To the Graduate Council:

I am submitting herewith a dissertation written by Patrick Shawn Moore entitled "An Investigation Into the Regulation of Tyrosine Aminotransferase." 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 Biochemistry.

John W. Koontz, Major Professor

We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Vice Provost and Dean of the Graduate School

AN INVESTIGATION INTO THE REGULATION OF TYROSINE AMINOTRANSFERASE

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Patrick Shawn Moore

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I first wish to thank my family for their support. It has been quite an interesting experience working in Dr. Koontz's lab, and I would like to express my appreciation to him for letting me have the freedom to carry out the work that I wanted to do, whether he wanted me to do it or not.

Although not quite a thanks is granted for the others (and you know who you are), I will say that I hope my life is never the same after I leave. I am sure that they share my attitude that we all feel like unwilling participants in our undertakings. Of course an acknowledgment wouldn't be complete without thanking Victor, who always was there when I needed his help most, usually every day.

ABSTRACT

Tyrosine aminotransferase (TAT) is a liver-specific enzyme that is regulated by glucocorticoids, agents that increase the intracellular concentration of cyclic AMP, and insulin. Glucocorticoids and cyclic AMP analogs increase TAT activity through transcriptional activation of the TAT gene. This same result has been demonstrated for insulin in adult rat liver and in Fao rat hepatoma cells. However, insulin will inhibit TAT activity in the fetal rat liver, and will inhibit TAT transcription in the neonatal rat liver. Previous work in this laboratory has shown that insulin will inhibit TAT transcription in the KRC-7 clone of H35 rat hepatoma cells, while at the same time causes an increase in TAT activity. This suggests that the KRC-7 clone represents a neonatal rat liver with respect to regulation of TAT. To test the hypothesis that insulin stabilizes TAT mRNA to contribute to the increase in activity, half-life determinations of TAT mRNA were made in the presence and absence of insulin. Although no effect of insulin on the half-life of TAT mRNA was observed, it was later shown that insulin changes the degradation rate of TAT (protein) which may account for the observed increase in enzyme activity after insulin treatment.

Our laboratory is interested in studying the regulation of TAT by insulin at the molecular level. Subclones of the TAT 5′ flanking region have been constructed and the sequence of the TAT 5′ flanking region has been determined to -1300 bp. A gel-shift assay has been used to study the DNA-protein interactions in the flanking region. The ability to detect DNA-protein interactions correlates well with regions of known DNase I hypersensitivity. By analysis of the gel-shift patterns obtained from different regions the proteins that interact with the TAT 5′ flanking region are found in non-hepatic cells. In addition, no differences in these interactions were observed after hormonal treatment.

Since TAT is expressed exclusively in the liver, it was of interest to determine if a known liver-specific trans-acting factor, APF, could be one of the protein(s) that is responsible for the tissue-specific expression of TAT. Although there are two APF consensus binding sites in the 5′ flanking region of the TAT gene, competition analysis demonstrated that APF does not interact with these consensus sites. Furthermore, KRC-7 cells express the adult form of APF, and not the variant form found in dedifferentiated hepatoma cells. These results are discussed.

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CHAPTER I

OVERVIEW

Gene expression is regulated at a variety of levels. These include, but are not limited to, tissue-specific and hormonal regulation. Much progress has been made in recent years in our understanding of the underlying mechanisms involved in selectivity of gene expression. It is generally accepted that cis-acting DNA sequences contained within or around a regulated gene are in part responsible for conferring specificity of gene expression. Trans-acting transcriptional factors are then believed to bind to a specific sequence and interact with the basic transcriptional machinery to facilitate a change in specific gene expression (for review see Jones, et al., 1988). The interaction of these factors with the transcriptional machinery, however, is not well-understood and the importance of protein-protein interaction has only recently been demonstrated (Gentz, et al., 1989; Turner and Tjian, 1989).

Studies of gene expression are typically carried out by deletion analysis and/or mutation of the flanking region (or the region of interest) of the gene being analyzed, followed by transient transfection of a mutated chimeric gene. The effect of these mutations on

transcription are then monitored by a reporter gene whose product is presumably independent of the effect that one is studying. In this way, cis-acting DNA elements can be identified that are important for the effect that is being studied. In some cases the use of transgenic animals has led to a further understanding of transcriptional specificity. However, what seems to be required for tissue-specific expression in cell-culture is often not sufficient in the animal (Pinkert, et al., 1987).

Several genes are known to be expressed in a tissue-specific manner, such as albumin (Cereghini, et al, 1987, 1988), growth hormone (Bodner and Karin, 1987), α-fetoprotein (Widen and Papaconstantinou, 1987), α and β fibrinogens (Courtois, et al., 1987), α-1-antitrypsin (Rheaume, et al., 1988), and tyrosine aminotransferase (Hargrove and Mackin, 1984), to name a few. Other genes are expressed in a tissue-selective manner, such as phosphoenolpyruvate carboxykinase (Benvenisty, et al., 1989), expressed primarily in the liver and kidney, and lipoprotein lipase (Kirchgessner, et al., 1989; Semenkovich et al., 1989), expressed primarily in the fat and heart.

The best understood example of tissue-specific gene expression is in the liver. Several liver-specific trans-acting factors have been discovered (Li, et al., 1988;

Sawadaishi, et al., 1988; Costa, et al., 1989; Baumheuter, et al., 1988). By in vitro transcriptional studies, it has been determined that one trans-acting factor (APF/hNF-1) in particular appears to be necessary for liver-specific transcription (Ryffel, et al., 1989; Cereghini, et al., 1988). The DNA sequence requirements for binding of this protein to DNA and the sequence requirements for liver-specific transcriptional expression in vitro have also been examined (see above references). Based on the consensus sequence to which APF binds, it would seem likely that these different labs are all characterizing the same protein.

