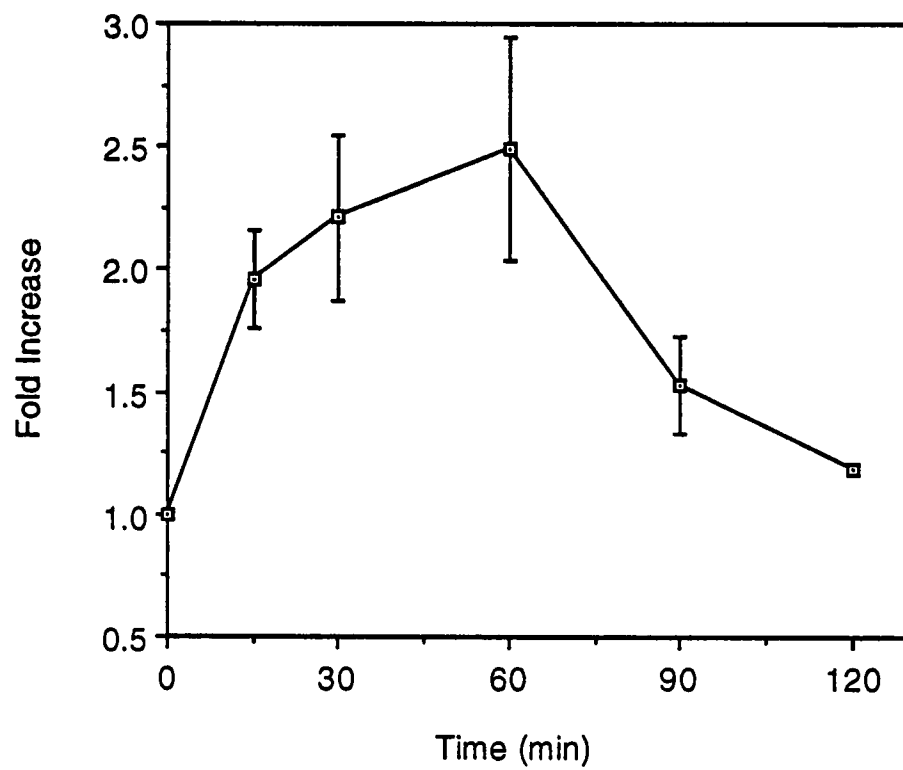


Figure II.2.

The rapidity of the transcriptional response to insulin suggests that the observed subsequent increase in hybridizable mRNA^{TAT} (Figure II-1) is primarily a result of insulin-mediated increases in the rate of transcription of the TAT gene.

Insulin's effect on dexamethasone and 8Br-cAMP-mediated induction of mRNA^{TAT}.

Insulin has been reported to inhibit both glucocorticoid- and cAMP-mediated enhanced transcription of TAT in the KRC-7 and H4 hepatoma clones (Moore and Koontz *et al.*, 1989; Messina *et al.*, 1992). In addition, insulin was found to antagonize cAMP-mediated increases in mRNA^{TAT} in FTO-2B cells (Schubart, 1986). In contrast, in Fao cells Crettaz *et al.* (1988) observed no significant effect of insulin on dexamethasone-mediated increases on mRNA^{TAT}. Crettaz's treatment regime consisted of 4 hours of insulin exposure to cells which had been preincubated for 16 hours in the presence of dexamethasone. In an effort to dissect potential converging and/or opposing molecular events which may lead to alterations in TAT gene expression, we chose to determine the effect of a simultaneous treatment with both insulin and dexamethasone. Cells were exposed to either agonist alone or in combination for 8 hr. Both dexamethasone and the cAMP analog, 8Br-cAMP, induce mRNA^{TAT} (Table II-1). However, it was observed that simultaneous treatment of Fao cells with dexamethasone together with insulin resulted in significantly less of an induction of the mRNA^{TAT} than from treatment with the dexamethasone alone. Similarly, simultaneous addition of insulin with 8Br-cAMP was found to inhibit 8Br-cAMP-mediated increases in mRNA^{TAT}. Inhibition of the dexamethasone or 8Br-cAMP response was 63% and 72% respectively (Table II-1). This observation is identical to that which has been

**Table II-1. Effect of Hormone Agonists Alone or Combination
on Endogenous mRNA^{TAT} in the Fao Hepatoma Clone.**

Hormone	mRNA ^{TAT} Fold Increase $\pm$ SE	% Inhibition [1-(Horm+Ins/Horm)]100
8Br-cAMP	3.2 $\pm$ 0.7	
8Br-cAMP + Ins	1.6 $\pm$ 0.5	72 %
8Br-cAMP + TPA	1.2 $\pm$ 0.5	90 %
Dex	22.0 $\pm$ 7.0	
Dex + Ins	8.6 $\pm$ 2.0	64 %
Dex + TPA	16.0 $\pm$ 5.0	N.S.
Insulin	1.50 $\pm$ 0.2	
TPA	0.50 $\pm$ 0.04	

Fao cells were treated with either 1.0 μ M dexamethasone, 0.5 mM 8Br-cAMP, 10 nM insulin, or 2.0 μ M TPA alone or together as indicated. 8Br-cAMP was replenished at 4 hours. Total RNA was harvested 8 hours after treatment. TAT mRNA levels were determined as described under Materials and Methods and were expressed as the fold change vs the vehicle control for each experiment. Each value represents the mean of three separate experiments. N.S., not statistically significant.

observed in the KRC-7 and H4 clones. It was unexpected, however, because any one of these compounds acting alone induces expression of the gene.

Effect of insulin on dexamethasone-mediated increases in TAT transcription rate.

Northern blot analyses indicate that insulin treatment alone increases expression of the TAT gene (Figures II-1 and II-2), while at the same time inhibiting either dexamethasone- or cAMP-mediated increases (Table II-1). In an effort to further characterize these paradoxical observations, we examined the effect of dexamethasone alone or in the presence of insulin on TAT transcription. When evaluated at time intervals 15 and 30 minutes after exposure, either dexamethasone (data not shown) or insulin (Figure II-2) alone rapidly increased TAT transcription rates when examined through nuclear run-off assay. Simultaneous treatment with both dexamethasone and insulin resulted in significantly reduced transcriptional increases when compared to dexamethasone treatment alone (Figure II-3).

Comparison of the effects of insulin or an activator of protein kinase C (PK-C) on dexamethasone and 8BrcAMP-mediated induction of mRNA^{TAT}.

Activators of PK-C have been shown to mimic several of insulin's actions (Messina *et al.*, 1992). Indeed, O'Brien *et al.* have reported that phorbol esters and insulin inhibit PEPCK gene transcription through the same 10-base pair promoter sequence (O'Brien *et al.*, 1991). We examined the effect of TPA on TAT expression in these cells as a possible indicator of the role of PK-C in the insulin signalling pathway leading to changes in TAT expression.

Figure II-3. Effect of insulin on dexamethasone-mediated increases in TAT transcription rate evaluated through nuclear run-off assay in Fao cells.

Cells were treated with either 1.0 uM dexamethasone or 1.0 μ M dexamethasone and 10^{-8} M insulin concurrently and harvested at the indicated times. TAT transcription levels were determined as described under Materials and Methods and are expressed as % Inhibition: $[1(\text{Dex}+\text{Ins}/\text{Dex})]100$.

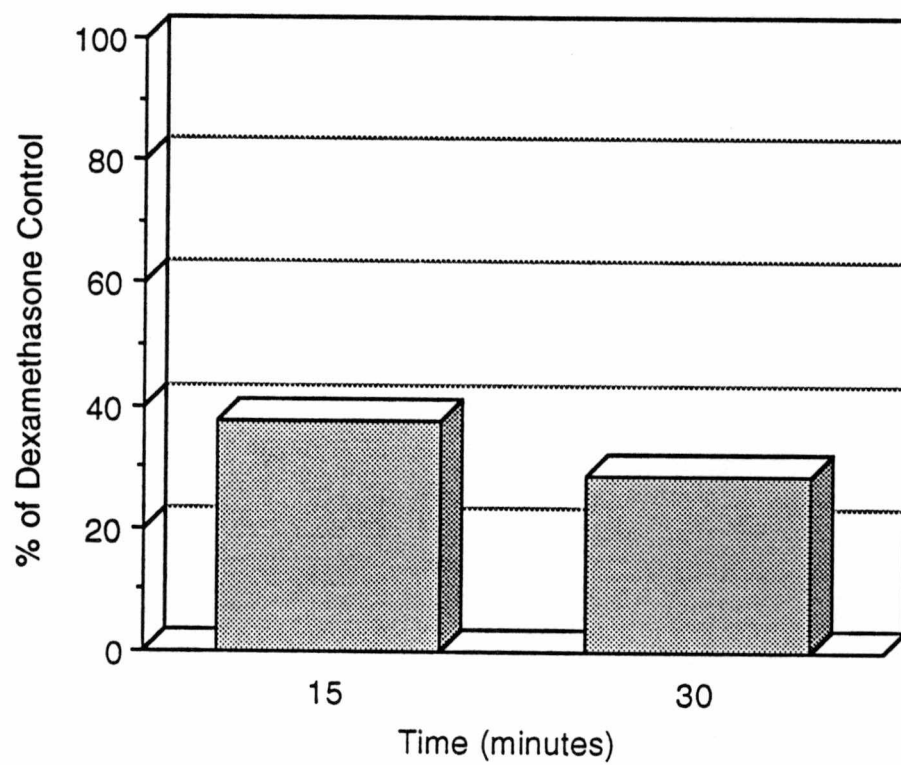


Figure II.3.

In contrast to the stimulatory effect of insulin on TAT mRNA [Figure II-1b.], an 8 hour TPA treatment significantly decreased the abundance of mRNA^{TAT} to ~50% of control levels (Table II-1).

TPA, like insulin, depressed the 8BrcAMP-mediated increase in mRNA^{TAT} (~90% inhibition) but, unlike insulin, had no statistically significant effect on dexamethasone-mediated increases in TAT mRNA ($P < 0.005$). The results suggest that the insulin signalling pathway affecting TAT expression in Fao cells is distinct from that of TPA.

In Vivo Steady State mRNA^{TAT} in Fao vs. KRC-7 Cells

As a prerequisite to an increased understanding of the opposing insulin action on basal TAT gene expression in KRC-7 and Fao cells (an inhibition vs. a stimulation respectively), was the determination of the mechanism by which the TAT promoter establishes basal TAT gene expression in each cell-line. The presence and/or abundance of a finite group of liver-enriched regulatory transcription factors might be the primary determinant for TAT basal expression in both the KRC-7 and Fao cells.

It was suspected that basal steady-state mRNA^{TAT} was reduced in the Fao clone due to the difficulties encountered obtaining strong mRNA^{TAT} signals while performing Northern blot analyses with the Fao clone when compared to that with the KRC-7 clone.

A comparative assessment of *in vivo* basal TAT gene expression in the KRC-7 and Fao clones could only be accomplished through parallel studies conducted simultaneously with both cell-lines. Therefore, basal mRNA^{TAT} in both cell-lines was examined in parallel through Northern analysis in two

separate experiments. The results indicate that *in vivo* steady-state mRNA^{TAT} is ~15-fold higher in the KRC-7 clone than in the Fao clone (data not shown).

Discussion

Previous studies from this laboratory have shown that in the rat hepatoma cell line, KRC-7, insulin inhibits TAT gene expression through a selective decrease in TAT transcription (Moore and Koontz, 1989). To further characterize the insulin response similar studies were conducted with the hepatoma cell line, Fao, which is characterized as representing a differentiated phenotype (Crettaz *et al.*, 1983). In agreement with a previous report (Crettaz *et al.*, 1988), it was found that insulin treatment resulted in an increased rate of TAT transcription followed by an increase in hybridizable mRNA^{TAT}.

Interestingly, the results indicate that while insulin alone rapidly and transiently increases basal TAT gene expression in Fao cells, it inhibits the increases observed with either dexamethasone or 8Br-cAMP when added simultaneously with either of these agonists. The results suggest that the KRC-7, H4, FTO-2B, and Fao hepatoma clones and cultured fetal hepatocytes share a common mechanism for an insulin-mediated dominant inhibition of enhancer-mediated induction. Experiments addressing the insulin-mediated inhibition of dexamethasone induction, which is the best characterized response for each of these clones, appear to support this conclusion.

The KRC-7 and Fao rat hepatoma cell lines were derived from the same parental clone, the Reuber H35 minimal-deviation rat hepatoma (Koontz and Iwahashi, 1981; Deshalette and Weiss, 1974). The contrasting effect of insulin on basal/constitutive expression in the two cell lines implies that each clone represents a stably-expressed and distinct phenotype. Interestingly, and

potentially significant, we find that basal steady-state mRNA^{TAT} levels are reproducibly 15-fold lower in the Fao clone as compared to the KRC-7 clone when examined in parallel through Northern blot analyses. The diminished basal TAT gene expression in the Fao clone may be the result of an absence or deficiency of a factor required for high-level TAT expression. In this regard, it may be postulated that this factor, deficient in the Fao clone, is an insulin-responsive target that is normally present or recruited to the promoter for both high-level basal and enhancer-mediated expression. The determination of the liver-specific phenotype is an area of recent intensive research by several laboratories (Tonjes *et al.*, 1992). It appears that both the abundance and class of several liver-enriched transcription factors together with binding sites for cell-type non-specific factors, confer the state of differentiation for any given liver-cell line (Kuo *et al.*, 1992; Lai and Darnell, 1991). It remains to be determined whether these proteins are regulated by insulin.

Results argue against a significant role for the activation of PK-C as an integral component of insulins' postreceptor intracellular signalling pathway for modulating TAT expression in Fao cells. TPA and insulin were observed to have opposing effects on mRNA^{TAT}. In addition, TPA did not significantly effect dexamethasone-mediated increases in TAT expression while insulin significantly inhibited dexamethasone-mediated increases.

We hypothesize that the conflicting reports regarding insulin regulation of TAT gene expression are dependent on the availability of the target(s) of insulin action the abundance of which is dependent on the state of hepatocyte growth, differentiation, and/or the presence of additional cytokines and hormones.

Chapter III

TRANSIENT TRANSFECTION ANALYSES

Introduction

In vivo studies reported in the previous chapter indicate that in the Fao clone insulin exerts its effect on TAT expression primarily at the level of transcription. We then sought to identify potential *cis*-acting sequence(s) contained in the TAT 5'-flanking sequence which may mediate the insulin response. This was undertaken through the analysis of a series of 5'-, 3'-, and internal deletion TAT•CAT chimeric constructs transiently transfected into Fao cells.

It should be cautioned that while most of the current information which has been obtained regarding the role of *cis*-acting elements in gene expression has been generated through the rapid and reproducible experimental system described herein (transient transfection), interpretations that result from this simplified "minimal segment" reductionist approach must ultimately be corroborated in the context of larger genomic segments encountering development and differentiation *in vivo*.

The -3.6 Kb liver-specific enhancer cAMP-response unit.

Chromatin from adult liver and well-differentiated hepatoma cells (those cells that maintain high-level expression of the TAT gene, FTO-2B) have strong Dnase I hypersensitivity (HS) at a position 3.6 Kb upstream relative to the TAT gene start site (Nitsch *et al.*, 1990). The -3.6 Kb HS site is absent in a TAT non-expressing hepatoma (HepG2), substantially diminished in the HTC

hepatoma (in which the *in vivo* steady-state mRNA^{TAT} is 10-fold lower than in FTO-2B), and is absent in non-liver cells (Nitsch *et al.*, 1990).

Expression of a -3341 to +62 bp TAT-CAT construct transfected into either FTO-2B, HTC, HepG2, and HeLa (no detectable *in vivo* TAT expression) resulted in very low CAT expression in all of the above cell lines (0.21 pm/min·mg in the FTO-2B) when compared to a transfected HSV TK promoter-CAT construct (pBLCAT2) (~3-fold greater) (Boshart *et al.*, 1990). Subsequent transfections with a construct that included the -3.6 Kb HS site(-3922 to +62 TAT-CAT) resulted in a more than 50-fold increase in CAT expression in the well-differentiated rat hepatoma FTO-2B, while no change in CAT expression was observed in any of the other cell lines tested.

Transfection analysis of a series of 5' and 3' internal deletion constructs had defined an 80 bp fragment (-3660 to -3580 bp, the 'B domain') as essential for the enhancer activity (Boshart *et al.*, 1990). A series of clustered point mutations allowed a systematic scan of the region. Two distinct motifs within the B domain, designated BI and BIII, were found to be required for enhancer function (refer to Figure III-1).

Tandem dimer oligonucleotides of BI or BIII were cloned in front of the HSV TK promoter and transfected into the FTO-2B clone (Boshart *et al.*, 1990). The uninduced BI dimer was found to increase TK promoter activity ~2.8-fold. In addition, the BI dimer, which contains an asymmetric (or degenerate) CRE motif, was found to be sufficient to confer cAMP responsiveness to the TK promoter (~1.5-fold induction). Three point mutations in the CRE-like sequence motif abolished cAMP responsiveness. A dimer, but not a monomer, of the BIII oligo strongly activated the TK promoter (~40-fold) and was unaffected by forskolin, a potent activator of PK-A.

Figure III-1. The TAT -3.6 Kb liver-specific enhancer and cAMP response unit.

BI and BIII are subset sequences of the entire enhancer unit that were used in transfection studies by Boshart *et al.* (1990).

Boxed DNA denote sequence similarity or identity to a consensus DNA recognition site for a particular DNA-binding protein. The arrows above the CRE sequence denote the presence of dyad symmetry. See the accompanying text for an explanation of sequences with designated identity assignments and individual DNA-binding protein identities. Taken from Boshart *et al.* (1990).