The study of hormonal regulation of gene expression has been performed in a similar manner. The best understood examples are those of cAMP mediated expression and steroid mediated expression. Two elements capable of conferring cAMP responsiveness to a normally unresponsive promoter have been found: the so-called CRE (Montminy et al., 1986) and AP-2 (Imagawa, et al., 1987). The transcription factors that bind to these minimal consensus sequences have also been characterized (Deutsch, et al., 1988). In particular, it would appear that a 43 kD protein (CREB/ATF) is a trans-activator of certain cAMP responsive genes (Andrisani, et al., 1989), based on in vitro transcriptional analysis.

Minimal cis-acting sequence requirements have also been defined for the activation of gene expression by glucocorticoid (Jantzen, et al., 1987) and estrogen (Burch, et al., 1988). Interestingly but perhaps expectedly, these sequences are quite homologous. It is expected since the steroid receptors themselves are also quite homologous with respect to their DNA binding domains (Evans, 1988). The triiodothyronine receptor is also classified as a member of this family of receptors based on sequence homology (Glass, et al., 1988).

The steroid receptors are thought to bind to their specific sequence through a "zinc finger" motif as a homodimer (Kumar and Chambon, 1988; Tsai, et al., 1988). The zinc finger was first suggested by analysis of the transcription factor TFIIIA (Miller, et al., 1985). The specificity of this DNA-protein interaction does not lie within the fingers themselves, but in the amino acids bordering the fingers (Mader, et al., 1989).

Although several laboratories have been studying the effects of insulin on gene expression, none has reported a minimal consensus sequence that will confer insulin-specific responsiveness to a normally unresponsive promoter. Such sequences have been implicated in the genes for glyceraldehyde-3-phosphate dehydrogenase (Alexander, et al., 1988), phosphoenolpyruvate carboxykinase (PEPCK)

(Magnuson, et al., 1987), α-amylase (Osborn, et al., 1987) and tyrosine aminotransferase (TAT) (Cheatham and Koontz, 1989). Positive regulation by insulin in a chimeric construct has been demonstrated for glyceraldehyde-3-phosphate dehydrogenase and α-amylase, and negative regulation for TAT and PEPCK.

So far only transcriptional regulation has been mentioned. However, it should be noted that regulation has been shown to take place at a variety of levels. Posttranscriptional and transcriptional mechanisms have been demonstrated for the c-myc gene (Piechaczyk, et al., 1985; Rabbitts, et al., 1985; Dean, et al., 1986). It has been shown that regulation of specific genes can also involve changes in mRNA stability. Estrogen can increase the half-life of vitellogenin mRNA from 16 to 500 hours (Brock and Shapiro, 1983). Rates of translation of specific gene products may also vary as a result of stimuli as is the case of iron regulation of ferritin H-chain (Hentze, et al., 1987). Lastly, it is well-known that certain events such as phosphorylation can alter the activity of proteins.

Tyrosine Aminotransferase

The TAT gene and its products have been a useful model system to study the regulation of gene expression at the transcriptional and translational levels. TAT is particularly interesting since its activity is regulated at many different levels. It is believed that TAT is a gluconeogenic enzyme since it is responsible for tyrosine catabolism (Tanaka and Ichihara, 1982). The ultimate products of the breakdown are fumarate and acetoacetate, thus its suggested relation to gluconeogenesis. TAT is regulated developmentally, by hormones, and is expressed only in the liver. Each of these will be discussed individually.

Developmental Regulation of TAT

It was first discovered in 1959 that TAT activity begins to appear shortly after birth, and continues to rise rapidly to adult levels after what has been called an induction phase (Sereni, et al., 1959). Pioneering studies in the late 1960's by Wicks showed that TAT activity could be induced prematurely by preparing fetal liver explants (Wicks, 1968). This suggested that TAT activity was suppressed during gestation, rather than activated after birth. It is now believed that insulin is the suppressing agent. This is supported by the observations that TAT activity can be induced prematurely *in utero* if the

pregnant dam is made diabetic (Cake, 1986) and that insulin will reduce glucocorticoid induction of TAT in fetal liver explants (Ho, et al., 1981). It has also been shown that insulin will inhibit transcription of TAT in the neonatal rat liver (Johnson, et al., 1988). TAT activity also correlates with plasma insulin levels, which are known to decrease after birth (when TAT activity appears).

It has also been shown that agents that hypomethylate DNA will prematurely increase TAT activity in the fetal rat liver (Rothrock, et al., 1983, 1988). DNA methylation is thought to be an important regulator of gene expression since genes that are highly methylated are often not expressed (Benvenisty, 1985). The correlation between gene expression and DNA methylation is not exact however. This is still a highly controversial area of research, and some investigators suggest that DNA methylation is a secondary event, and not a primary regulator of gene expression (Enver, et al., 1988).

Hormonal Regulation of TAT

Glucocorticoids. Since it was first suggested that TAT synthesis increases in adrenalectomized animals treated with hydrocortisone (Kenney, 1962), much progress

has been made in elucidating the mechanism of action of glucocorticoids. Following the observation that inhibitors of RNA synthesis prevent the induction of TAT by steroid (Butcher, et al., 1972), which suggests that steroids cause an increase in transcription to increase TAT activity, it was later shown that there was an increase in the level of TAT mRNA after hydrocortisone treatment (Nickol, et al., 1978). It is now known that glucocorticoids increase TAT activity by transcriptional activation of the TAT gene (Schmid, et al., 1987). As alluded to earlier, there are specific sequences of DNA in the 5′ flanking region of the TAT gene termed glucocorticoid responsive elements (GRE) to which the activated glucocorticoid receptor binds (Jantzen, et al., 1987). The GREs from TAT are able to confer glucocorticoid responsiveness to a normally unresponsive promoter. It is still not understood how the binding of glucocorticoid receptor to these GREs can enhance transcription, although it has been demonstrated that the glucocorticoid receptor can interact in a synergistic manner with other transcription factors (Strahle, et al., 1988). Nonetheless, the GREs of TAT remain among the best characterized.

Cyclic AMP. Two pancreatic hormones, insulin and glucagon, increase the activity of TAT (Holten and Kenney, 1967). These will be discussed separately. Since it is now known that glucagon acts by increasing the intracellular concentration of cAMP, analogs of cAMP have been extensively used to study the induction of TAT. Although much progress has been made in other systems as to the mechanism of action of cAMP, its mechanism of action on TAT remains less clear. No consensus CRE or AP-2 sites are located within the first 1300 bp of 5′ flanking sequence (P. Moore, unpublished observation). Apart from the transcriptional effects that cAMP exerts on TAT, it was discovered early on that cAMP analogs appeared to have dual effects on TAT (Wicks and McKibbin, 1972; Nakamura, et al., 1981). This result has been confirmed by other investigators (Granner, et al., 1981; Noguchi, et al., 1982). In addition although TAT is known to be phosphorylated on a serine residue, it was concluded that this event is probably not related to hormonally induced increases in activity (Wicks and Su, 1978). However, evidence exists that the catalytic subunit of cAMP dependent protein kinase is responsible for the increase in TAT activity (Boney, et al., 1983).

The aforementioned dual effects by cAMP result from the observation that although an initial dose of cAMP will

cause an increase in TAT transcription and activity, subsequent doses of cAMP fail to cause an increase in synthesis of TAT mRNA, but will still elicit an increase in activity. This phenomenon is referred to as desensitization or the deinduction phase. It was initially observed that the decay of the rate of synthesis of TAT is lower in cells treated with cAMP compared to those treated with glucocorticoid (Lewis, et al., 1982). This observation has led to investigation into the effects of cAMP on the stability of TAT mRNA. Two groups have carried out such studies. Spielholz, et al. (1988) reported that cAMP does not effect the stability of TAT mRNA, while Smith and Liu (1988) reported that cAMP causes increased turnover of TAT mRNA during deinduction. This discrepancy cannot be accounted for at present.

It has been shown that cAMP will increase transcription of TAT in rat liver (Hashimoto, et al., 1984) and in hepatoma cells (Smith and Liu, 1988; Moore and Koontz, submitted). This increase in transcription is reflected in an increase in TAT mRNA (Schubart, 1986; Moore, 1986, submitted). However, the increase in transcription cannot fully account for the increase in activity, thus more work remains to be done on elucidating the mechanism of action of cAMP in regulating TAT. To further complicate the matter, different hepatoma cell

lines have differential sensitivities toward cAMP analogs (Wasner and Simons, 1987).

Insulin. In general, it would appear that the mechanism of insulin action has remained more elusive than other regulators of gene expression. With regards to TAT, it was first demonstrated that insulin would increase TAT activity in the same report that demonstrated glucagon increases TAT activity (Holten and Kenney, 1967). It was later shown that insulin can increase TAT activity in HTC rat hepatoma cells in the presence of inhibitors of RNA synthesis. This suggested that insulin controls TAT activity by a posttranscriptional mechanism (Gelehrter and Tomkins, 1970). Further experimentation in the same cell-line demonstrated that insulin decreases the degradation rate of TAT to account for the increase in TAT activity (Spencer, et al., 1978). This result was confirmed by Strinden and Stellwagen (1982).

More recent reports have shown that insulin will increase transcription of the TAT gene in rat liver (Lee, et al., 1986) and in Fao rat hepatoma cells (Crettaz, et al., 1988). However, to further complicate matters, Moore and Koontz (submitted) have demonstrated conclusively that insulin will inhibit transcription of TAT in KRC-7 rat hepatoma cells. In short, the mechanism of insulin action

depends on the system being analyzed, particularly on the the cell-type that one is using for the studies. One interesting possibility is that the various responses of insulin on TAT are related to the state of differentiation of the cell under examination. As mentioned earlier, it has been shown that insulin will inhibit TAT activity in fetal rat liver, and that insulin will inhibit TAT transcription in the neonatal liver (a time when TAT activity begins to appear). It is an appealing hypothesis that the KRC-7 cells represent a liver in the neonatal stage of development. This is, however, based solely on an examination and comparison of the developmental regulation of TAT, and not on other markers of differentiation.