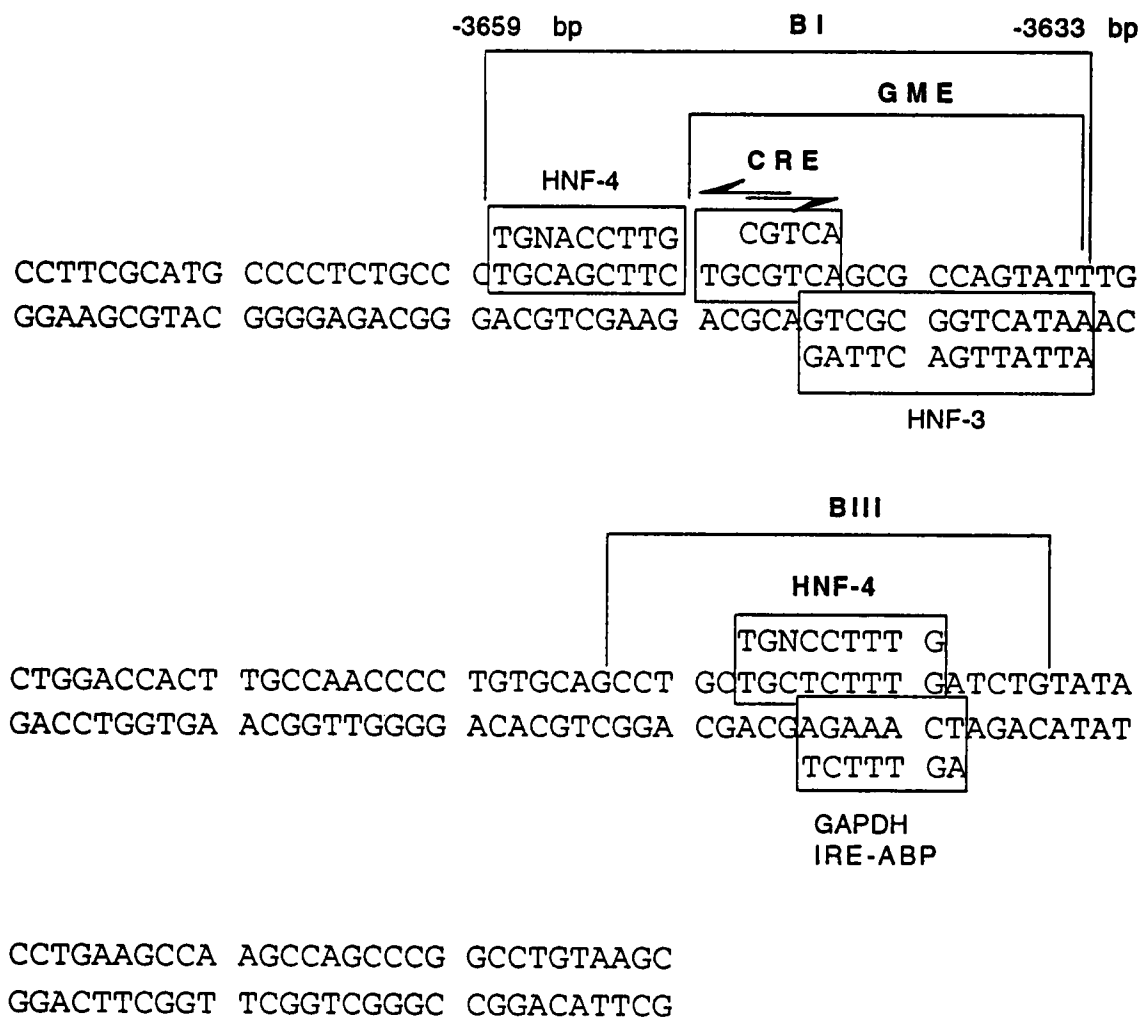


Figure III-1.

The above findings suggest a synergism between a CRE (BI) and a constitutive element (BIII) for the intact -3.6 Kb HS site B domain enhancer function.

To establish the role of the BI and BIII elements in determining cell-type specificity of the enhancer the above constructs were transfected into the four cell-lines described earlier (FTO-2B, HTC, HepG2, and HeLa) (Boshart *et al.*, 1990). The intact B domain conferred strong activation (~40-fold) onto the TK promoter in FTO-2B cells (possessing high-level *in vivo* TAT expression), a minimal effect (~2-fold) in HTC cells (possessing low-level *in vivo* TAT expression), and no effect in either HepG2 (no detectable *in vivo* TAT expression) or HeLa. Thus, the B domain of the enhancer confers specificity for highly-differentiated hepatoma cells.

The CRE motif alone could not account for the restriction of the enhancer activity to FTO-2B cells. The 2xBI-TK construct was found to result in strong constitutive expression in HeLa cells. In addition, replacement of the TAT-CRE with a dimer of the somatostatin CRE (a consensus CRE motif), construct 2xCRE-TK, paralleled the activity of the TAT CRE in the different cell-lines.

The BIII motif was found to be active in hepatoma cell-lines only. In addition, 2xBIII-TK CAT expression in each of the three hepatoma cell-lines closely paralleled TAT gene expression *in vivo*.

It appears that the specificity of the intact B domain enhancer is generated by the combination of a CRE element and a hepatocyte-specific element, both of which are fully interdependent, and, together, provide a dominant role in establishing hepatoma-specific basal TAT gene expression.

Interestingly, the existence of a second binding activity has recently been demonstrated within the BI region (Nichols *et al.*, 1992). Both methylation interference and band shift analyses have demonstrated that the 'A complex' protein specifically binds the TAT CRE, but not to a mutated version. Whereas the 'BC complex' (rigorously demonstrated to be CREB α through several criteria) also binds the symmetrical somatostatin CRE, 'A complex' protein does not. Furthermore, the BC complex was found to possess an increased affinity for the TAT CRE and an altered mobility subsequent to PK-A treatment (due to an increase in phosphorylation state), whereas 'A complex' remains completely unaffected.

Although the identity of the 'A complex' binding activity is presently unknown, inspection of the TAT CRE DNA sequence reveals significant homology to PEPCK CRE-1 and proenkephalin CRE-2 (see Figure III-2). Both PEPCK CRE-1 and proenkephalin CRE-2 have been reported to bind several members of the bZip family of transcription factors including LAP, DBP, C/EBP α , and Jun- and Fos-family members (Kobierski *et al.*, 1991; Gurney *et al.*, 1992).

In addition, a DNA-binding activity, located in the region designated BI, has recently been reported to modulate the induction properties of glucocorticoid acting on the distant -2.5 Kb glucocorticoid response unit (Oshima and Simons, 1992).

Close inspection of the BI DNA sequence reveals a region with sequence similarity to the consensus recognition site for HNF-3. The putative HNF-3 site can be located in the non-coding strand immediately adjacent to the TAT CRE [see Figure III-1]. HNF-3 is a liver-enriched transcription factor family containing three similar yet distinct proteins designated HNF-3 α , β , and γ

Figure III-2. The asymmetric TAT CRE contained in DNA sequence designated BI.

The boxed DNA sequences denote sequence similarity of the TAT CRE to previously characterized CRE/AP-1 sites present in other promoters. AP-1 and CRE-2 of proenkephalin taken from Kobierski *et al.* (1991). CRE-1 of PEPCK taken from Gurney *et al.* (1992). Arrows above the CRE denote the presence of dyad symmetry. See the accompanying text for an explanation of DNA-binding protein identities.

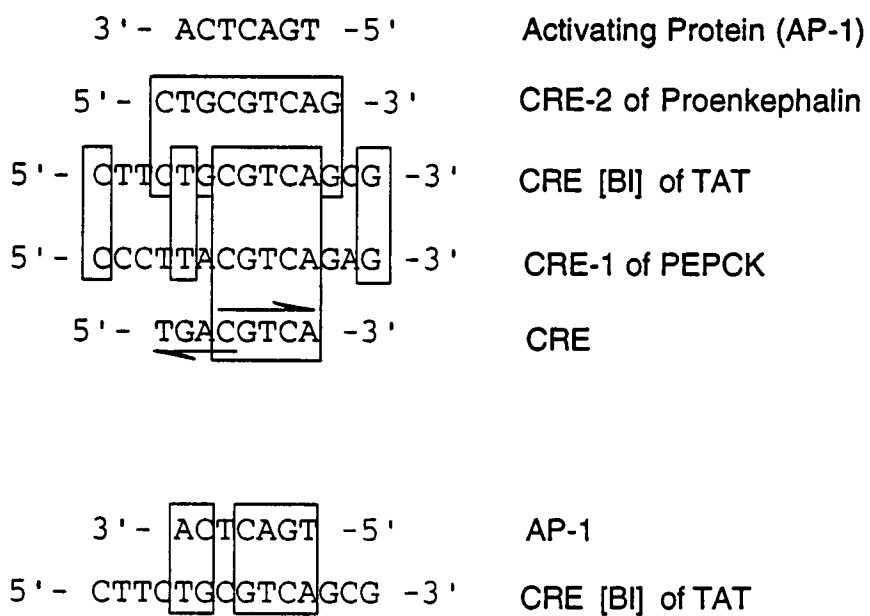


Figure III-2.

(Pani *et al.*, 1992). The HNF-3 proteins bind DNA as a monomer. Most interesting of the three members of this family is HNF-3 β . In addition to being rich in the hydroxylated amino acids serine and tyrosine (there are two putative casein kinase I and II phosphorylation sites present), HNF-3 β possesses a unique stretch of histidines in its novel activation domain. Interestingly, 2-D SDS PAGE studies indicate that HNF-3 β exists as a multiple phosphorylated species in liver nuclear extracts (Pani *et al.*, 1992).

The presence of a target sequence for a liver-enriched DNA-binding activity (a putative HNF-3 site), immediately adjacent to an asymmetric/degenerate enhancer response element, is a recurring theme present in all three promoter units required for TAT gene expression *in vivo*:

- the -3.6 Kb cAMP response unit $\rightarrow$ CRE•HNF-3
- the -2.5 Kb glucocorticoid response unit $\rightarrow$ HNF-3•GRE
- the -350 bp minimal-basal unit $\rightarrow$ HNF-3•HNF-1, HNF-3•HNF-4, and HNF-3•TFIID

See Figure III-3 for a diagram which illustrates the redundancy of a liver-enriched DNA-binding activity associated with a non-cell-type specific enhancer element in each of the three TAT 5'-flanking sequence regulatory units.

In support of a functional significance to this observation, a report from Zaret's laboratory demonstrates the formation of a novel, composite regulatory element whose activity is essential for the liver-specific activity of the albumin promoter (Jackson *et al.*, 1993). The element consists of an HNF-3 site closely associated with an NF-1/CTF site which, upon DNA binding, leads to higher-order interactions affecting the local DNA sequence environment.

Figure III-3. Diagram demonstrating the hypothetical redundancy of putative and/or reported HNF-3 and HNF-4 recognition sites in the modular units which together comprise the determinants of TAT expression.

A detailed discussion of DNA-binding identities and appropriate references is contained in the accompanying text. Arrows denote DNA sequences possessing dyad symmetry.

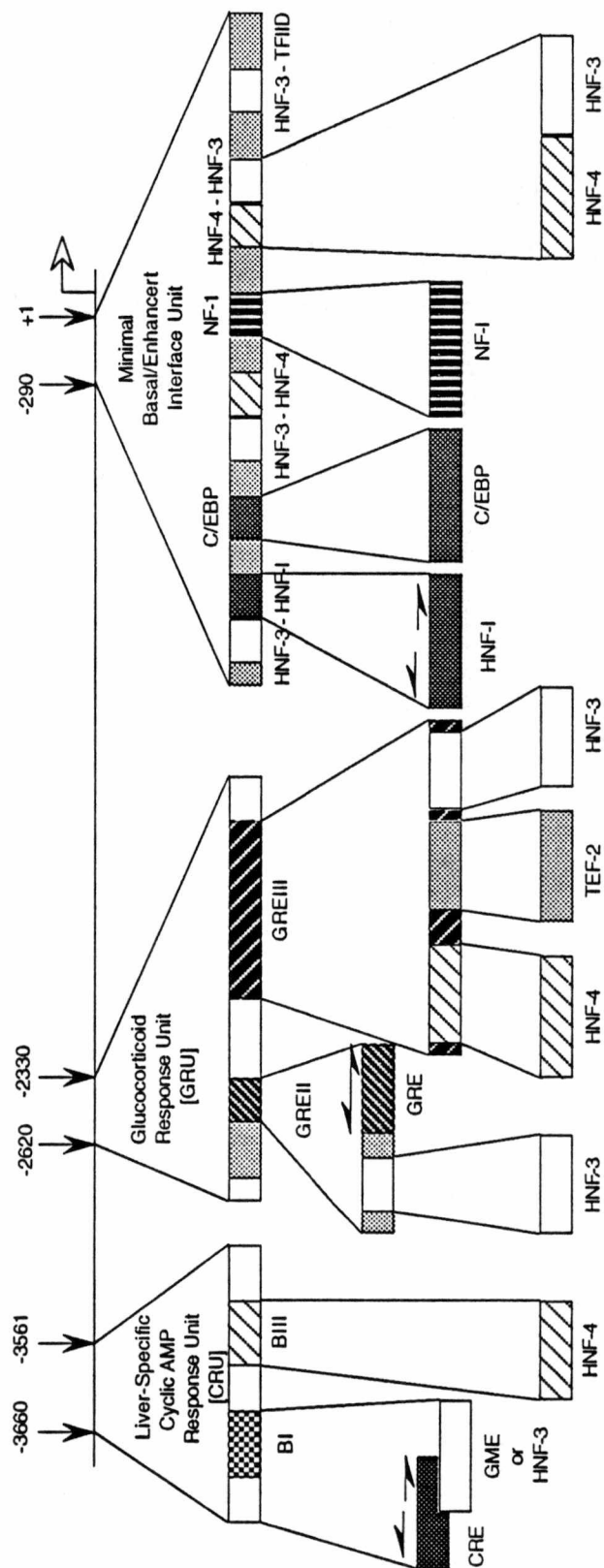


Figure III-3.

The authors state that the TGTTT-class of HNF-3 binding sites are often found in close association with binding sites for other transcription factors in liver-specific promoters (e.g., transferrin, α -fetoprotein, glucokinase, and apolipoprotein A1). Furthermore, the authors suggest that the CCAAT-motif DNA-binding activity, located immediately adjacent to the TAT GRE, is HNF-3.

The -2.5 Kb glucocorticoid responsive unit.

Typically, enhancer-binding elements exist in assemblies, referred to as hormone response units (HRU's), either as multiple copies and/or associated with additional *cis*-elements which serve to augment the response and/or as obligatory components (Lucas and Granner, 1992).

DNA sequences required for conferring glucocorticoid-induction to the TAT gene were mapped through transfection of a series of TAT-CAT 5'- and 3'-deletion constructs (Jantzen *et al.*, 1987). The reported glucocorticoid-responsive region included DNA sequences -2527 to -2419 bp and was contained fully within the region of hormone-induced DNase I hypersensitivity (refer to Figure III-4).

Early DNA binding studies with partially-purified glucocorticoid receptor (GR) revealed GR binding to two sites, -2527 to -2493 bp and -2474 to -2308 bp, designated GRE-II and GRE-III. Significantly, a GRE-III/TAT promoter/CAT construct (-2474 to -2308 bp ligated to the enhancer-interface region -351 to +62 bp) was totally non-responsive to glucocorticoid treatment when examined through transfection analysis [(-)Dex 143 pm·min·mg vs. (+)Dex 121 pm·min·mg] (Jantzen *et al.*, 1987).

In addition, the -2527 to -2493 bp region, GRE-II, (which contains a discrete GRE and is immediately preceded by a second DNA-binding activity

Figure III-4. The -2.5 Kb TAT glucocorticoid response unit.

Boxed DNA sequences that are labeled in bold type denote identified DNA-binding *cis*-element identities. Taken from Grange et al. (1991). Boxed DNA sequences that are labeled in normal type denote DNA sequence similarity to a consensus DNA recognition site. The 3'-boundary for the region designated GRE III continues until -2308 bp and is not shown in its entirety.

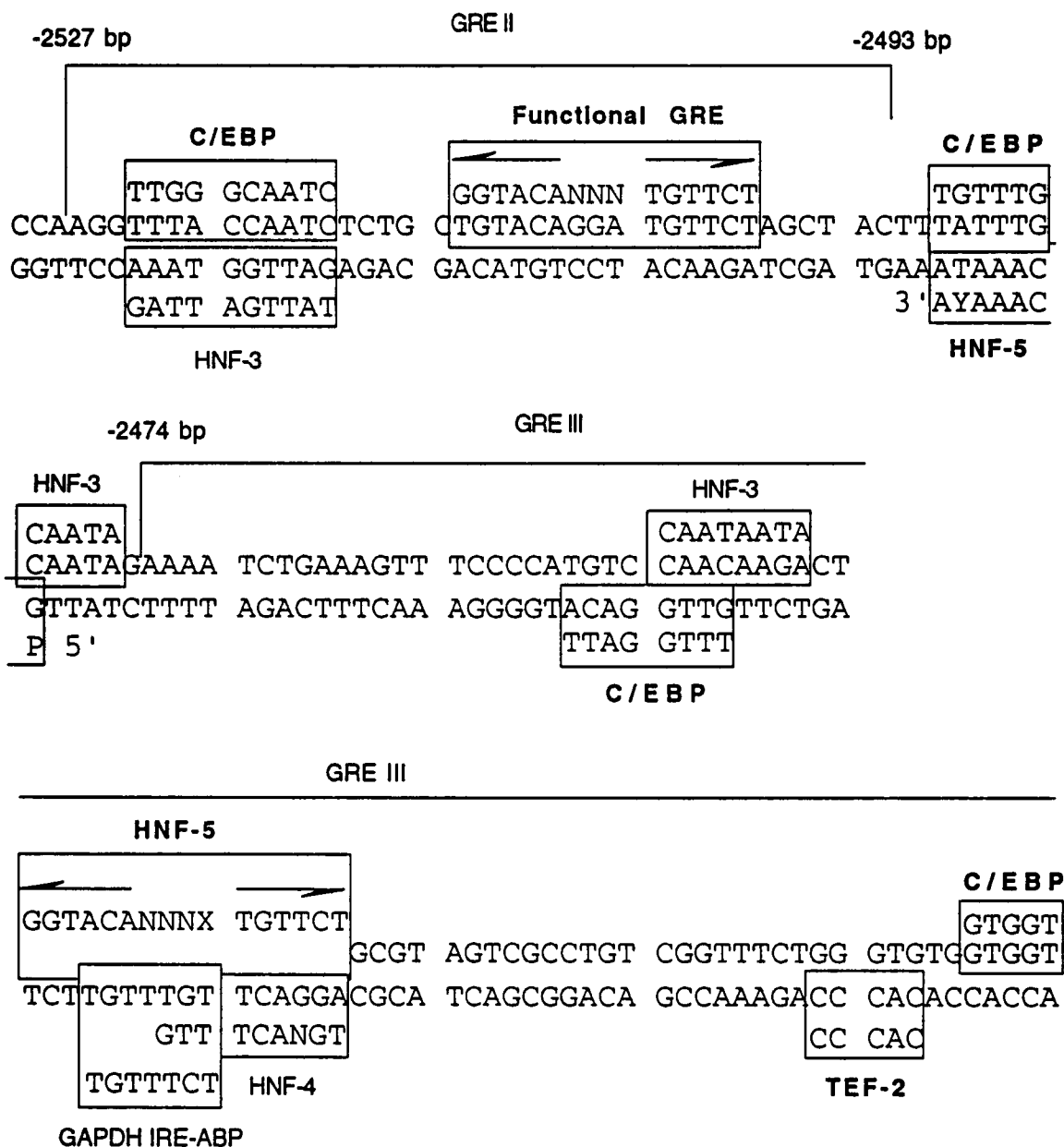


Figure III-4.

which contains a CCAAT motif) resulted in only 15-fold induction whereas the intact glucocorticoid-responsive sequences -2527 to -2308 resulted in 44-fold induction.