KRC-7 Cells

The Reuber H-35 cells were originally isolated from a chemically induced rat liver carcinoma (Reuber, 1961). Since that time they have been utilized in culture as both a cloned and an uncloned population. Clones have been isolated that represent differentiated (Fao, Deschatrette and Weiss (1974)), partially dedifferentiated (HTC, Thompson, et al. (1966)), and dedifferentiated (C2, Deschatrette and Weiss (1974)) liver cells. The KRC-7 clone was selected for a low basal level of TAT and was originally chosen for use in this laboratory based on its

ability to arrest growth and to respond to insulin as a growth factor (Koontz and Iwahashi, 1981). It was also shown that insulin exerts its effects in these cells through the insulin receptor (Koontz, 1984). The markers used for differentiation are few, but generally include the ability to express various liver-specific proteins such as albumin, α-fetoprotein, TAT, and gluconeogenic enzymes, as well as morphological appearance. It should be apparent that the attempt to categorize one cell-type as differentiated or dedifferentiated is not exact. The KRC-7 clone would apparently represent a cell-line that may not fit into either category, as long as the definitions are strict. They express neither albumin or α-fetoprotein, but they do express TAT and gluconeogenic enzymes, based on the ability to grow in glucose-free media.

These cells have been used to study hormonal regulation of ornithine decarboxylase (Goodman, et al., 1988) and TAT (Wicks, et al., 1980; Spielholz, et al., 1988; Moore and Koontz, submitted).

Previous Work With KRC-7 Cells and TAT

As alluded to earlier, one criterion used to choose KRC-7 cells was by their ability to induce TAT as a result of hormonal treatment. At that time there were many conflicting reports as to the mechanism of action on TAT. Moore and Koontz (1986) carried out studies to

characterize the induction of TAT by insulin in KRC-7 cells. It was initially shown that insulin can induce TAT activity in the presence of an inhibitor of RNA synthesis. This suggested a posttranscriptional mechanism of action of the hormone. However after measuring hybridizable TAT mRNA it was found, quite paradoxically, that insulin causes a decrease of TAT mRNA. This decrease caused by insulin was also observed in the presence of glucocorticoid or a cAMP analog, which both increase TAT transcription, mRNA, and activity. Transcriptional run-off studies were performed to directly test the hypothesis that insulin inhibits transcription of TAT to account for the decrease in mRNA. It was found that insulin does indeed inhibit transcription of TAT rapidly (within 15 minutes) by at least 50%. This decrease in the rate of transcription of TAT is sufficient to account for the decrease in hybridizable mRNA. To the author's knowledge this remains the only unusual example of a hormone inhibiting the transcription of the mRNA for an enzyme while at the same time increasing the activity of that enzyme.

Although interesting, these results did not explain how insulin causes an increase in TAT activity. After it was determined that insulin had no effect on TAT mRNA half-life (this manuscript), it was later shown that

insulin increases the half-life of TAT protein, which may account for the increase in activity (Moore and Koontz, submitted). This result is similar to what has previously been found in HTC Cells (Spencer, 1978).

The results regarding inhibition of transcription while increasing activity were unexpected. TAT catalyzes the first step in the pathway of the catabolism of tyrosine, and has been classified as a gluconeogenic enzyme (Tanaka and Ichihara, 1975). As such one would predict that insulin would decrease TAT activity. Thus one would expect that insulin inhibits TAT transcription; however, it is obvious that this is not the case in adult rat liver. In KRC-7 cells, it is clear that insulin has two effects on TAT: transcriptional and posttranslational.

Experimental Overview

One interest of our laboratory has been to use molecular approaches to study the regulation of TAT by insulin. The work presented in this manuscript is a continuance of earlier studies. At that time, measurements of the half-life of TAT in the presence and absence of insulin had not been performed, so it was still unclear how insulin increased TAT activity in KRC-7 cells. One possibility was that insulin increases the half-life of TAT mRNA to account for the increase in TAT activity, or

causes changes in the half-life of TAT mRNA, but by itself, these changes would be insufficient to account for the observed increase in TAT activity. Nonetheless, a determination of the half-life of TAT mRNA in the presence and absence of insulin would be a useful piece of kinetic information with which to elucidate the mechanism of insulin action on TAT.

The half-life of TAT mRNA has been measured in the presence and absence of insulin. Although these experiments show that there is no change in the half-life of TAT mRNA, they were important in two respects. First, this could now be ruled out as a possible mechanism by which insulin increases TAT activity, and second, they provided a cornerstone on which to guide further experimentation.

Another member of the laboratory has performed transient transfection assays and determined that insulin will regulate a chimeric gene containing 3012 bp of 5′ flanking sequence fused to the chloramphenicol acetyltransferase reporter gene. These results also suggest that putative insulin regulatory elements analogous to the glucocorticoid regulatory elements exist in the 5′ flanking region of TAT. Since it is believed that a hormonal stimulus can be transmitted into a change in gene expression through trans-acting transcriptional

factors, it might be possible to detect insulin inducible changes in DNA-protein interactions in the flanking region, assuming such changes occur. In addition, it would be of interest to examine these interactions in cells in which it is known that the regulation of TAT by insulin is different, i.e. positive, negative, or not expressed at all. The results of such experiments will be discussed.

CHAPTER II

MATERIALS AND METHODS

Materials

DRB, PMSF, and benzamidine were purchased from Sigma (St. Louis, MO). Leupeptin, pepstatin A, $\alpha 2$ macroglobulin, and poly [d(I-C)] were from Boehringer-Mannheim (Indianapolis, IN). Isotopes were from ICN (Irvine, CA) or New England Nuclear (Boston, MA). Restriction enzymes and modifying enzymes were usually from Promega (Madison, WI), New England Biolabs (Beverly, MA), or US Biochemical (Cleveland, OH). The oligonucleotides were synthesized by the Analytical Services Facility at the University of Tennessee. All other materials used were generally of the highest quality available.