Subsequent studies by the same group confirmed a functional significance to the CCAAT-containing motif by selectively destroying the CCAAT sequence with a cluster of five point mutations (Strahle *et al.*, 1988). These mutations abolished the glucocorticoid-induction mediated by GRE-II when placed at -351 of the TAT promoter.

The identity of the CCAAT-motif DNA-binding activity has been attributed to the liver-enriched transcription factor, C/EBP (Grange *et al.*, 1991). This was concluded following gel-shift competitions with high-affinity recognition sites for C/EBP (the SV 40 enhancer core), NF-Y (albumin promoter), and NF-1 (the adenovirus 2 origin of replication). Further evidence was the observation that the *in vitro* DNase I footprint protection was not destroyed when nuclear extract was heated at 90°C for 10 minutes. Previous studies, reported by Trus *et al.*, (1990), had shown that C/EBP is resistant to denaturation when exposed to high temperatures for prolonged periods of time.

Nordeen *et al.* (1990) have methodically examined every position of the literature-derived consensus 15 bp GRE through substitution-mutagenesis in an effort to define the critical base-contacts and spatial-requirements. Results indicate that the 15 bp TAT GRE (contained within region GRE-II) alone confers severely compromised glucocorticoid-responsiveness to a CAT-reporter construct.

Mutant GRE's were cloned into a truncated basal MMTV promoter at position -109 which had been shown to be uninducible by hormone.

Glucocorticoid-responsiveness was evaluated by transfection into glucocorticoid-responsive mouse Ltk-fibroblasts. The preferred, wild type (WT) GRE, GGNACA NNN TGTNCC, closely resembled the literature-derived consensus, GGTACA NNN TGTTCT (note an underlined difference, +7 C to T). The 15-bp TAT GRE-II sequence TGTACA NNN TGTTCT (note -7 G to T) was shown to be severely compromised in its ability to mediate glucocorticoid-inducibility. The induction index = 2 for the GRE contained within TAT GRE-II. Induction index = $[(R_M - 1)/(R_{WT} - 1)] \times 100$ where R_M and R_{WT} are the luciferase-corrected induction ratios for the mutant and the WT GRE. $I = 100$ implies that the mutation has no signalling impairment. $I = 0$ implies that the mutation abolishes all hormone inducibility.

TAT GRE-III deviates from the WT GRE in that it possesses an additional bp insertion within the central 3 bp, regarded as critical for proper alignment of the palindromic 6-bp half-sites. Experimentally, an insertion within the central 3 bp was shown to abolish glucocorticoid inducibility. Indeed, it can be postulated that the binding activity attributed to GR in the region delineated GRE-III is most likely due to the binding of a related member of the steroid receptor super-family (e.g., HNF-4; Sladek *et al.*, 1990)) present in the partially-purified GR preparation. Upon examination of the GRE-III region one can locate a DNA sequence with significant homology to the consensus HNF-4 binding site (refer to Figure III-3). This inference has been further substantiated by a recent report from Schutz's laboratory in which it is stated only one functional GRE is present in the -2.5 Kb GRU (the GRE contained in region GRE-II - see Figure III-4) (Nitsch *et al.*, 1993).

The -2.5 Kb TAT GRU is structured similar to the -3.6 Kb cAMP response unit. It appears that the intact GRU is generated by the combination of an

asymmetric/degenerate GRE that is immediately adjacent to a liver-enriched DNA-binding activity that recognizes a core CCAAT-motif (a putative C/EBP or HNF-3 recognition site), and a third element present in region GRE-III (an HNF-4 binding-site), all of which functionally contribute to full glucocorticoid-inducibility of the TAT gene.

A significant difference between the -2.5 Kb TAT GRU and the -3.6 Kb cAMP response unit is that the *cis*-element binding activity of those *trans*-factors which serve to augment the glucocorticoid response *in vivo* is completely hormone-dependent (Reik *et al.*, 1991).

Upon glucocorticoid treatment there is a rapid hormone-induced alteration in chromatin architecture the result of which is the displacement of two nucleosomes positioned between -2680 and -2500, and -2500 and -2310. Previously repressed *cis*-element binding-activities (e.g., HNF-4, a CACCC-motif DNA-binding activity, transcriptional enhancer factor (TEF)-2, and C/EBP) are then accessible to their respective recognition sites (Grange *et al.*, 1991).

Considering the above, *in vivo* glucocorticoid induction can be viewed as a bimodal mechanism including both de-repression and true activation, as has been described for the MMTV promoter by Hager's laboratory (Archer *et al.*, 1992).

Importantly, the hormone-dependent -2.5 Kb HS has been observed only in cells and tissues of hepatic origin (Reik *et al.*, 1991). The HS response in endogenous chromatin fails to occur in TAT non-expressing cells (e.g., fibroblasts) that are able to activate, upon glucocorticoid treatment, transiently transfected constructs containing the intact TAT GRU.

A cause-and-effect relationship between changes in transcription and alterations in chromatin architecture was first established with studies of the MMTV promoter (Archer *et al.*, 1992). A stably transfected MMTV promoter attains a phased array of six nucleosomes. This architecture excludes NF1/CTF from the promoter prior to glucocorticoid treatment and hormone-dependent access of nucleolytic agents to promoter DNA. In contrast, when the promoter is transiently introduced into cells, NF1/CTF is bound constitutively and nucleolytic attack is hormone-independent.

The above observations suggest the following important conclusions. First, the -2.5 Kb HS response requires a hepatocyte-specific priming event (e.g., locus activation). A likely candidate is the CCAAT motif-core DNA-binding site which is immediately adjacent to the asymmetric TAT GRE. Earlier studies had demonstrated its obligatory role for glucocorticoid responsiveness of the TAT gene in cells of hepatic origin (Strahle *et al.*, 1988).

Second, studies of glucocorticoid-responsiveness employing transiently-transfected TAT constructs may not necessarily reflect the *in vivo* response. Transiently-transfected GRU-containing constructs appear not to attain the highly-ordered *in vivo* chromatin architecture which is a prerequisite of the repressed state. Thus, TAT GRU *cis*-element binding activities may only reflect the presence and abundance of binding-competent soluble *trans*-factors present in the cell line being transfected (e.g., constitutive de-repression). This observation is directly applicable to the interpretation of my transfection studies and has allowed me to reconcile data which might otherwise appear ambiguous.

Materials and Methods

Transient Transfection Analyses.

Transient DNA transfections were performed in solution using an optimized DEAE-dextran co-precipitation (Cheatham *et al.*, 1992) with 20 µg/ml test plasmid and 5 µg/ml pSV2aPAP (as an internal control for plate-to-plate transfection efficiency). Supercoiled test and control plasmids were co-precipitated, washed extensively, dried and resuspended in 500 µl sterile Tris buffer saline (TBS) - 154 mM NaCl, 50 mM Tris, pH 7.5, and 1 mM MgCl₂. The DNA solution was then added drop-by-drop while swirling into a 50 ml conical polypropylene falcon tube containing 250 µl 10 mg/ml DEAE/dextran and 750 µl TBS prewarmed to 37°C (note: no precipitate should form). The DEAE/dextran/DNA solution was then brought to 5 ml volume with sterile TBS and maintained at 37°C. Approximately 3×10^8 Fao cells (~15x100 mm dishes 70% confluent and actively growing) are required to seed 13x100 mm dishes [4 treatments (3 replicates/treatment) and 1 mock]/plasmid tested. Cells were harvested into a 50 ml conical polypropylene tube and loosely pelleted with a table top centrifuge at 1/2 speed for 4 minutes. The loose cell pellet was then immediately and carefully resuspended in the DEAE/dextran/DNA solution, slowly swirled on a rocker platform at room temperature for exactly 25 minutes, diluted to 12 ml volume with serum containing medium, and aliquoted (1 ml/dish) to dishes already containing 5 ml serum containing DMEM. After the cells had adhered to culture plates (about 6 hours) the cells were shocked with serum-free medium containing 20% DMSO v/v for 5 minutes at room temperature, washed twice with TBS, and placed on serum-containing medium for ~24 hours. Cells were then placed on serum-free medium for >18 hours, treated with hormones, and harvested 18

hours later. Cells to be harvested were washed two times with TBS, scraped into 1 ml TBS, pelleted, resuspended in 100 μ l ice cold 250 mM TRIS, pH 7.8, 5 mM EDTA, disrupted through three successive freeze/thaw cycles, incubated for 10 minutes in a 55°C H₂O bath, cooled on ice and centrifuged at 11000 g for 10 minutes at 4°C. The supernatant was then quick-frozen with dry ice after a 5 μ l aliquot was removed for the Bradford protein assay. CAT activity was evaluated through a non-chromatographic CAT assay as described by M. Sleight (1986). Placental alkaline phosphatase (PAP) activity was assayed by the method described by Henthorn *et al.* (1988).

Results

GRE-containing Constructs.

TAT 5'-flanking sequences contained in the full-length construct, pTC 3.92, are sufficient for hormonal regulation which reflects that observed for TAT expression *in vivo* (see Figures III-5 and III-6 and Figure III-7). Compare hormone-regulated CAT reporter expression to hormone-regulated mRNA^{TAT} of the endogenous gene.

Deletion of 5'-flanking sequence to -2561 bp (the upstream boundary of the -2.5 Kb GRU), construct pTC 2.6, results in the loss of a constitutive liver-specific enhancer activity (an ~10-fold decrease in basal expression) This can be attributed to the loss of BI (bZip family member) • BIII (HNF-4) synergism associated with the -3.6 Kb cAMP response unit (Boshart *et al.*, 1990). Note, however, there is no loss in either appropriate glucocorticoid- or insulin-mediated regulation.

An internal deletion of sequences -2308 to -351 bp (the 3'-downstream boundary of the -2.5 Kb GRU ligated to the 5'-boundary of the enhancer-

Figure III-5. TAT•CAT and TAT•TK•CAT constructs used in transient transfection studies.

pTC3.92 and pTC3.92 Δ were obtained from G. Schutz's laboratory (Boshart et al. 1990). Remaining constructs were derived from pBLCAT3 and/or pBLCAT2 (Luckow and Schutz, 1987).

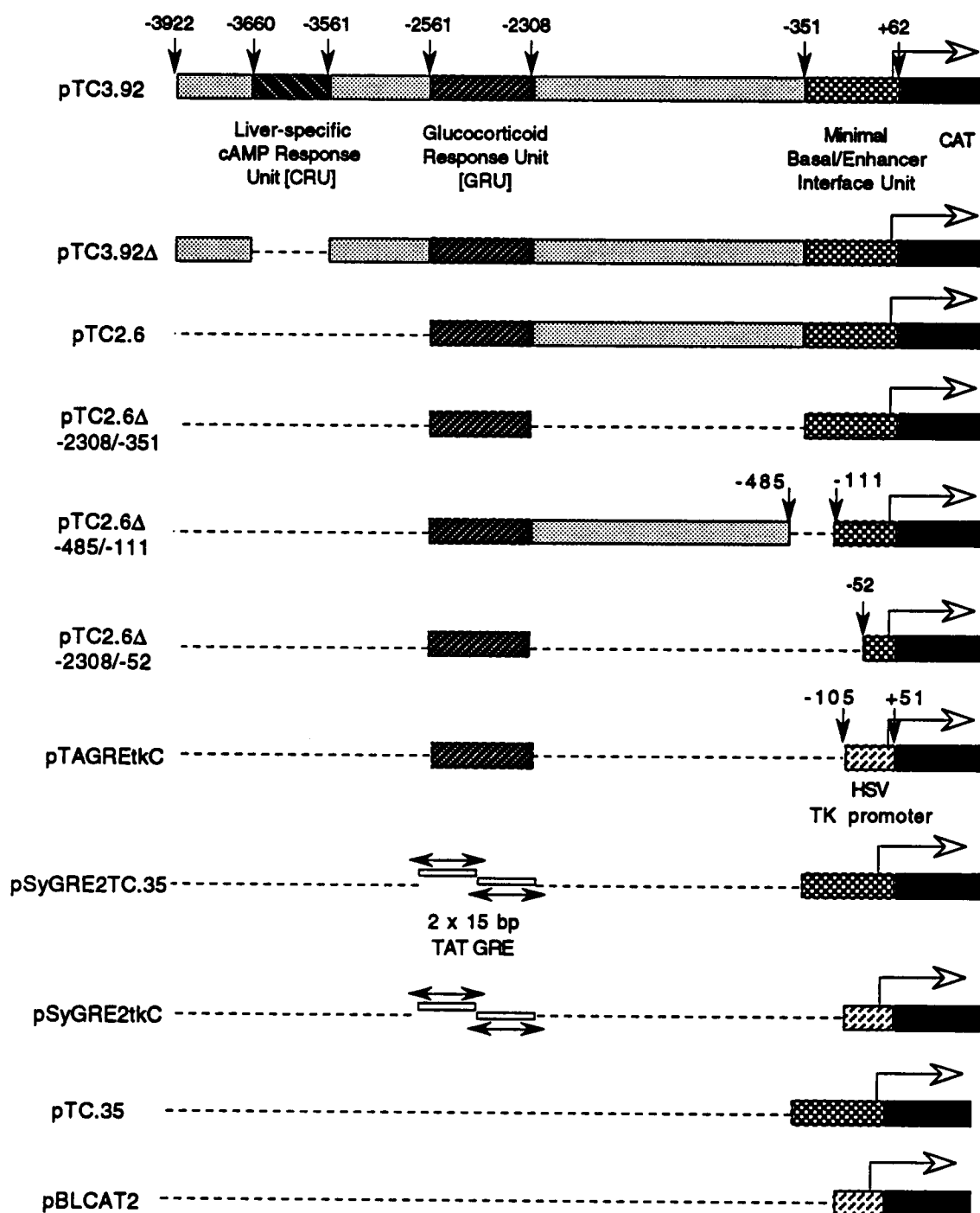


Figure III-5.

Figure III-6. Transient transfection fold induction results summary.

Transfection experiments were conducted as described in Materials and Methods. Each row of results is the data from one experiment. Results for each treatment are reported as the average fold induction of three replicates/treatment plus or minus one standard deviation. Insulin inhibition of dexamethasone-mediated induction is expressed as % Inhibition: $[1 - (\text{Dex} + \text{Ins} / \text{Dex})]100$. $\uparrow$ designates a dexamethasone + insulin increase in fold induction rather than an inhibition for that particular TAT•TK construct.

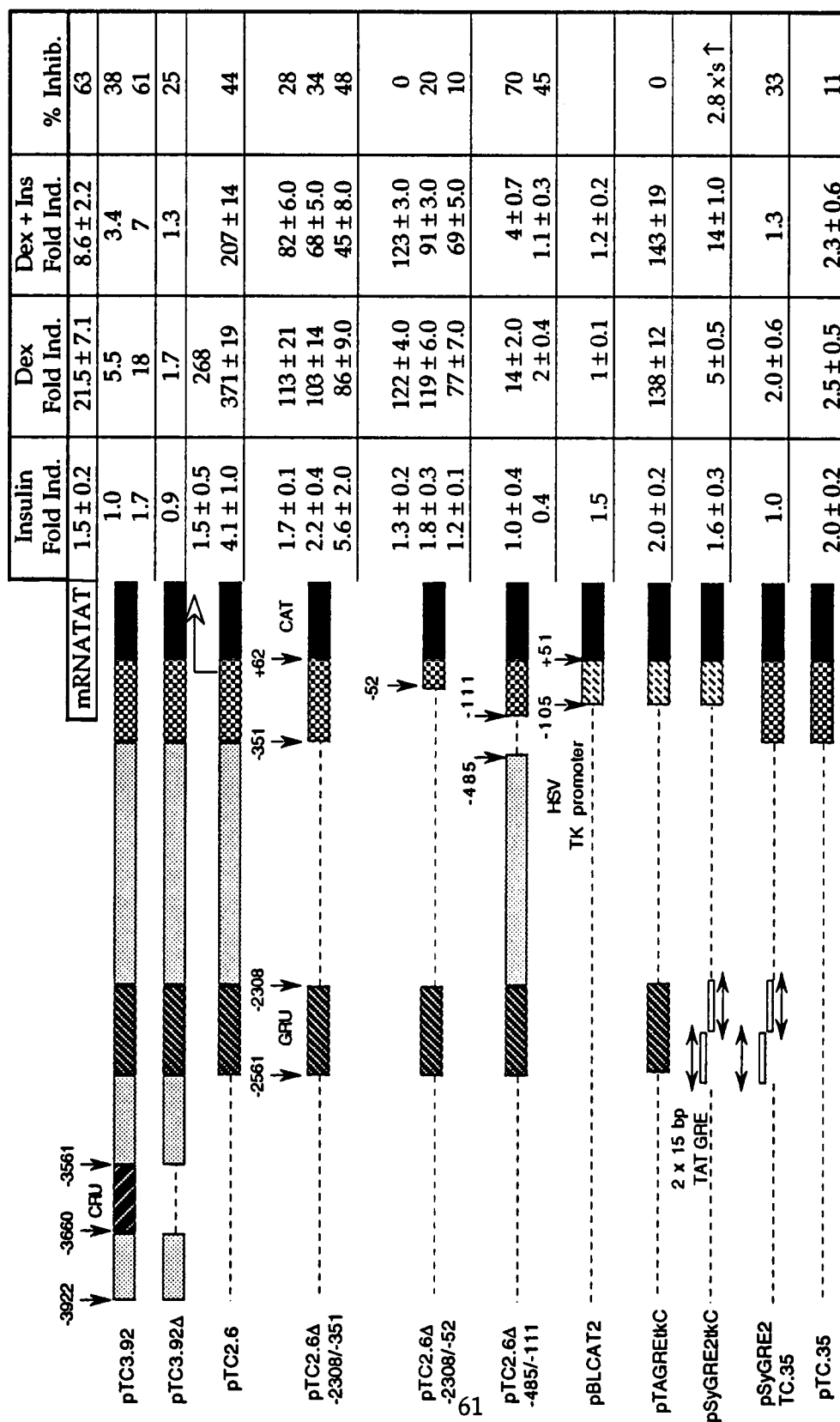


Figure III-6.

Figure III-7. Transient transfection analysis results summary.

Transfection experiments were conducted as described in Materials and Methods. Each row of results is the data from one experiment. Results for each treatment are reported as the average of three replicates/treatment plus or minus one standard deviation. Absolute CAT reporter activity is expressed as picomoles/minute/mg protein. Fold induction is reported as the average fold induction of three replicates/treatment plus or minus one standard deviation. Insulin inhibition of dexamethasone-mediated induction is expressed as % Inhibition: $[1 - (\text{Dex} + \text{Ins} / \text{Dex})]100$. $\uparrow$ designates a dexamethasone + insulin increase in fold induction rather than an inhibition for that particular TAT•TK construct.

Tfx No.	Construct	Control pM·min·mg	Insulin pM·min·mg	Fold Ind.	Dex pM·min·mg	Fold Ind.	Dex + Ins pM·min·mg	Fold Ind.	% Inhib.
T8 Hr	mRNA ^{TAT}	1.0		1.5 ± 0.2		21.0 ± 7.0		9.0 ± 2.0	63
22	pTC3.92	7.0 ± 2.0	7.0 ± 3.0	1.0	38 ± 5	5.5	24.0 ± 3	3.4	38
20			6.0	2.0	63	18	24.0	7	61
20	pTC3.92Δ	1.0	0.60	1.0	1.2	1.7	1.0	1.3	25
10	pTC2.6	0.7 ± 0.2	1.0 ± 0.3	1.5	184	268			
9		0.2	0.6 ± 0.2	4.0	56 ± 3	371	31.0 ± 2	207	44
13	pTC2.6Δ- 2308/-351	1.20 ± 0.0	2.0 ± 0.4	1.7	130 ± 25	113	95.0 ± 7.0	82	28
12		0.10 ± 0.0	0.3 ± 0.5	2.2	13 ± 1.8	103	9.0 ± 1.0	68	34
10		3.80 ± 1.4	9.0 ± 0.1	2.4	183	48			
7		0.03 ± 0.0	0.2 ± 0.1	5.6	2 ± 0.2	86	1.2 ± 0.2	45	48
12	pTC2.6Δ- 2308/-52	0.3 ± 0.0	0.3 ± 0.4	1.3	31 ± 1.0	122	31.0 ± 0.7	123	0
10		2.1	2.8 ± 0.5	1.3	185	86			
8		0.4 ± 0.0	0.7 ± 0.1	1.8	52 ± 3.0	119	40.0 ± 1.0	91	20
7		0.1 ± 0.0	0.2 ± 0.0	1.2	10 ± 1.0	77	9.0 ± 0.6	69	10
17	pTC2.6Δ- 485/-111	0.7 ± 0.4	0.8 ± 0.3	1.1	10 ± 1.0	14	3.0 ± 0.5	4	70
18		8.2	3.5 ± 2.5	0.4	17 ± 3.0	2	9.3 ± 2.2	1.1	45
16	pBLCAT2	1.3 ± 0.2	2.0 ± 1.0	1.5	1 ± 0.1	1	1.6 ± 0.2	1.2	
16	pTAGREtk C	1.3 ± 0.2	2.4 ± 0.2	1.9	180 ± 15	138	183.0 ± 24	143	0
15	pSyGRE2tk C	4.3 ± 0.8	8.0 ± 1.3	1.6	23 ± 2.3	5	58.0 ± 6	14	2.8 x's ↑
21	pSyGRE2T C.35	0.3 ± 0.8	0.3 ± 0.2	1.0	0.6 ± 0.2	1.8	0.4 ± 0.3	1.3	33
21	pTC.35	0.7 ± 0.2	1.5 ± 0.2	2.0	2 ± 0.4	2.5	1.7 ± 0.4	2.3	11

Figure III-7.

interface/minimal-basal promoter), pTC 2.6Δ -2308/-351, retains those sequences necessary for appropriate regulation by glucocorticoid or insulin.

Construct pTC 2.6Δ -2308/-351 deletes a constitutive liver-specific DNase I hypersensitive site (at -1 Kb) that is present in TAT-expressing cells only. Two distinct DNA-binding activities (-1083 to -1065 bp and -1048 to -1035 bp) have been demonstrated through *in vivo* footprinting. The functional role and identity of the binding activities, associated with the -1 Kb DNase I hypersensitive site, is presently unknown.

Significantly, a further deletion of sequences -351 to -52 bp, construct pTC 2.6Δ -2308/-52, results in the loss of insulin regulation while retaining an appropriate glucocorticoid response. This result indicates sequences necessary for appropriate insulin-responsiveness are contained in the region -351 to -52 bp.

To demonstrate the insulin-response specificity of TAT proximal promoter 5'-flanking sequences -351 to -52 bp, and eliminate potential *cis*-element positional-effect ambiguities, the intact -2.5 Kb TAT GRU (sequences -2561 to -2308 bp) was cloned into pBLCAT2 (construct pTAGRE tkC). pBLCAT2 contains the Herpes simplex virus thymidine kinase (HSV TK) promoter sequences -105 to +51 bp. See Figure III-8 for the HSV TK promoter sequence and reported DNA-binding activities. The presense of the HSV TK promoter in pBLCAT2 permits the analysis of the effects of putative regulatory elements on a heterologous eukaryotic promoter.

The control construct, pBLCAT2, is totally unresponsive to glucocorticoids while insulin treatment reproducibly results in a modest increase in CAT expression (~1.5-fold).

Figure III-8. The Herpes simplex virus thymidine kinase promoter DNA sequences -105 to -18 bp contained in pBLCAT2 and present in all TAT•TK constructs tested in transient transfection studies.

The boxed DNA sequences denote characterized DNA-binding *cis*-elements. SP 1 and CTF/NF-1 are the predominate DNA-binding proteins present in HeLa cells (Jones *et al.* 1985). The predominate DNA-binding protein present in hepatocytes that recognizes the SP 1 designated *cis*-element is TEF-2 (DeVack *et al.* 1993). The arrows above the SP 1 recognition sites denotes the orientation of each site relative to the other.

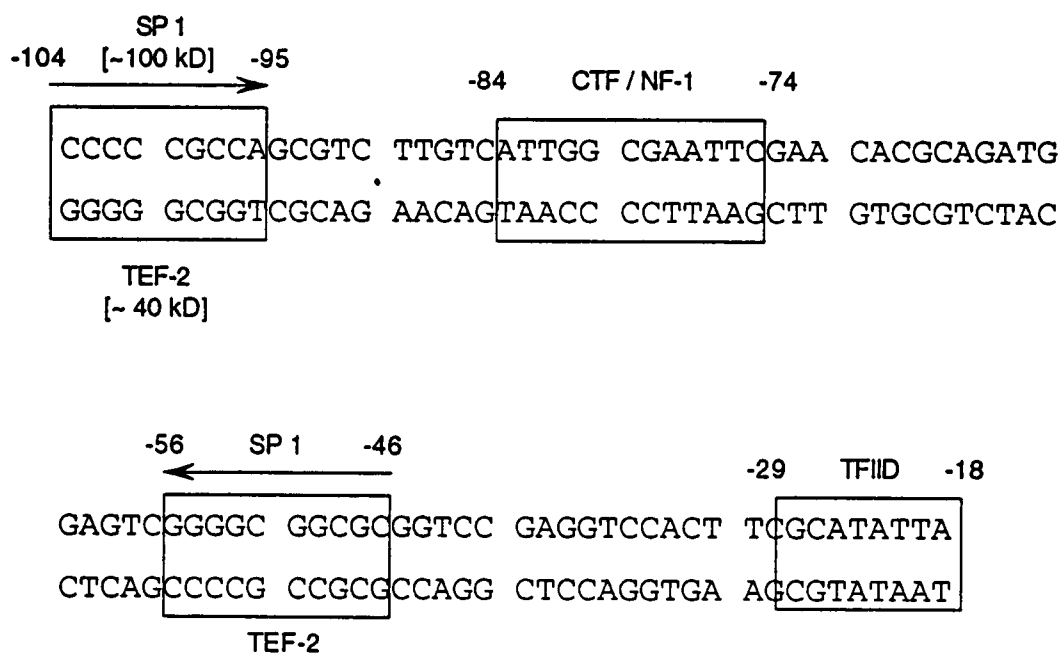


Figure III-8.

Construct pTAGRE tkC was tested for glucocorticoid and insulin responsiveness. Glucocorticoid treatment increases reporter expression substantially. Insulin does not inhibit the glucocorticoid-mediated induction in CAT expression. This observation allows the following two conclusions. First, the HSV TK promoter, with its associated DNA-binding activities, cannot substitute for TAT promoter sequences -351 to 52 bp in mediating an insulin inhibition of the glucocorticoid response. Second, sequences contained in the TAT GRU (sequences -2561 to -2308 bp) are, alone, insufficient for the insulin inhibition. Therefore, TAT promoter sequences -351 to 52 bp are an obligatory requirement for the insulin effect.

The above results do not exclude a requirement for the intact -2.5 Kb TAT GRU (sequences -2561 to -2308 bp) for insulin action. Therefore, test construct pSyGRE2TC.35 and control construct pSyGRE2tkC were examined. pSyGRE2TC.35 contains tandem 25-mer oligos of the 15-bp TAT GRE ligated to TAT promoter sequences -351 to -52 bp (see Figure III-1). The objective is to retain glucocorticoid-responsiveness while eliminating the neighboring DNA-binding activities present in the intact TAT GRU. This would then allow direct assessment of the requirement for TAT sequences -2561 to -2308 bp in insulin action. Construct pSyGRE2tkC contains tandem 25-mer oligos of the 15-bp TAT GRE ligated to the heterologous HSV TK promoter.

Dexamethasone increases control construct (pSyGRE2tkC) expression 5-fold while simultaneous dexamethasone and insulin exposure increases expression 14-fold (a 180% increase over dexamethasone alone). Test construct (pSyGRE2TC.35) expression increased 1.8-fold after dexamethasone treatment while insulin was found to inhibit glucocorticoid-mediated induction to 1.3-fold (a 33% inhibition). The above data support the following conclusion. TAT

sequences -2561 to -2308 bp are not a mandatory requirement to observe the insulin response. Therefore, an insulin response element(s) is contained in TAT promoter sequences -351 to -52 bp.

Construct pTC2.6 Δ -485/-111 has an internal deletion of TAT promoter sequences -485 to -111 bp. Paradoxically, insulin induction of basal reporter expression is completely abolished while insulin inhibition of glucocorticoid-mediated induction is retained (see Figure III-7). This data suggests the following conclusions. First, insulin's stimulatory effect on basal expression and insulin's inhibitory effect on glucocorticoid-mediated induction are mediated through different regions of TAT 5'-flanking sequence -351 to -52 bp. Insulin's stimulatory effect on basal expression requires TAT promoter sequences -351 to -111 bp. Insulin's inhibitory effect on glucocorticoid-mediated induction requires TAT promoter sequences -110 to -52 bp.

Construct pTC.35 (TAT 5'-flanking sequences -351 to +62 bp) expression is increased 2-fold by insulin (sd=0.2). Interestingly, this construct is responsive to glucocorticoid treatment (2.5-fold induction, sd=0.5). These results appear to support the conclusion that TAT 5'-flanking sequences -351 to -52 bp contain sequences functionally important for glucocorticoid regulation of TAT gene expression.

Functional Role of the -3.6 Kb cAMP Response Unit in Fao and KRC-7 cells.

Basal expression of construct pTC2.6 (upstream 5'-boundary at -2561 bp) in Fao or KRC-7 cells is extremely low (~ 0.6 pm $\cdot$ min $\cdot$ mg) (Cheatham *et al.*, 1992). Transfection of construct pTC3.92 into the KRC-7 clone increases basal expression 17500-fold over that attained with pTC2.6 (10,500 vs. 0.6 pm $\cdot$ min $\cdot$ mg). Transfection of construct pTC3.92 into the Fao clone increases

basal expression 10-fold over that attained with pTC2.6 (6.0 vs. 0.6 pm·min·mg). These results suggest that the Fao clone is deficient in either the BI or BIII binding activities or both. In addition, when compared to the TAT-expressing FTO-2B rat hepatoma (see Introduction), the -3.6 Kb enhancer is extremely active in the KRC-7 clone (50-fold increase vs. 17500-fold increase respectively).

To address the possibility that the absence of the -3.6 Kb enhancer activity in the Fao clone was due to a non-functioning BI element, pTC3.92 was transfected into Fao cells and evaluated for cAMP inducibility. In addition, cAMP inducibility of the endogenous gene was examined through Northern analysis (see Table III-1). Cells were treated with 8Br-cAMP at T0Hr and again at T4Hour and harvested at T8Hour. CAT reporter expression increased 2.1-fold (sd=0.5). In good agreement, the endogenous mRNA^{TAT} increased 3.2-fold (sd=0.7). These results indicate that the TAT CRE (BI) is functional in the Fao clone. Furthermore, *in vivo* cAMP induction in Fao cells closely paralleled that which has been documented in the KRC-7 clone (Moore and Koontz, 1989). cAMP acutely and transiently increases mRNA^{TAT} to a comparable extent in both the Fao and KRC-7 clones.

The above considerations suggest that the BIII binding activity might be, at least in part, responsible for the decreased enhancer activity observed in Fao cells.

Table III-1. Parallel study relating the effect of 8Br-cAMP treatment on pTC3.92 expression and endogenous mRNA^{TAT} in Fao Cells.

Treatment	Fold Increase	
	mRNA ^{TAT}	CAT reporter
8Br-cAMP	3.2 ± 0.7	2.1 ± 0.5

Fao cells were treated with 0.5 mM 8Br-cAMP at time zero. 8Br-cAMP was replenished at 4 hour. Total RNA was harvested 8 hours after treatment. CAT reporter expression and TAT mRNA levels were determined as described under Materials and Methods and were expressed as the fold change vs the vehicle control for each experiment. Each value represents the mean of three separate experiments.

Discussion

The objective of the experiments described here was to identify *cis*-acting elements in the TAT promoter which functionally contribute to the insulin response in Fao cells. This was undertaken through a series of 5'-, 3'-, and internal deletion TAT•CAT chimeric constructs transiently transfected into Fao cells.

The results suggest the following conclusions. First, insulin's stimulatory effect on basal expression and insulin's inhibitory effect on glucocorticoid-mediated induction are mediated through different regions of TAT 5'-flanking sequence -351 to -52 bp. The loss of insulin induction with construct pTC2.6Δ-485/-111 suggests that insulin's stimulatory effect on basal expression requires TAT promoter sequences -351 to -111 bp. Construct pTC2.6Δ-485/-111 retains insulin's inhibitory effect on glucocorticoid-mediated induction while pTC2.6Δ-2308/-52 does not. The results suggest insulin's inhibitory effect on glucocorticoid-mediated induction requires TAT promoter sequences -110 to -52 bp.

Construct pTC.35 (TAT 5'-flanking sequences -351 to +62 bp) expression is increased 2-fold by insulin (sd=0.2). Interestingly, this construct is responsive to glucocorticoid treatment (2.5-fold induction, sd=0.5) while insulin appears to modestly antagonize the glucocorticoid-mediated induction (an 11% inhibition). These results support the conclusion that TAT 5'-flanking sequences -351 to -52 bp are insulin-responsive.

Do the sequences -351 to -52 bp contain two discrete and separable insulin-response elements one of which mediates insulin's stimulatory effect on basal expression, and the other, insulin's inhibitory effect on glucocorticoid-mediated induction? The following evidence may support this interpretation.

Since there is no GRE present in construct pTC.35, the only mechanism by which glucocorticoids can mediate induction would be through recruitment of a pre-existing DNA-binding activity present in very low amounts to a site contained in TAT 5'-flanking sequences -351 to +62 bp which then acts to increase expression. Increased DNA-binding could be attributed to a glucocorticoid-mediated increased expression of a putative accessory factor which in turn leads to its increased abundance which is then available for functional promoter interactions. Therefore, a corollary of this postulate may include a requirement for ongoing protein synthesis for glucocorticoid-mediated induction of TAT gene expression.

In support of this postulate, two reports demonstrate a requirement for ongoing protein synthesis for glucocorticoid induction of TAT. In the Fao rat hepatoma, a 1 hour preincubation with 3 μ M cycloheximide (CHX) abolishes completely the increased TAT mRNA levels observed after a 6 hour treatment with 0.1 μ M dexamethasone (Barouki *et al.*, 1989). In a second report, J. Messina's laboratory has shown that simultaneous addition of 10 nM dexamethasone with 50 μ g/ml CHX to serum-deprived H4 cells completely antagonizes dexamethasone-mediated transcription of TAT (Messina *et al.*, 1992).

The above reports prompted an investigation of literature regarding the characterization of glucocorticoid-mediated induction of TAT *in vivo*. Both glucocorticoids and cAMP increase TAT gene expression in the adult rat liver. A maximum 5-fold increase in mRNA^{TAT} is attained 45 minutes after BT2-cAMP injection while a maximum 6-fold increase in mRNA^{TAT} is attained 2 hours after dexamethasone injection (Hashimoto *et al.*, 1984). Maximum dexamethasone induction required an additional 75 minutes. In primary

hepatocytes, the increased TAT expression has been attributed to a transcriptional activation. Interestingly, and in agreement with the *in vivo* studies, the induction kinetics of the two agonists differ. CPT-cAMP rapidly enhanced TAT gene transcription 13-fold within 60 minutes whereas dexamethasone progressively increased transcription to 8-fold after 120 minutes (Schmid *et al.*, 1987). It was concluded that cAMP induction does not require *de novo* protein synthesis. No similar statement was made for glucocorticoid induction.

Taken together, the CHX studies and the *in vivo* glucocorticoid induction kinetics support a mechanism which may include the recruitment of a DNA-binding activity which may serve to augment the glucocorticoid response.

The TAT CRE (BI) has been shown to be functional in the Fao clone while the liver-specific enhancer activity, also associated with the -3.6 Kb unit, is not. The data suggests that the BIII binding activity might be, at least in part, responsible for the decreased enhancer activity in Fao cells when compared to that observed in KRC-7 cells. Indeed, the BIII binding activity might be viewed as a potential insulin-responsive target mechanistically involved in insulin-mediated inhibition of both basal and cAMP-induced TAT gene expression in the KRC-7 clone.

Although no published empirical evidence demonstrating the identity of the BIII-binding activity currently exists, two recently published reports from Schutz's laboratory state that the BIII element DNA-binding activity is HNF-4 (citing a personal communication within the Schutz laboratory) (Ruppert *et al.*, 1992). The BIII DNA sequence possesses 89% sequence identity (8/9) to the consensus HNF-4 binding site. Interestingly, BIII possesses 100% sequence identity (7/7) to the HMG family core recognition motif. As stated earlier, the

HMG class of transcription factors regulate expression of genes that initiate and maintain the differentiated phenotype (Alexander-Bridges, 1992).