Cell Culture

KRC-7 (Koontz and Iwahashi, 1981), Fao (kindly provided by M. Weiss), C2 (kindly provided by M. Weiss), PC12 (kindly provided by W.D. Wicks), and 3T3 (kindly provided by L. Huang) cells were cultured in DMEM containing 10% bovine serum supplemented with iron (Hyclone, Logan, UT). C6 (kindly provided by W.D. Wicks) cells were cultured in F-10 media with 12.5% horse serum (Gibco, Grand Island, NY), 2.5% fetal calf serum (Gibco). For most experiments, cells were changed to media without

serum for 24 to 48 hours prior to treatments and/or harvesting. All cells were maintained in a humidified environment with 10% CO_2.

RNA Isolation and Analysis

RNA was isolated by the method of Chirgwin, et al. (1979). Briefly, cells were washed in ice-cold PBS, and then dissolved in homogenization buffer containing 4M guanidine thiocyanate. The homogenate was then layered over a 5.7M CsCl cushion and centrifuged in a SW 50.1 rotor (Beckman) for about 16 hours at 36,000 rpm. The RNA pellet was then dissolved in DEPC-treated H_2O. This was followed by addition of 0.1 volumes of 3M NaCl and 2.5 volumes of ethanol, incubated overnight at -20°, and the RNA was pelleted by microcentrifugation. For Northern analysis, 15 μg RNA aliquots were run on 1% agarose gels containing formaldehyde and then transferred to nitrocellulose according to the method of Thomas (1980). Following transfer, the blots were baked for 1 hour at 80° in a vacuum oven and prehybridized overnight at 42° in 10X Denhardt's, 5X SSPE, 0.1% SDS, 50% deionized formamide, and 100 μg/ml denatured salmon sperm DNA (Thomas, 1980). Nick-translated probe was then added and the blot was allowed to hybridize overnight at 42°. The blot was then washed twice in 2X SSC, 0.1% SDS at room temperature, 15

minutes each, followed by two washes at 55° in 0.1X SSC, 0.1% SDS, 15 minutes each. The blot was then autoradiographed until the desired exposure was achieved, usually 24 to 48 hours. Kodak XRP film was used with a Dupont Cronex intensifing screen. Bands were quantitated by laser-scanning densitometry (LKB). For most quantitation dot-blots were used, which were prepared in a similar manner. Three different concentrations of RNA were used, 12, 5, and 2.5 μg.

Subcloning and DNA Sequencing

DNA subcloning employed standard protocols (Maniatis, et al., 1982). Normally, DNA fragments were recovered by electroelution (International Biotechnologies Incorporated, New Haven, CT) after restriction enzyme digestion. Preparation of plasmids for screening was performed by a standard mini-prepation procedure (Maniatis, et al., 1982). Larger scale plasmid preparation utilized pZ523 columns (5'-3', Paoli, PA) and the protocol provided by the manufacturer. Yields were typically 10 mg/liter of starting culture.

DNA sequencing was performed with the Sequenase kit (US Biochemical) and instructions provided by the manufacturer. The label was ^{35}S-α-dATP (NEN), which

provided a higher resolution than could be obtained with ^{32}P as the label. In some cases ITP was used to reduce secondary structure. When necessary, single-strand binding protein (US Biochemical) was also used to reduce sequencing artifacts. Sequencing gels were fixed in 10% methanol, 10% acetic acid to remove the urea, dried, and autoradiographed overnight using Kodak XAR film. Typically, 200 bases could be read from a single gel using wedge-spacers and two loadings.

Purification of Oligonucleotides

Synthesized oligonucleotides were purified by standard protocols. Briefly, one-fourth of a preparation was lyophilized, resuspended in formamide, heated at 90° for 4 minutes, and applied to a 2mm thick 15% denaturing polyacrylamide gel. The gel was run for about two hours at around 500 V. The band containing the oligo was identified by shadow-casting, cut out, and placed in a 15 ml conical tube. The slice was crushed and then incubated overnight in 0.3M NaOAc, pH 5.0, with shaking at 37°. The mixture was then pushed through a syringe filter and the resultant liquid phase was precipitated with ethanol. The pellet was then resuspended in DEPC-H_2O and quantitated by absorbance at 260 nm. This was followed by then combining and annealing the complementary strands at 37°.

Nuclear Extract Preparation

Nuclear extracts were prepared essentially as described by Wildeman, et al. (1984) with slight modification. Briefly, cells were pelleted in ice-cold PBS, and resuspended in 10mM HEPES, pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, and allowed to swell on ice for 10 minutes The cells were then homogenized using a dounce apparatus (pestal B) for 10 strokes and then centrifuged at 1000 x g for 10 minutes at 4°. The pellet was resuspended in 20mM HEPES, pH 7.9, 25% glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT, 1mM PMSF, 2mM benzamidine. This suspension was stirred for 30 minutes on a rocker platform at 4° followed by centrifugation for 20 minutes at 4°, 25,000 x g. $(NH_4)_2SO_4$ was added to the supernatant (0.33 g/ml) and the suspension was stirred for 30 minutes at 4°, again followed by centrifugation for 20 minutes at 4°, 25,000 x g. The protein pellet was resuspended in 20mM HEPES, pH 7.9, 20% glycerol, 50mM KCl, 2mM DTT, 2mM $MgCl_2$, 2mM benzamidine and dialyzed against the same buffer for at least 2 hours at 4°.

Some extracts were prepared in the presence of the protease inhibitors pepstatin A, leupeptin, and α_2 macroglobulin, all at 1μg/ml. The extract was aliquoted and stored at -70°. Protein concentration was determined

by the Bradford assay (Bio-Rad), using bovine serum albumin as the standard. Once the samples were thawed they were not refrozen and used again.