Chapter IV

IN VITRO STUDIES

GEL-SHIFT AND DNASE I FOOTPRINT ANALYSES

Introduction

Transfection results have identified an insulin response element(s) in TAT 5'-flanking sequences -351 to -52 bp. The prevailing second-messenger signalling cascade paradigm includes hormone signal transduction to the nucleus which leads to rapid changes in targeted gene expression. Typically, there is no requirement for *de novo* protein synthesis. This implies that changes in targeted gene expression are the result of covalent modification of pre-existing *cis*-element DNA-binding activities which in turn alters their functional interaction with DNA recognition sites and/or other factors required for the formation of a committed transcriptional initiation complex.

Therefore, we next initiated a series of *in vitro* DNase I footprint analyses of TAT 5'-flanking sequences -351 to -52 bp in an effort to detect insulin-dependent alterations in footprint protection patterns. We sought to correlate insulin-responsive regions of TAT 5'-flanking sequence defined functionally through transient transfection to insulin-dependent alteration in DNA-binding activity in coinciding promoter sequences.

The -351 to +1 bp enhancer-interface/minimal-basal unit.

Functional characterization of the TAT proximal promoter has included DNase I Hypersensitive site mapping (Becker *et al.*, 1987). Two strong Dnase I HS sites occur within the first 200 nucleotides (nts) of the TAT cap site in adult

liver and TAT-expressing cells only. Further characterization has included *in vivo* footprinting of the region from -350 to +1 bp (Becker *et al.*, 1987). Two regions containing CCAAT-box homologies (putative HNF-3 binding sites), centered at -75 bp and -40 bp (refer to figure IV-1), were strongly protected in the TAT-expressing FTO-2B hepatoma, but not the TAT non-expressing H5 hepatoma or the rat fibroblast, XC. Two additional regions, one centered at -212 bp (containing putative HNF-3 and HNF-4 DNA-recognition motifs) and -162 bp (putative liver-enriched NF-1), also demonstrated protection from methylation *in vivo*.

Importantly, and central to the interpretation of my investigations, no indications of further *in vivo* protein-DNA interactions were detected in an analysis of the neighboring DNA From -230 to -350 bp (data not shown). Furthermore, within the text of the report, the authors discuss *in vitro* DNase I footprinting of the region -351 to +81 bp which had revealed an array of protected regions that suggest a close spacing of several protein-binding sites in all three cell lines examined. Interestingly, data presented in the accompanying figure did not include results from the region -351 to -230 bp (Becker *et al.*, 1987).

Recent *in vitro* transcription studies , employing non-reconstituted (implying the absence of nucleosomes) DNA templates (therefore, in a constitutive-binding or de-repressed state), have demonstrated that the TAT promoter region (-350 to +1 bp) can, alone, drive liver-specific TAT gene expression (crude liver nuclear protein extract vs. spleen) (Schweizer-Groyer *et al.*, 1992). Two DNA-binding activities, recognizing TAT 5'-flanking sequence -291 to -266 bp, were shown to contribute up to 90% of the total expression obtained when using the -351 to +1 bp TAT promoter template. To reconcile

Figure IV-1. TAT 5'-flanking sequence -310 to -11 bp.

Boxed DNA sequences that are labeled in bold type denote identified/characterized DNA-binding *cis*-element identities. Taken from Schweizer-Groyer *et al.* (1992) and Boshart *et al.* (1987). Boxed DNA sequences that are labeled in normal type denote DNA sequence similarity to a consensus DNA recognition site. Black squares denote bases that are protected from DMS methylation *in vivo* (Boshart *et al.* 1987). Open triangles denote hypersensitive bases when examined through *in vivo* footprinting. Oligo sequence designations are from Schweizer-Groyer *et al.* (1992). The percentages indicate the relative contribution of the DNA-binding activities associated with a particular oligo sequence to -350 to +1 bp TAT template-driven basal transcription as evaluated through *in vitro* transcription competition experiments (Schweizer-Groyer *et al.* 1992).

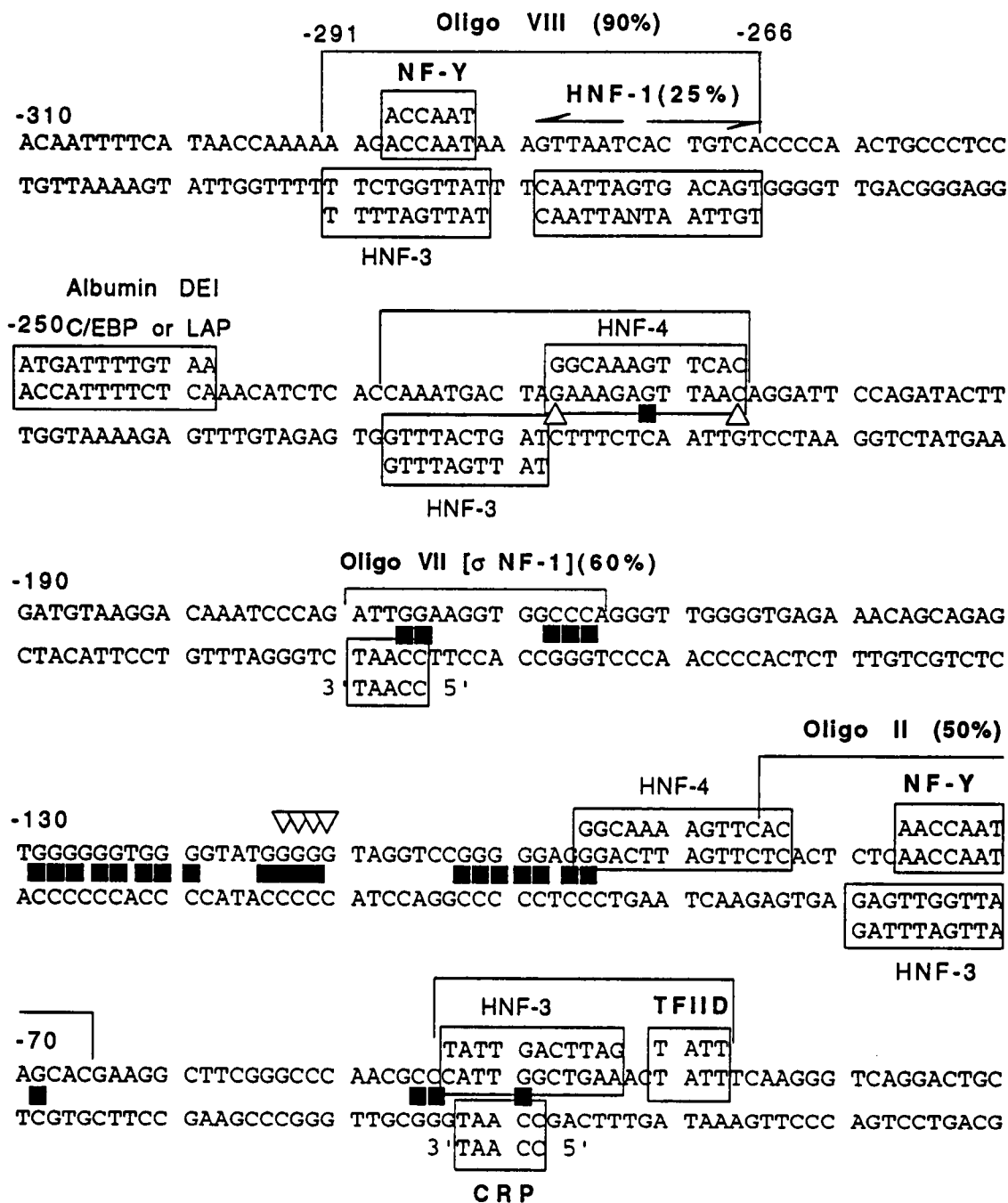


Figure IV-1.

this observation with *in vivo* footprinting results, it might be suggested that the *in vivo* TAT proximal-promoter chromatin architecture precludes *cis*-element binding of a liver-enriched enhancer(s) in the region spanning nts -350 to -230 which might contribute to basal TAT gene expression.

In an effort to identify those DNA-binding activities contributing to liver-specific TAT gene expression, and also, define their functional role, a series of 20-bp ds-oligos spanning the TAT 5'-flanking sequence from -300 to -30 bp were synthesized. Labeled ds oligo probes were competed with well-defined consensus recognition sites (and mutated, non-binding versions) for several ubiquitous and liver-enriched DNA-binding factors in gel-shift studies. A liver-enriched NF1 site derived from the Albumin promoter (DEII) competed strongly for binding with TAT 5'-flanking sequence -176 to -155 bp (oligo VII), a region previously shown to be protected from methylation in *in vivo* footprinting (Schweizer-Groyer *et al.*, 1992).

Of particular interest, a region of the TAT 5'-flanking sequence -290 to -265 bp (oligo VIII), was shown to possess two distinct DNA-binding activities in *in vitro* gel-shift studies (using adult rat liver nuclear protein extract) while the *in vivo* footprint studies (discussed above) indicated no DNA-binding activities in the region -230 to -351 bp. An HNF-1 site derived from the albumin promoter (PE 56) competed strongly with one of the two specific complexes (Schweizer-Groyer *et al.*, 1992).

Upon inspection of the DNA sequence in the region -230 to -351 bp, one can identify a motif with 79% sequence identity (11/14) to the HNF-1 consensus recognition site (Meidel and Crabtree, 1991). In addition, immediately preceding the degenerate HNF-1 site is a region (containing a core CCAAT motif) with sequence identity to the HNF-3 recognition site (HNF-

3•HNF-1). A putative HNF-3 binding activity could account for the second specific complex observed in gel-shifts. The significance of this finding will be discussed together with my footprinting results.

In addition, the -350 to +1 bp TAT promoter template was competed with oligo VII (-176 to -155 bp, the putative NF-1 site) and oligo VIII (-290 to -265 bp, the putative HNF-3•HNF-1 sites) in *in vitro* transcription assays in an effort to determine the functional contribution of each DNA-binding activity to 'basal' TAT gene expression. A 50-fold molar excess of oligo VII (-176 to -155 bp, the putative NF-1 site) reduced transcription ~60%.

Oligo II (-85 to -65 bp) spans a region that is protected *in vivo* and *in vitro*, and contains a core CCAAT motif. Schweizer-Groyer *et al.* (1992) attribute this observed DNA-binding activity to NF-Y, a ubiquitous CCAAT-specific transcription factor with sequence similarity to the yeast factor HAP2/3 (data not shown) (Li *et al.*, 1992). Competition with this oligo reduced transcription ~50%. 90-fold molar excess of PE56 (a well characterized HNF-1 site present in the albumin promoter) reduced transcription ~25% (Cereghini *et al.*, 1988).

A 90-fold molar excess of oligo VIII (-290 to -265 bp, the putative HNF-3•HNF-1 sites) reduced transcription >90%. This result could be interpreted as follows. First, there exists a functional interaction between HNF-1 and the CCAAT-motif DNA-binding activity (note how this possibility is reminiscent of the TAT GRU region GRE-II, which contains a CCAAT-motif DNA-binding binding activity immediately adjacent to a degenerate GRE, HNF-3•GRE). Alternatively, oligo VIII (-290 to -265 bp, the putative HNF-3•HNF-1 sites) sequesters DNA-binding activitie(s) relevant elsewhere for TAT promoter functions. Support for the second interpretation is that oligo VIII competed

strongly with oligo II (-85 to -65 bp, a single DNA-binding site that contains a core CCAAT motif) in gel-shift studies.

Materials and Methods

Nuclear Extract Preparation.

Nuclear extracts were prepared as described by Lee *et al.* (1988). All solutions were made fresh and amended with 1x protease inhibitors (1.0 mM PMSF, 0.7 ug/ml Pepstatin A, 0.5 ug/ml leupeptin, and 0.6 ug/ml α_2 Macroglobulin). All solutions were kept on ice and all procedures were performed at 4°C. After hormone treatment, Fao or KRC-7 cells were washed two times with ice-cold PBS, lysed in Solution 2 [1x Homogenization Buffer (HB) (10mM Hepes, pH7.5, 0.5 mM spermidine tri-HCl, 0.15 mM spermine tetra-HCl, 5.0 mM EDTA (diNa salt), 0.25 mM EGTA, 50 mM NaF, 7.0 mM 2-mercaptoethanol) containing 0.5 M sucrose and 0.05% NP-40]. The nuclei were sedimented by centrifugation at 12000 g for 15 minutes at 4°C. After washing with Solution 3 (1x HB plus 0.35 M sucrose) nuclei were suspended in Solution 4 (1x HB, 10% glycerol, 50 mM NaCl), gently mixed on a rocker platform in the cold room, and then centrifuged 20 minutes/4°C/12000 g. The nuclei were then extracted with Solution 5 (1x HB, 16% Glycerol, 300mM NaCl) by gentle agitation on a rocker platform in the cold room for 1 hour, followed by centrifugation for 30 minutes at 2000 g. The supernatant was brought to 45% ammonium sulfate and mixed on a rocker platform for 30 minutes. Nuclear proteins were collected by centrifugation at 17000 g for 30 minutes, resuspended in Solution 6 (1x HB, 10% Glycerol, 50 mM NaCl, and 2 mM MgCl₂) and dialyzed against solution 6 for no less than two hours and no more than four hours. Dialyzed extract was then pooled for homogeneity, aliquoted

(10 μ l/tube), quick frozen on dry ice, and stored at -80°C . Protein concentration was determined by the Bradford assay. Aliquots were thawed on ice for experimental use. Any unused thawed aliquots were discarded.

Probe Preparation.

Labelling of the non-coding strand of TAT sequences -351 to -2 bp or -208 to -2 bp was done by double-digestion of plasmid pTAT KS.35 or pTAT HS206 with restriction enzymes Sac I and Hind III followed by filling in at the 3'-recessed end of the Hind III site using the Klenow fragment of DNA polymerase with [$\alpha^{32}\text{P}$]-dCTP. The coding strand was prepared by first digesting with Hind III (12u) followed by calf intestinal alkaline phosphatase (CIAP). Linearized plasmid (12.2 pmol 5' ends) was then extracted with phenol/chloroform and redigested with CIAP in an optimal buffer supplemented with 1.0 mM spermidine, 0.1 mM ZnCl_2 , 1.0 mM MgCl_2 , 50 mM TRIS, pH 8.0 to ensure complete 5' phosphate removal. After phenol/chloroform extraction the Hind III generated 5'-overhang was labeled using T4 polynucleotide kinase (T4 PNK) in the presence of [$\gamma^{32}\text{P}$]ATP (~200 uCi or > 50 pM). After phenol/chloroform extraction the linearized 5'- ^{32}P -labeled plasmid was digested with Sac I (20u). The desired Hind III/Sac I fragment was separated from endlabeled cloning vector by electrophoresis in a 2% agarose gel. The probe was then recovered by electroelution, precipitation, resuspended in TE, counted, and stored at -20°C for future use. Specific activity calculations were performed as indicated by Gibco BRL Focus® technical bulletin 8004-1.

Gel mobility shift assay.

Gel shifts were performed as described by Fried and Crothers (1981). The binding reactions each contained 10,000 cpm of end-labeled double-stranded DNA probe, 1.25 µg poly[d(I-C)], 100 ng pUC-18 DNA and variable amounts of nuclear extract in 1x HB containing 0.05% NP-40, 0.35 M sucrose, 50 mM NaCl, 2mM MgCl₂ and 1 mM PMSF in a final volume of 20 µl.

Alternatively, binding reaction components and parameters were exactly as that used for DNase I footprint reactions (see below). The reaction mixture was incubated at 30°C for 30 minutes, and then loaded directly onto a 5% polyacrylamide (acrylamide:bisacrylamide, 49:0.6) gel in a buffer of 50 mM Tris-borate (pH 8.0), 1 mM EDTA. After prerunning the gel for no longer than one hour, samples were run at 200 V for 2-3 hours at room temperature. The wet gels were visualized by autoradiography after ~16 hour exposure.

In vitro Dnase I Footprinting.

DNase I footprints were performed as described by Zhang *et al* (1990) with modifications. Nuclear extract protein was incubated with 1.25 µg poly [d(I-C)], 500 ng pGEM-3Z, and 6.5 femtomoles (~30,000 cpm) of DNA probe at 30°C for 30 minutes in a 100 µl reaction mixture containing 25 mM HEPES (pH ~6.0), 50 mM NaCl, 1 mM dithiothreitol, 10 %glycerol, and 1 mM EDTA. After the incubation period, MgCl₂ was added to 5 mM and the samples were digested with 20 to 200 ng DNase I for 1 minute at room temperature. The digestion was terminated with the addition of 20 µg glycogen carrier, 1/10 volume 3 M sodium acetate, 2.5 volumes ethanol, vortexed, and immediately placed on dry ice for >30 minutes. After a 20 minute 12000g centrifugation at 4°C, the pellets were washed two times with 70 % ethanol, dried, and

resuspended in 4 µl of loading buffer (80% formamide, 5 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). The digestion fragments were denatured at 90°C for no greater than 2 minutes, vortexed, microfuged, and immediately loaded onto a 6 % polyacrylamide/1:2 w/v urea (~7 M) sequencing gel which had been prerun at 90 watts for 1 hour, electrophoresed for 4-6 hours at 70 watts (1700 volt/45 mA by end of run). The gels were then immediately wrapped with saran wrap and placed in cassettes with an intensifying screen at -80°C for a 2.5 to 3.5 day exposure and visualized through autoradiography. G and G/A sequencing ladders were performed according to the method of Maxam and Gilbert (1980).

Results

Initially, gel shift studies were performed in order to evaluate the binding integrity of the nuclear-extract protein employing binding-parameters to be used in DNase I footprint analyses. In addition, we sought to identify potential hormone-dependent alterations in mobility and/or band(s) intensity. Figure IV-2 is a gel shift with TAT 5'-flanking sequences -208 to -2 bp. Although no hormone-dependent alterations were discerned, the determination of appropriate conditions for DNase I footprint analyses had been accomplished.

Figure IV-3 is a footprint with pTATKS.35 (-351 to -2 bp) non-coding (template) strand. This footprint demonstrates a direct correlation between nuclear-extract protein concentration and protected regions which are flanked by DNase I hypersensitive nicks. The results indicate the presence of multiple protected regions suggesting a close-spacing of several DNA-binding activities.

Figure IV-2. Representative gel shift of TAT 5'-flanking sequence -208 to -2 bp (pTAT HS206).

Fao or KRC-7 cells were treated with either 1.0 μ M dexamethasone or 10 nM insulin alone or together as indicated. Nuclear protein extraction was initiated after cells had been exposed to hormone for 1 hour. Nuclear extract preparation and gelshift procedure were as indicated in Materials and Methods. Each lane contains 1.7 ng probe (specific activity of 6×10^6 cpm/ μ g) and 10 μ g nuclear extract protein. The gel was run for 3 hours at 250 volts and had been exposed for 18 hours.

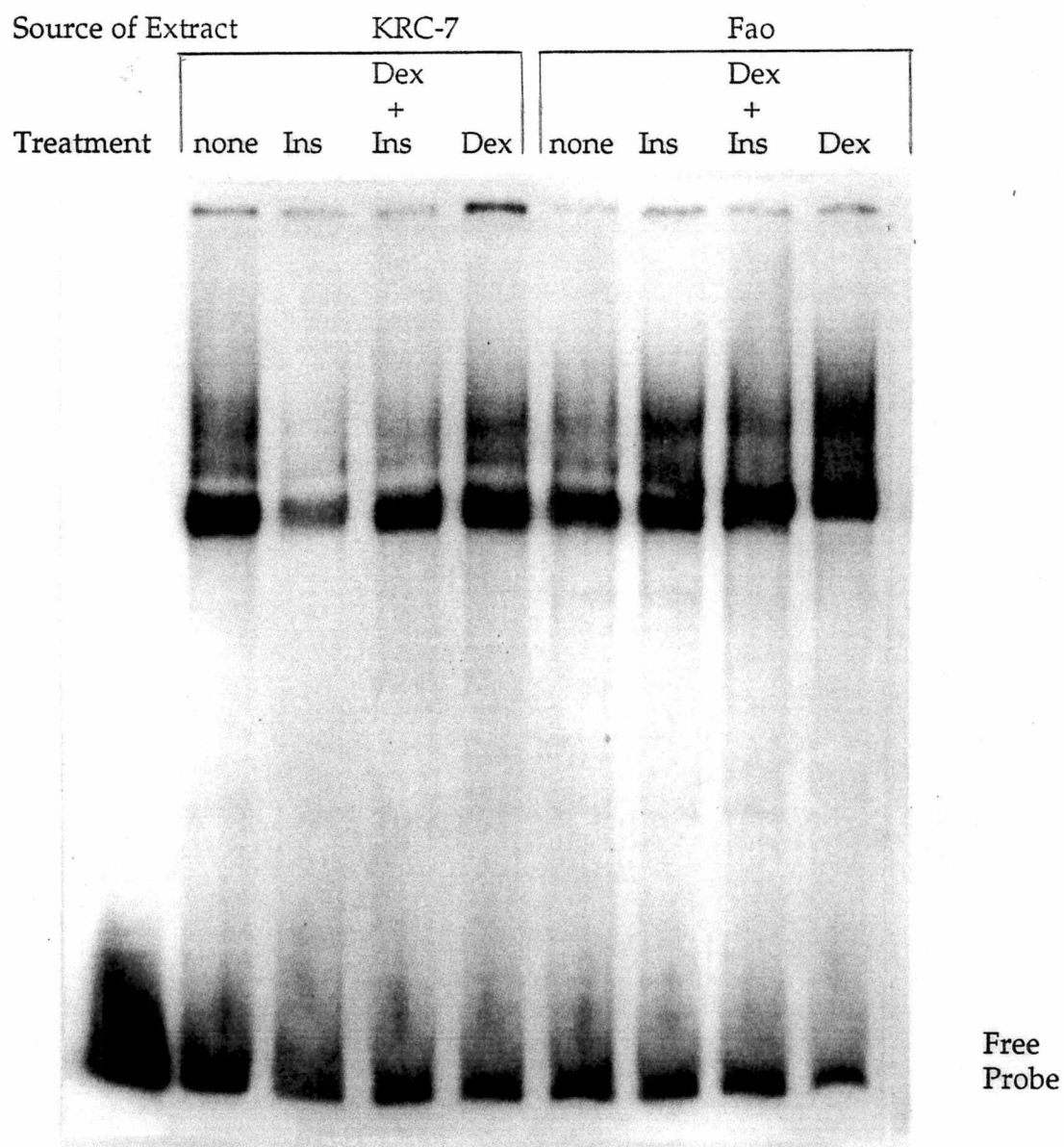


Figure IV-2.

Figure IV-3. Representative *in vitro* DNase I footprint of TAT promoter non-coding strand sequence -351 to -2 bp.

Labelled probe was incubated with untreated nuclear extract protein derived from Fao cells as indicated in the Material and Methods section. The protein-bound probe was then partially digested with increasing amounts of DNase I as indicated and resolved on a 6% acrylamide gel after a 4 day exposure. 1 unit (U) DNase I equates to 200 ng (stock concentration was 200 ng/ μ l). G and G/A lanes are chemical cleavage sequence markers for the probe (Maxam and Gilbert, 1980). Filled triangles indicate DNase I hypersensitive sites which are aligned to the appropriate image on the gel as indicated.

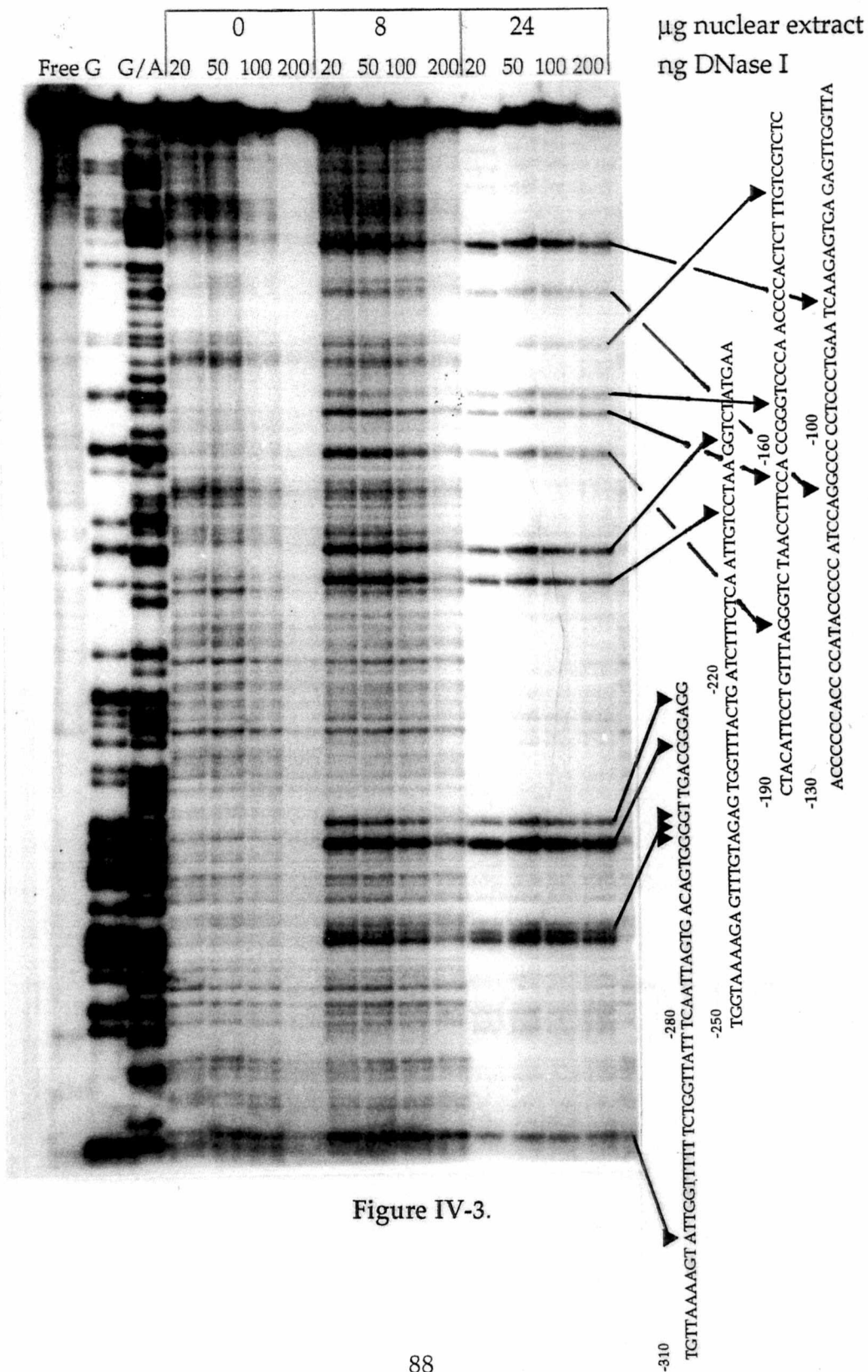


Figure IV-3.

Figure IV-4 is a footprint analysis comparing protection/hypersensitive site patterns when using untreated-control vs. 10^{-8} M insulin-treated nuclear extract protein. Insulin treatment was for 1 hour since earlier studies had shown that this time point is when maximal insulin-mediated transcriptional effects are apparent on the endogenous gene (Chapter II - Figure II-2). No insulin-dependent alteration in protection/hypersensitive site pattern(s) could be discerned in any region of TAT 5'-flanking sequences -351 to -2 bp in analyses with either coding or non-coding template strands (Figure IV-9 and Figure IV-3 respectively).

A summary of *in vitro* DNase I footprint analyses can be viewed in Figure IV-5. Contrary to a report from G. Schutz's laboratory (Becker *et al.*, 1987), but in agreement with that reported by Pictet's group (Schweizer-Groyer *et al.*, 1992), the results suggest a potential role for DNA-binding activities associated with TAT 5'-flanking sequences -300 to -220 bp.

Figure IV-6 is a footprint analysis of pTATKS.35 coding strand using nuclear-extract protein derived from the Fao rat hepatoma or pig brain. The distinct differences in Fao rat hepatoma vs. pig brain footprint protection/hypersensitive-site patterns suggests that DNA-binding activities in this region *in vivo* can be attributed primarily to liver-enriched DNA-binding factors.

In an effort to further characterize TAT proximal-promoter DNA-binding activities, footprint analyses were performed with nuclear extract protein which had been heated at 85°C for 10 minutes (Figure IV-7). A similar experiment was done by Pictet's group in an effort to provide corroborative evidence that the liver-enriched DNA-binding factor, C/EBP, binds to TAT GRU sequences (Grange *et al.*, 1991). Previous studies by Trus *et al.* (1990) had

Figure IV-4. *In vitro* DNase I footprint of TAT promoter non-coding strand sequence -351 to -2 bp.

Labelled probe was incubated with increasing amounts of untreated or 10^{-8} M insulin treated nuclear extract protein derived from Fao cells as indicated in the Material and Methods section. Cells were exposed to vehicle control or hormone for 1 hour. The protein-bound probe was then partially digested with 1 unit (200 ng) DNase I as indicated and resolved on a 6% acrylamide gel after a 4 day exposure. G and G/A lanes are chemical cleavage sequence markers for the probe (Maxam and Gilbert, 1980).

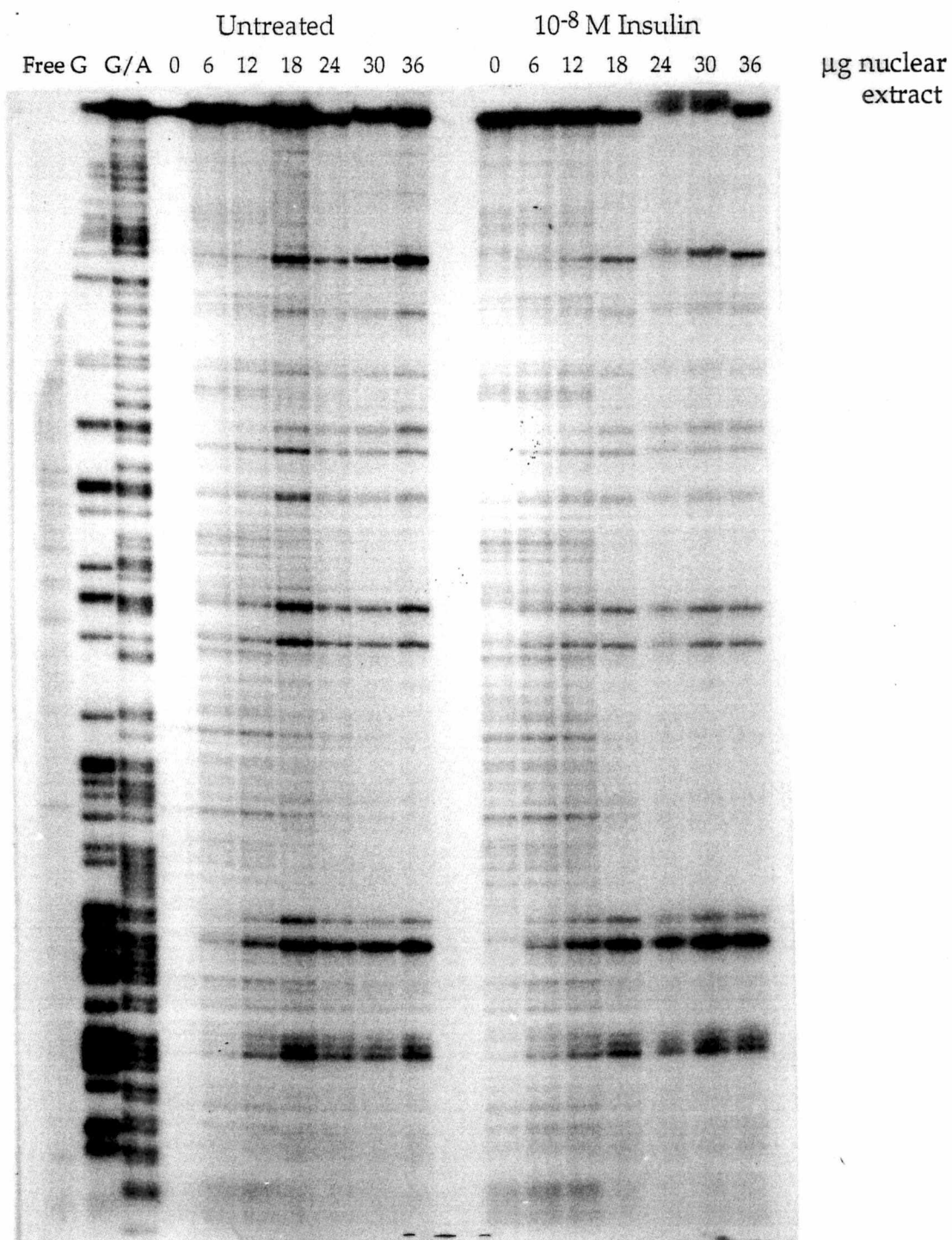


Figure IV-4.

Figure IV-5. *In vitro* DNase I footprint results summary of TAT 5'-flanking sequence -300 to -30 bp.

The summary is taken from results obtained in experiments using nuclear protein extract derived from Fao rat hepatoma cells. Shaded bars, above the coding (upper) strand or beneath the template (lower) strand, indicate TAT 5'-flanking sequences that are strongly protected from DNase I digestion *in vitro*. Filled triangles indicate DNase I hypersensitive sites. Boxed DNA sequences that are labeled in bold type denote identified/characterized DNA-binding *cis*-element identities. Taken from Schweizer-Groyer *et al.* (1992). Boxed DNA sequences that are labeled in normal type denote DNA sequence similarity to a consensus DNA recognition site.

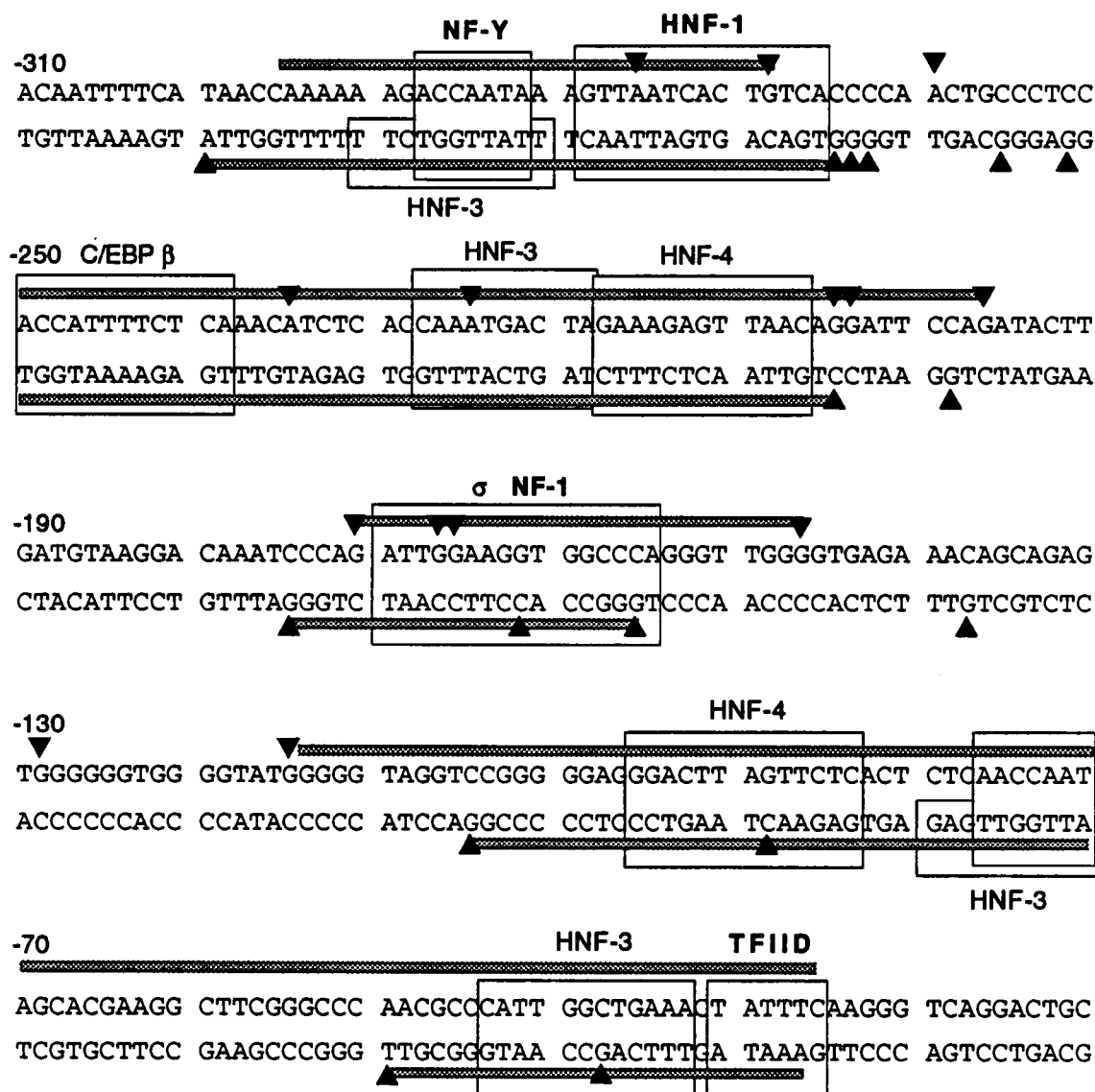


Figure IV-5.