Gel Mobility-Shift Assay

Most fragments were labeled by first cutting the plasmids at the Xba I site (in the polylinker) and filling in the overhang with Klenow fragment using α-^{32}P-dCTP as the label. The second resriction cut was done after heat inactivation of the Klenow fragment. The DNA was then loaded on a standard agarose gel, and electrophoresis was carried out. The band containing the fragment of interest was then excised from the gel (as a slice) and electroeluted. Typically the specific activity was around 1×10^7 cpm/μg.

The mobility shift assay was carried out as described by Fried and Crothers (1981) and Schneider, et al. (1986) with slight modifications. Binding reactions contained 10mM HEPES, pH 8.0, 5mM $MgCl_2$, 10% glycerol, 100 mM KCl, 1mM spermidine, 0.05% NP-40, 8 μg/ml pUC18, and 100 μg/ml poly [d(I-C)] (unless otherwise indicated) in a final volume of 25 μl. Usually, 30,000 cpm (around 3 ng) of labeled probe and 5-10 μg of nuclear extract protein was used. The protein was added last and the binding reaction was carried out for 15 minutes at room temperature. The samples were then loaded onto low-ionic strength (0.5X

TBE) 5% polyacrylamide gels and ran at 150 volts until the desired separation was achieved. The wet gels were then placed in plastic wrap and autoradiographed at -70° with an intensifying screen, for 12-20 hours.

Transient Transfection Assay

Cells were transfected by modification of the DEAE-dextran procedure as described by Cheatham and Koontz (1989). Briefly, cells were harvested in serum-free media and spun in a clinical centrifuge for 5 minutes. The cell pellet was resuspended in DNA solution (0.5 mg/ml DEAE-dextran, 25 μg/ml plasmid DNA in TBS) and placed on a rocker platform for 20 minutes at room temperature. TBS is 25mM Tris, pH 7.4, 137mM NaCl, 5mM KCl, 0.6mM Na_2HPO_4, 0.7mM$CaCl_2$, 0.5mM $MgCl_2$. The mixture was then plated out in media containing 1% serum and allowed to recover for 4-6 hours. The cells were then shocked with 20% DMSO in serum-free media for 5 minutes, washed once with TBS, and fresh serum-free media was then added along with various hormonal treatments.

About 18 hours later, cells were harvested in PBS (0.9% NaCl, 20mM K_2HPO_4, pH 7.4) and split for assay for CAT (chloramphenicol acetyltransferase) (Gorman, et al., 1982) or PAP (placental alkaline phosphatase) (Henthorn, et al., 1988) activity. When harvesting for CAT activity,

the cells were pelleted and resuspended in 250mM Tris, pH 7.5, 5mM EDTA followed by a 10 minute incubation at 55°. It was found that the typical freeze-thaw step was unnecessary, since extracts prepared by either method gave identical activity. This was followed by another centrifugation for 10 minutes at 4° at 10,000 rpm. The supernatant was transferred to a clean tube and assayed for protein by the Bio-Rad assay. Cells were prepared for PAP activity by pelleting the cells, resuspending in TBS, and incubating at 65° for one hour. An appropiate amount of this extract was assayed at 30° until the desired activity was achieved, usually one to two hours.

CHAPTER III

TAT mRNA HALF-LIFE

One way that the cell has of controlling the level of a particular mRNA is by changing the rate of decay of that mRNA. Half-lives of various mRNA species can vary dramatically. For example, the half-life of c-fos mRNA is about 15 minutes (Muller, et al., 1984), while that of β-globin mRNA is around 18 hours (Volloch and Housman, 1981). Little is known about the degradation process itself or how it is controlled. The development of a cell-free assay for RNA degradation is beginning to provide insight into these mechanisms (Peltz, et al., 1987; Ross, et al., 1987; Brewer and Ross, 1988). This is particularly true for c-myc mRNA, which is known to be regulated posttranscriptionally (Rabbitts, et al., 1985). Much progress has also been made in determining the mechanism of instability of c-fos mRNA (Shyu, et al., 1989). In particular, a conserved AU rich sequence appears to be involved (Wilson and Treisman, 1988). This AU rich sequence is found in several other RNA species (Shaw and Kamen, 1986). It has been observed that RNA containing this sequence are superinducible by cycloheximide (Shaw and Kamen, 1986), which also correlates with the

observation that translation may be required for RNA degradation (Graves, et al., 1987). TAT mRNA also contains such an AU rich sequence (P. Moore, unpublished observation), is superinducible by cycloheximide (Ernest, 1982), and has a relatively short half-life of about 2 hours (Spielholz, et al., 1988; Smith and Liu, 1988; Moore, this manuscript).

There are reports that hormonal stimulation can change the half-life of a particular mRNA. Vitellogenin mRNA is stabilized by estrogen (Brock and Shapiro, 1983) and c-myc mRNA is transiently stabilized by mitogens (Kindy and Sonnensheim, 1986; Levin, et al., 1986). As mentioned earlier, it is unclear how insulin increases TAT activity in KRC-7 cells. One possibility is that insulin stabilizes TAT mRNA. Following insulin treatment, TAT mRNA levels apparently reach a new steady-state by 4 hours (Moore, 1986). Due to the low-abundance of TAT mRNA however, especially following insulin treatment, extremely accurate numbers on the decrease in TAT mRNA have been difficult to determine. Thus, it is possible that insulin causes an increase in the half-life of TAT mRNA while at the same time causing an decrease in TAT mRNA synthesis. It will also be essential to determine if insulin has an effect on the half-life of TAT mRNA, even if it could not be

responsible for the observed increase in TAT activity, in order to study the kinetics of the insulin effect on TAT.

Half-life determinations of TAT mRNA were made in the presence and absence of insulin. The strategy was to inhibit transcription and measure the amount of TAT mRNA remaining at various times thereafter. The assumption was that if insulin does have an effect on TAT mRNA stability, then the rate of decay of TAT mRNA should be different in cells that had been treated with insulin compared with those that had not; i.e. less in insulin treated cells if insulin stabilized TAT mRNA.

The inhibitor of RNA synthesis chosen was 5,6-dichloro-1-β-ribofuranosyl benzimidazole (DRB). DRB has been hypothesized to act by increasing the rate of premature chain termination or initiation (Tamm and Kikuchi, 1979; Laub, et al., 1980). However, recent evidence would suggest that DRB may interfere with an elongation factor (Chodosh, et al., 1989) (rather than as a nucleotide analog) since the extent of DRB inhibition of transcription is proportional to the length of a transcript. In additional support of this hypothesis, DRB will not inhibit transcription of all mRNA's (for example interferon), and will not inhibit transcription in at least one cell-free system (Zandomeni, et al., 1983). It has been demonstrated that DRB will inhibit transcription

of TAT, as evidenced by the observation that DRB will completely block the induction of TAT by glucocorticoids which increase TAT activity through transcriptional activiation of the TAT gene (Moore, 1986). Thus DRB is a valid tool with which to inhibit TAT transcription.

Half-life determinations were made by preincubating cells with DRB for 30 minutes, which has been shown to be more than sufficient to allow the effects of DRB to become apparent (Tamm and Kikuchi, 1979). Following the preincubation, half of the dishes received insulin, while the other half received no further additions. As a control to ensure that insulin was having an effect, some dishes received no DRB but only insulin. At various times after the addition of insulin, cells were harvested and RNA was purified and analyzed. Quantitation of the RNA was performed by dot-blot analysis probing using a nick-translated full-length cDNA probe directed against TAT (Grange, et al., 1985). RNA integrity was examined by performing electrophoresis on the RNA using a denaturing agarose gel and determining if the 28S and 18S ribosomal RNA bands are intact. The use of the dot-blot apparatus allowed one to not have to correct for differences in transfer of the RNA that can occur in Northern analysis, normally done by use of a cDNA for a second mRNA. The

autoradiographs were scanned and first-order regression analysis was carried out.

The results of three independent experiments are shown in Table III-1[1]. Using this technique, the half-life of TAT mRNA is measured to be 1.5 to 2 hours. This is in good agreement with previous values on the half-life of TAT mRNA (Smith and Liu, 1988; Spielholz, et al., 1988). It is also apparent that the half-life is the same in cells that have been treated with insulin compared with those that have not. Although the experiment to experiment variation was higher than one would desire, it is clear that insulin had no effect on TAT mRNA in any single experiment. Lastly, for this type of analysis, an experiment to experiment standard deviation of 15% is not unreasonable. In summary, no treatment of these cells could result in any significant change in the half-life of TAT mRNA.

From visual examination of an actual plot of the rate of decay of TAT mRNA (Figure III-1), one notices that there is a slight apparent initial stabilization. Although this may be real, it should not be taken into consideration for two reasons. First, the standard deviation of about 15% would easily overlap points that are close together, thereby diminishing the effect. Second, if this slight initial stabilization were

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[1]All figures and tables are found in the appendix.

occuring, it could not account for the observed increase in TAT activity following insulin treatment.

Since we are also interested in the effects of other hormones on TAT, the half-life of TAT mRNA was measured in the presence of dexamethasone (a non-metabolizable glucocorticoid analog) and dibutyryl cAMP (a cyclic AMP analog that will cross the cell membrane and increase the intracellular concentration of cAMP). Table III-2 shows that the half-life of TAT mRNA in the presence of these inducing agents is the same as that in the presence of insulin or no inducing agent. As mentioned earlier, the value of 2 hours is in good agreement with the half-life of TAT mRNA reported by Spielholz, et al. (1988), but slightly less than that reported by Smith and Liu (1988), who find that the half-life of TAT mRNA is around 3 hours. The latter investigators did use an uncloned H35 cell population, however. Thus, none of the agents tested have any effect on TAT mRNA stability.

To test the possibility that DRB was inhibiting the production of a factor (made in response to insulin) that was required for the stabilization of TAT mRNA, an experiment was done by first preinducing cells with insulin for one hour. DRB was then added and the decay of TAT mRNA was followed. Apparently this is not the case

since the half-life in either the presence or absence of insulin was about 2 hours (Table III-2).

Later experiments in the laboratory have found that the rate of degradation of TAT protein is changed by insulin about two-fold. This may be sufficient to account for the observed increase in activity. The rate of synthesis of TAT would also have to increase for this mechanism to be able to explain the increase in enzyme activity since TAT mRNA levels decrease after insulin treatment. One might suggest that if the rate of TAT synthesis increases proportionally to total protein synthesis in response to insulin, there must still be a specific effect of insulin on TAT synthesis. Hypothetically, if insulin causes TAT mRNA levels to decrease by 50% and the rate of TAT synthesis increases two fold (as does total protein) then the rate of TAT synthesis per molecule of TAT mRNA must increase four fold (or even more since insulin causes an increase in general RNA synthesis). In this case insulin would have three effects on TAT: transcriptional, translational, and posttranslational. At any rate the experiments measuring the rate of TAT synthesis are in progress.