Figure IV-6. *In vitro* DNase I footprint of TAT coding strand sequence -290 to -40 bp with untreated Fao rat hepatoma vs. pig brain nuclear extract protein.

The coding strand probe was incubated with increasing concentrations of untreated Fao rat hepatoma or pig brain nuclear extract protein as indicated. Vertical double lines indicate sequences protected from DNase I digestion. Encircled asterisks denote DNase I hypersensitive sites. G and G/A lanes are chemical cleavage sequence markers for the probe (Maxam and Gilbert, 1980).

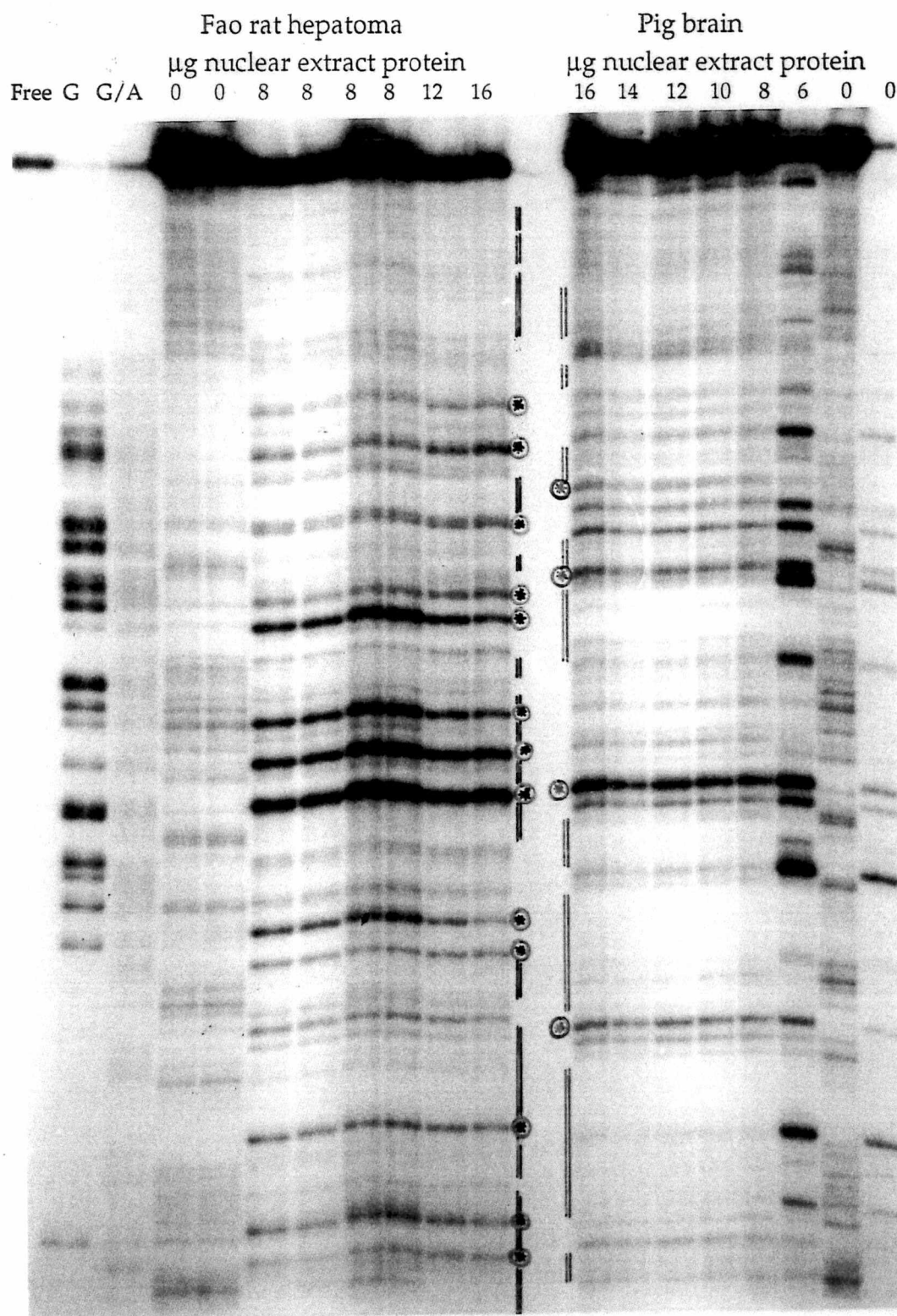


Figure IV-6.

Figure IV-7. *In vitro* DNase I footprint of TAT coding strand sequence -351 to -2 bp with Fao-derived untreated vs. Fao-derived heat-treated (10 minutes/85°C) vs. pig brain-derived nuclear extract protein.

Coding strand probe was incubated with increasing concentrations of nuclear extract protein derived from the described sources as indicated. Rationale for an experiment using heat-treated nuclear extract protein in the binding reaction is described in Grange *et al.* (1991) and the accompanying text. Vertical lines indicate sequences protected from DNase I digestion. Encircled points (bulls eye) denote DNase I hypersensitive sites. G and G/A lanes are chemical cleavage sequence markers for the probe (Maxam and Gilbert, 1980).

	Untreated Control								10'/85°C								Pig Brain													
			μg nuclear protein										μg nuclear protein										μg nuclear protein							
Free G	G/A	0	0	6	12	18	24	0	0	6	12	18	24	0	0	6	12	18	24	G/A	G									

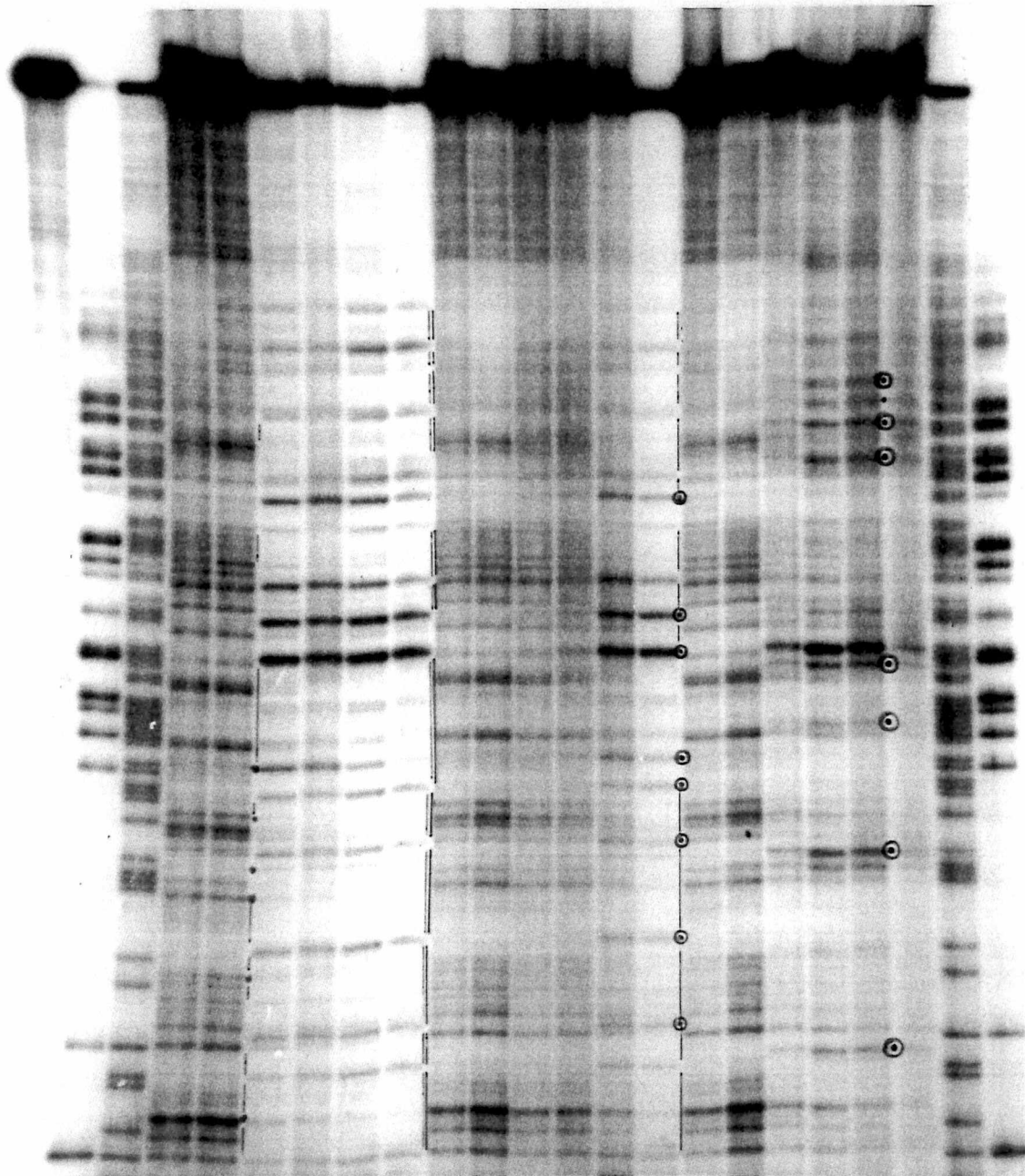


Figure IV-7.

shown that C/EBP is resistant to denaturation when exposed to high temperatures for prolonged periods of time.

Further efforts to assign identity to TAT 5'-flanking sequence -351 to -2 bp DNA-binding activities included competition analyses with literature-derived, well-characterized liver-enriched DNA-binding recognition sites and, in addition, promoter sequences from other genes which had been reported to be insulin-responsive. Included were ds oligo recognition sites for HNF-3 (Costa and Grayson, 1991), HNF-4 (Sladek *et al.*, 1990), C/EBP (Cao *et al.*, 1991), HNF-1 (Cereghini *et al.*, 1988), the GAPDH IRE (Nasrin *et al.*, 1990), and the PEPCK IRE (O'Brien *et al.*, 1990). For these experiments, nuclear extract protein from Fao cells was incubated for 15 minutes at 30°C in the presence of 500-fold molar excess of unlabeled competitor prior to the addition of labeled probe. Several attempts to obtain competitor binding leading to removal of DNA-binding activities associated with TAT 5'-flanking sequence -351 to -2 bp was unsuccessful (see Figure IV-8).

Finally it was postulated that parallel DNase I footprinting of KRC-7- vs. Fao-derived nuclear protein extract might reveal differences in the footprint patterns. Parallel transfection analysis of pTC3.92 in KRC-7 and Fao cell lines indicates a diminished -3.6Kb liver-specific enhancer activity in the Fao clone. The results suggest a deficiency in BIII component DNA-binding activity (see Chapter III). Although no differences in protection pattern were observed, the extent of protection observed with the lowest amount of KRC-7 nuclear protein used in the binding reaction (6 µg) greatly exceeded that observed with the highest amount of Fao nuclear protein (24 µg) (see Figure IV-9).

Together with the parallel transfection results obtained with construct -3922 TAT-CAT, the above observation suggests the possibility that the KRC-7

Figure IV-8. *In vitro* DNase I footprint competition of TAT coding strand sequence -351 to -2 bp with 500-fold molar excess competitors.

Double stranded oligo recognition sites for HNF-3 (Costa and Grayson, 1991), HNF-4 (Sladek *et al.*, 1990), C/EBP (Cao *et al.*, 1991), HNF-1 (Cereghini *et al.*, 1988), the G33 CCAAT motif (Yin, 1991), the GAPDH IRE (Nasrin *et al.*, 1990), and the PEPCK IRE (O'Brien *et al.*, 1990) were incubated with 8 μ g of nuclear protein extract derived from Fao cells which had been exposed to 10^{-8} M insulin for 1 hour. For these experiments, varying amounts of nuclear extract protein (8-24 μ g as indicated) from insulin-treated or untreated Fao cells was incubated for 15 minutes at 30°C in the presence of 500-fold molar excess of unlabeled competitor (3.2 picomoles for the above experiment) prior to the addition of labeled probe (6.4 femtomoles/ 2.5×10^4 cpm for the above experiment) for an additional 15 minutes. Also included in this figure is the coding strand footprint patterns obtained when using increasing amounts of Fao-derived control vs. 10^{-8} M insulin treated nuclear extract protein.

Control						10 ⁻⁸ M Insulin						Competitor DNA							
		μg nuclear protein				μg nuclear protein													
Free	G	G/A	0	0	6	16	24	0	0	8	16	24	HNF-3	HNF-4	C-EBP	G33	GAPDH	PEPCK	HNF-1

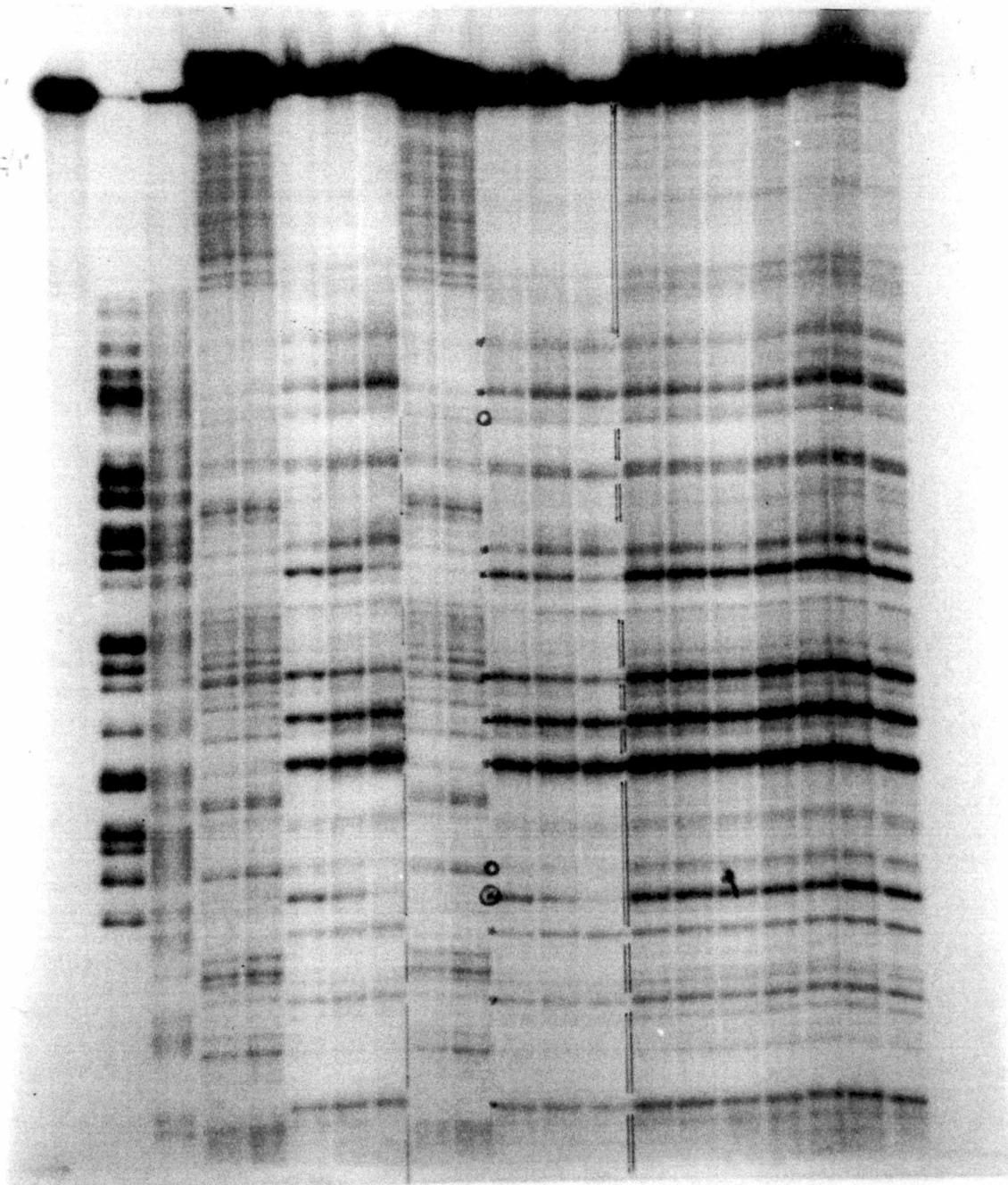


Figure IV-8.

Figure IV-9. *In vitro* DNase I footprint of TAT 5'-flanking sequence -351 to -2 bp with KRC-7 vs. Fao rat hepatoma derived nuclear extract protein.

Labelled probe was incubated with increasing amounts of untreated or 10^{-8} M insulin treated KRC-7 or Fao derived nuclear extract protein as indicated. The protein bound labelled probe was digested with 0.025 units [U] (5 ng) DNase I and resolved on a 6% acrylamide sequencing gel after a 3.5 day exposure. Weak protection associated with nuclear protein extract derived from Fao cells may be attributed to intentional over exposure and, also, a decreased amount of DNase I used to partially digest protein-bound labelled probe when compared to that used typically (0.025 U vs. 1.0 U, respectively).

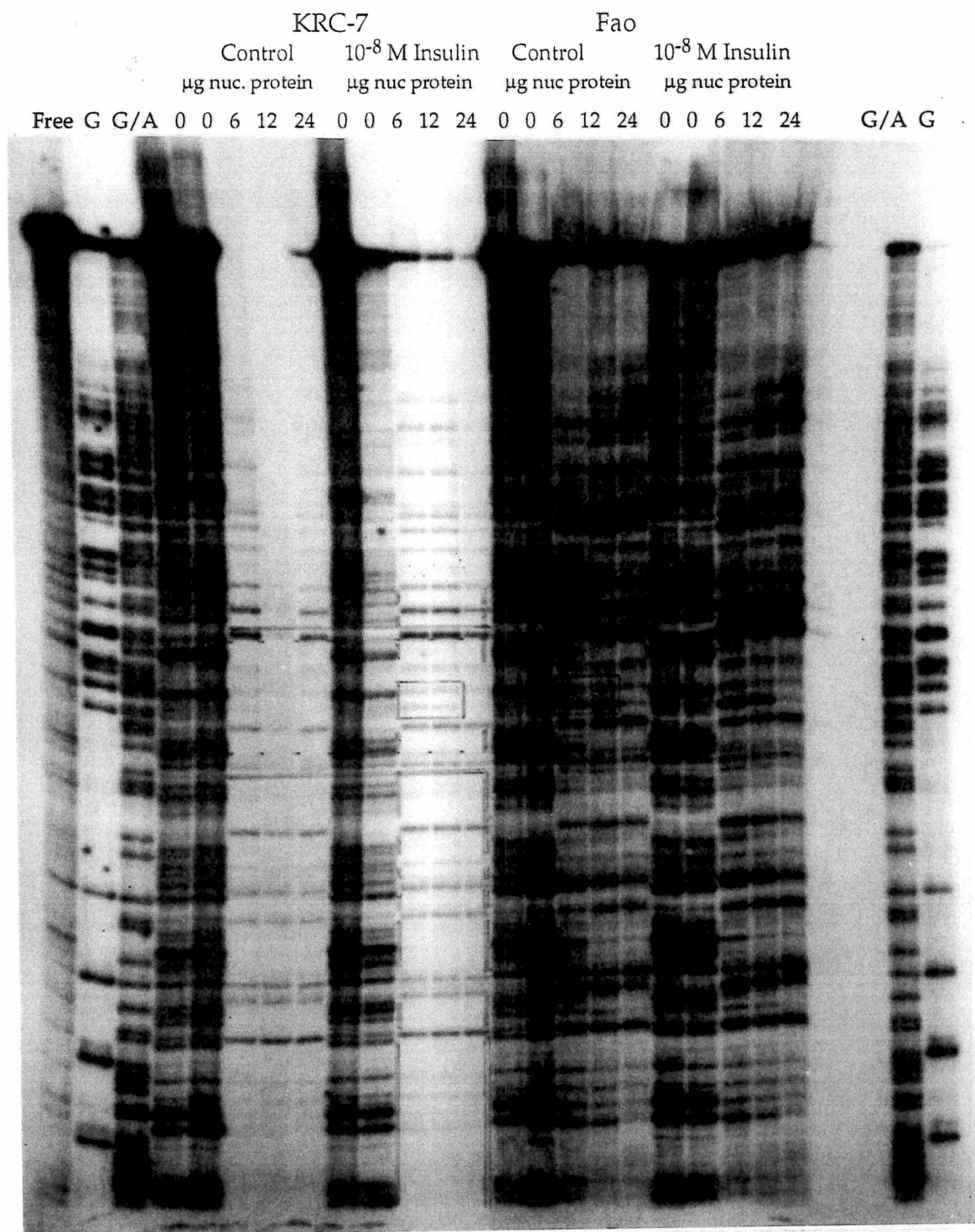


Figure IV-9.

clone possesses elevated levels of the liver-specific transcription factors (not just the liver-specific BIII DNA-binding activity, HNF-4) when compared to that present in the Fao clone.

Discussion

A question central to any *in vitro* analysis is does it truly reflect the *in vivo* system. In this regard, interpretation of *in vitro* DNase I footprint analyses has been demonstrated to accurately reflect both the presence of *cis*-element recognition sites in the DNA fragment examined and, also, the presence and abundance of binding-competent soluble *trans*-factors present in the nuclear protein extract. However, *in vivo* accessibility to DNA-sequence recognition sites is determined by the added complexity of chromatin structure and/or DNA modification.

With this in mind, how do we know the results presented reflect binding of specific factors? First, the regions of protection and hypersensitive sites patterns are both protein concentration dependent, and are reproducible, both between experiments and batches of nuclear extract. Second, protection patterns differ significantly when using nuclear extract derived from pig brain compared to that obtained with nuclear extract derived from rat hepatoma clones KRC-7 or Fao (e.g., tissue-specific vs. ubiquitous binding activities). Third, only the highest concentration of heat-treated nuclear extract retained binding/protection activities (10 minutes/85°C). And last, a strong correlation exists between my *in vitro* DNase I footprint analyses and gel-shift/*in vitro* transcription studies performed by Pictet's group (Schwizer-Groyer *et al.*, 1992).

A comprehensive summary of the *in vitro* DNase I footprint analyses can be viewed in Figure IV-5. Those DNA-binding assignments that appear in

bold-type confirm that which has been reported in the literature, those that are followed by a '?' are putative assignments. Unfortunately, several attempts to empirically validate recognition-site assignments through competition with 500-fold molar excess of consensus recognition *cis*-element ds oligos has failed. It is believed, by this investigator, that the 385-bp probe (pTAT KS.35) or the 242-bp probe (pTAT HS206), with their multiple and adjacent *cis*-element binding sites (refer to the Introduction section pertaining to modular structure), was too formidable a sink for DNA-binding activities when compared to a 500-fold molar excess of a consensus 25-bp recognition motif in solution, not TAT 5'-flanking sequence-derived competitors, as was the circumstance with Pictet's *in vitro* transcription studies (Schweizer-Groyer 1992). In addition, *in vitro* transcription adds a second kinetic component, the repeated formation of a stable preinitiation complex, which may be unstable (e.g., more sensitive) in the presence of competitor DNA.

Parallel DNase I footprint analyses using KRC-7- vs. Fao-derived nuclear-extract protein confirmed earlier related parallel studies. Although no differences in protection pattern were observed, the extent of protection observed with the lowest amount of KRC-7 nuclear-extract protein used in the binding reaction (6 µg) greatly exceeded that observed with the highest amount of Fao nuclear-extract protein (24 µg).

The above results, together with the parallel transfection results obtained with construct pTC3.92 and *in vivo* mRNA^{TAT} analyses, suggest the possibility that the KRC-7 clone possesses elevated levels of the liver-specific transcription factors (not just the liver-specific BIII DNA-binding activity, HNF-4) when compared to those present in the Fao clone.

It could be suggested that the abundance of liver-specific transcription factors in the KRC-7 clone is comparable to that which has been observed in the Rev7 clone (Cuo *et al.*, 1992) (see Table IV-1). Note, in particular, how the Rev7 is highly enriched in both HNF-4 and HNF-3 DNA-binding activities.

**Table IV-1. Relative Abundance of Liver-Enriched Transcription Factors
in the Adult Rat Liver and Three Hepatoma Clones.^a**

liver -enriched Transactivator	DNA-Binding Activity			
	Adult Rat Liver	C2	Fao	Rev7
HNF-1 β	^b M	+	M	M
HNF-1 α	+++	0	+++	++
HNF-4	+++	0	++	++++
HNF-3 α	+	0	M	++
HNF-3 β	++	++	++	+++
HNF-3 γ	+	0	0	+
C/EBP- $\alpha/\beta/\gamma$	++++	++	+++	++++

^a Source: Nature (1992)355:457

^b M : marginal/scarcely detected

0 : no detectable binding activity

Chapter V

CONCLUDING REMARKS AND FUTURE EXPERIMENTS

A Model for Insulin Inhibition of Glucocorticoid-mediated Induction.

Several reports from the literature appear to support the probability that a principle contributing determinant of TAT expression *in vivo* is the liver-enriched transcription factor, HNF-4.

HNF-4 has been reported to be an integral component of the - 3.6 Kb cAMP response unit (BIII) and the -2.5 Kb glucocorticoid response unit (GRE-III). In addition, the *alf/hsdr-1* phenotype is associated with a deficiency in HNF-4. Furthermore, a recent report from Schutz's laboratory has shown that a concatamerized HNF-4 site cloned into pBLCAT2 dramatically increases CAT reporter expression when transiently transfected into a TAT-expressing rat hepatoma, FTO-2B (Nitsch *et al.*, 1993).

Parallel Northern blot, transient transfection, and *in vitro* DNase I footprint analyses in Fao vs. KRC-7 cells suggest that a deficiency in BIII DNA-binding activity (HNF-4) in the Fao clone contributes to the diminished TAT expression *in vivo*. Furthermore, construct pTC.35 is responsive to glucocorticoids while no functional GRE is present (2.5-fold induction). Two reports discussed in Chapter II further support a possibility that glucocorticoid may indirectly recruit a DNA-binding activity to TAT 5'-flanking sequence -351 to +1 bp (along with that recruited to TAT -2.5 Kb GRU region GRE-III) that contributes to enhanced TAT gene expression.

PEPCK expression is similarly affected by the *alf/hsdr-1* phenotype. Interestingly, HNF-4 (Accessory Factor 1), has been shown to be an obligatory

DNA-binding activity, along with accessory factor 2 (AF-2), required for full glucocorticoid inducibility of the PEPCK gene (Hall *et al.*, 1991). The identities of the multiple DNA-binding activities associated with the AF-2 recognition site (PEPCK 5'-flanking sequence -416 to -407 bp) are currently unknown although it has been firmly established that the AF-2 recognition site is an insulin response element (IRE) (O'Brien *et al.*, 1990). Inspection of the PEPCK-IRE sequence shows a region with sequence homology to the HMG class core recognition motif and the HNF-3 recognition site.

Although the mechanism by which insulin inhibits glucocorticoid-mediated induction is unclear, the following information is known. Complete inhibition of glucocorticoid-mediated induction requires two separate regions of the PEPCK promoter, a sequence contained within the PEPCK GRU (5'-flanking sequence -416 to -407 bp, the AF-2 recognition site) and a region in the proximal promoter (5'-flanking sequence -271 to +61 bp) (Forest *et al.*, 1990).

Significantly, the functional identity of the IRE in the PEPCK GRU (5'-flanking sequence -416 to -407 bp, the AF-2 recognition site) could only be established through stable transfection analyses. Furthermore, *in vitro* gel-shift analyses with the PEPCK IRE (5'-flanking sequence -416 to -402 bp) demonstrated multiple DNA-binding activities. No observable differences in protein-DNA complex intensities and/or mobilities were evident when using nuclear extract from control vs. insulin-treated cells (O'Brien *et al.*, 1990).

It has been suggested that the IRE present in the PEPCK GRU mechanistically operates by prohibiting appropriate AF-2 function (O'Brien *et al.*, 1990).

It is believed that a similar mechanism is conserved and operating to allow insulin inhibition of glucocorticoid-mediated induction of TAT gene

expression. The DNA-binding activity (containing a core CCAAT motif) immediately adjacent to the asymmetric TAT GRE has been demonstrated to be an essential component for full glucocorticoid inducibility. This component may be functionally equivalent to the PEPCK AF-2 recognition site. GRE III DNA-binding activity may be functionally equivalent to AF-1 (aka., HNF-4). The TAT CCAAT motif DNA-binding activity may function to allow perinatal acquired competence of the TAT gene to glucocorticoid inducibility. Insulin action may then mechanistically operate by prohibiting appropriate CCAAT motif DNA-binding activity functional interaction with the TAT GRE.

The result is to short-circuit a hepatocyte-specific priming activity that facilitates enhancer-mediated TAT gene expression. Under some circumstances (such as the TAT GRU), it can be suggested the hepatocyte-specific priming activity (insulin target) is part of an enhancer-mediated bimodal induction mechanism involving both de-repression and true activation.

In support of this hypothesis, a recent review entitled, "Regulation of gene expression by insulin", authored by D. Granner describes a requirement to employ stable transfection studies, "in cases where chromatin structure is important for hormonal regulation of gene expression" (O'Brien and Granner, 1991). Indeed, his group, employing stable transfection studies (after having no success with transient transfection studies), has identified an insulin response element within the GRU of the liver-specific PEPCK promoter (O'Brien *et al.*, 1990).

The added complexity imposed by chromatin architecture may account for the inability to detect insulin-dependent altered gel-shifts and/or DNase I footprint protections using naked DNA templates.

A likely candidate for the insulin-responsive DNA-binding activity is HNF-3. Putative HNF-3 recognition sites are immediately adjacent to both HNF-4 sites present in the enhancer-interface (-351 to +1) region. In addition, putative HNF-3 sites can be located immediately adjacent to an asymmetric/degenerate enhancer response element in all three promoter units essential for appropriate TAT gene expression (see Figure III-3).

A Model for Insulin-mediated enhancement of Basal TAT Gene Expression in the Fao, (a terminally differentiated hepatoma)

How, then, does insulin enhance TAT gene expression in the Fao clone? Several lines of evidence suggest a role for a distinct C/EBP-related protein, C/EBP β . First, the -351 TAT-CAT construct is insulin-responsive (~2-fold induction). A sequence with significant homology to the albumin promoter sequence (DE1) that binds Liver-enriched Activator Protein, LAP (a bZip-family member) (Descombes *et al.*, 1990) (Note: LAP = C/EBP β = NFIL-6), is centered at position -245 of the TAT promoter (see Figure IV-1).

LAP accumulates to maximal levels only in terminally differentiated parenchymal hepatocytes (Descombes and Schibler, 1991). Serum, v-src, and IL-6 have been shown to increase trans-activation activities of LAP through phosphorylation of Ser-276 (Hunter and Karin, 1992; Dutta *et al.*, 1990).

Second, insulin treatment alone, reproducibly increased construct pTC2.6 Δ -2308/-351 expression >2.5-fold. The presence of multiple C/EBP binding sites has been demonstrated through *in vitro* DNase I footprint studies of the TAT GRU using purified C/EBP protein (Grange *et al.*, 1991). Therefore, this construct may have created a modular unit containing adjacent and multiple (>5) C/EBP, insulin-responsive *cis*-elements.

In conclusion, it may be suggested that insulin-mediated regulation of hepatic gene expression is dependent on the availability of those liver-enriched *trans*-factor(s) that serve as the target of insulin action the abundance of which is dependent on the state of hepatocyte growth, differentiation, and the presence of cytokines and hormones.

Future Experiments

The hypothesis is that targets of insulin action include the liver-enriched *trans*-factors HNF-3, and a DNA-binding activity present in terminally differentiated hepatocytes only (e.g., Fao), LAP. The following considerations must be taken into account when designing experiments. First, it is suggested that HNF-3 is an indispensable participant in both basal/liver-specific TAT gene expression and enhancer-mediated TAT gene expression (cAMP and glucocorticoid). Second, multiple HNF-3 sites are contained in the three regions of the TAT promoter required for appropriate expression (see Figure III-3). Third, both the HNF-3 and LAP DNA recognition sequences contain a core CAAT-motif (aka. TGT3-motif). Consequently, each factor could feasibly compete for binding to the same site.

A suitable hepatoma that can be used to confirm a functional role for HNF-3 and LAP in insulin-action is the de-differentiated derivative of the Fao, C2. C2 cells possess barely detectable levels of basal TAT gene expression and are lacking in both HNF-1 and LAP (Cereghini *et al.*, 1990). Significantly, C2 cells are inducible by glucocorticoid (Deschatrette and Weiss, 1974). This implies the absence of DNA-modification (e.g., methylation) at putative HNF-3 (immediately adjacent to the functional TAT GRE) and HNF-4 sites (contained in the GRE-III and enhancer-interface regions). Furthermore, HNF-4 DNA-

binding activity is undetectable in this clone (as determined through gel-shift studies) whereas HNF-3 β binding activity is qualitatively similar to that observed in the Fao (comparatively low) (Kuo *et al.*, 1992).

In their entirety, the above observations suggest that C2 cells possess extremely limited, if any, -3.6 Kb liver-specific enhancer activity.

To confirm a functional role for HNF-3 β in insulin-action, the following transient transfection study is proposed. Co-transfect C2 cells with the full-length pTC3.92 construct together with an HNF-3 β expression vector, or an HNF-4 expression vector, or both an HNF-3 β and HNF-4 expression vector. As a control, pTC3.92 will be co-transfected with a reverse-orientation HNF-3 β expression vector. For each transfection arrangement cells will be treated with insulin, glucocorticoid, both insulin and glucocorticoid, and vehicle control.

Parameters to be monitored for each transfection include basal TAT expression, fold-induction with glucocorticoid treatment, and the % inhibition conferred by simultaneous insulin treatment. Theoretically, expression of transfected HNF-3 β expression vector, alone, would increase basal TAT gene expression marginally (lacking HNF-4 activity). Glucocorticoid-mediated fold-induction would be similar to that observed without introduction of HNF-3 β . If the hypothesis is correct, insulin will dramatically inhibit a significantly increased glucocorticoid-mediated fold-induction observed when both HNF-3 β and HNF-4 are introduced. The presumed functional role of HNF-3 β is to allow HNF-4 *trans*-activation. Insulin may disallow appropriate HNF-3 β • HNF-4 interaction thus preventing *trans*-activation of composite HNF-3 • [second 'enhancer' component] elements. HNF-4, alone, would also result in increased basal TAT expression, while insulin inhibition of glucocorticoid-mediated fold-induction would decrease.

A similar transfection study would be performed in C2 cells in an effort to define a functional role for Lap in insulin action. pTC3.92 will be co-transfected with or without a LAP and/or an HNF-4 expression vector. Treatments would include vehicle-control vs. insulin.

The following strategy was employed by Granner's group to verify the existence of an IRE in the PEPCK promoter GRU and, therefore, a similar experiment is proposed (O'Brien *et al.*, 1990). Four tandem copies of a 22-mer oligo, representing TAT 5'-flanking sequence -84 to -63 (a putative insulin-responsive HNF-3 recognition sequence containing a core CCAAT-motif) and containing BamH1 compatible ends, will be cloned into TKC-VI. TKC-VI is a BamH1 linker-scanning mutant of pBLCAT2. The BamH1 linker is positioned between -48 and -32. This construct will be transiently transfected into the KRC-7 clone. It is predicted the construct will possess high basal CAT reporter activity when compared to TKC-VI or a construct containing the tandem 22-mers with point mutations in the core CCAAT-motif. Insulin treatment will strongly inhibit (~50%) the observed increased CAT expression.

To confirm the identity of HNF-3 β and LAP as insulin-responsive factors the following experiments are proposed.

Two 22-mer oligos, one representing TAT 5'-flanking sequence -84 to -63 bp, the other TAT 5'-flanking sequence -256 to -235 bp would be synthesized along with mutated, non-binding versions. The WT oligo will be end-labelled and used in gelshift studies with control vs insulin-treated nuclear protein extract from KRC-7, Fao, and fibroblast (TAT non-expressing) cells. In addition, polyclonal antibodies directed at unique domains contained within HNF-3 β or LAP will be included resulting in a super-shift if, for example, antibodies recognizing HNF-3 β are bound to HNF-3 β bound to the probe.

In addition, bacterially-expressed, purified HNF-3 β and/or LAP proteins can be used in *in vitro* DNase I Fping experiments using the TAT 5'-flanking sequence -350 to -2, 385-bp probe.

To summarize, the novel findings of my research include the following:

- Insulin inhibits both cAMP- and glucocorticoid-mediated induction of TAT gene expression in the Fao cells.
- Transfection studies confirm the identity of an insulin response element(s) contained in TAT 5'-flanking sequence -351 to -52 bp.
- The -3.6 Kb liver-specific enhancer activity is compromised in the Fao clone and may be attributed to a deficiency of BIII-component functional contribution to overall B domain-directed basal TAT expression.
- Characterization of TAT 5'-flanking sequence -300 to -230 bp through *In vitro* DNase I footprint analysis.

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LIST OF REFERENCES

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