Since our laboratory is interested in studying the effects of insulin at the molecular level, it was decided

to follow up on the aforementioned transcriptional inhibition of TAT by insulin. While another member of the laboratory began transient transfection assays using the chimeric gene, the thrust of this research was to examine the DNA-protein interactions in the 5′ flanking region of TAT. This flanking region was obtained as the entire chimeric gene, so it first had to be subcloned and characterized.

CHAPTER IV

CLONING AND SEQUENCING OF THE FLANKING REGION

The plasmid pTATCAT3 (Jantzen, et al., 1987) was used to construct subclones. It contains the TAT 5′ flanking region from -2950 to +62. At +62 the flanking region is fused to the bacterial gene for CAT, which is used as a reporter in transient transfections. The CAT gene is fused at the 3′ end to the SV40 splicing and polyadenylation signals. The remainder of the plasmid contains pUC8 sequences. The sequence of the TAT 5′ flanking region has been published only to -600 bp (Shinomiya, et al., 1984). The sequence of the rest of the TAT region was unknown, except for a small portion around the GRE's (Jantzen, et al., 1987) and a small portion around -1000 (Becker, et al., 1987). It was decided that pGEM-3-blue (Promega) would be used as the cloning vector. pGEM-3-blue has both T7 and SP6 RNA polymerase promoters that flank the multiple cloning site, and primers are commercially available that recognize these promoters and can thus be used for sequencing. In addition, the vector has the gene for the lac-Z α-peptide which is used for an easy color detection of colonies harboring plasmids that contain an insert. The only five known restriction sites (excluding the region from -600 to +62) were initially used to

construct subclones: Sal I (-2950), Xba I (-2561), Tth111 I (-2308), Bam HI (-1300), and Pst I (-800). A schematic diagram of the flanking region and of the clones constructed is shown in Figure IV-1. Further information follows and is also given in Table IV-1.

The plasmids pTATKS.35, pTATKP.45, pTATSP.8, pTATBX1.3, pTATPB500, and pTATBX400 were constructed by forced directional insertion into pGEM-3-blue. The restriction sites utilized are also found in the multiple cloning site of pGEM-3-blue. pTATSS.28 was obtained by digestion of the insert from pTATPB500 with Sau3A and the 281 bp fragment was cloned into the compatible Bam HI site. The plasmid pTATSS199 was obtained by digestion of the insert from pTATSS.28 with Sau3A and Sau96 I, blunt-ending with Klenow, addition of Bam HI linkers, and inserted in the Bam HI site of pGEM-3-blue. pTATHH104 was made by digestion of the insert from pTATKS.35 with Hpa I and the 104 bp fragment cloned in the compatible Sma I site of pGEM-3-blue. pTATBX1.3 was digested with Tth111 I and Sma I, blunt-ended, and religated to yield pTATXT250. pTATKS.35 was digested with Hpa I and Sma I, and religated to construct pTATHS206. Finally, pTATHS206 was digested with Apa I and Sst I, blunt-ended with T4 DNA polymerase, and religated to produce pTATHA156.

The plasmids used for sequencing are listed in Table IV-1. Again, the SP6 and T7 promoter primers were used so both ends of the insert could be sequenced. The compiled sequence from -1300 bp to -600 bp is shown in Figure IV-2. The sequence from -600 to -2 agrees with that published (Shinomiya, et al., 1984). This information was utilized for the construction of smaller subclones (Figure IV-2 and Table IV-1). A restriction map of this area is shown in Figure IV-3.

A computer search for homology to several known functional regions was performed. There is a consensus recognition site for DNA Topoisomerase II, as reported by Becker, et al., (1984). In addition there is a consensus sequence for albumin promixal factor (Cereghini, et al., 1988). This will be discussed in the next chapter. It does not seem unusual that there are few regions of homology in the region from -1300 to -600 since there are only two DNase I hypersensitive sites located at about -1000 (Becker, et al., 1984). The possible function of the DNA-protein interactions in this region will be discussed later as will the results of gel-shift experiments performed with the clones that have been constructed.

CHAPTER V

DNA-PROTEIN INTERACTIONS IN THE 5′ FLANKING REGION OF TAT

The electrophoretic gel-mobility shift assay is now a widely used tool with which to study DNA-protein interactions. It is based on the fact that a DNA-protein complex has a lower mobility in a polyacrylamide gel (due to the increased mass) as compared to a free, uncomplexed, fragment of DNA used as a probe in this assay. Thus, a DNA-protein complex is observed as a retarded band after electrophoresis and autoradiography. The gel-shift assay has successfully been used to detect a wide variety of trans-acting factors which bind to their target sequence in an inducible manner (Prywes and Roeder, 1986), factors that are tissue-specific (Cereghini, et al., 1988), as well as general transcription factors (Raymondjean, et al., 1988).

It was decided that an analysis of the DNA-protein interactions in the 5′ flanking region of TAT would be undertaken to determine if hormonal treatment (in particular insulin) would induce any detectable changes in these interactions. It would also be determined if these DNA-protein interactions were different in cell-lines in which TAT is regulated in an opposite manner by insulin or

in which the TAT gene is not expressed at all (i.e. non-hepatic cells). In the KRC-7 rat hepatoma clone insulin inhibits TAT transcription (Moore and Koontz, submitted) while in the Fao rat hepatoma clone insulin increases TAT transcription (Crettaz, et al., 1988). Two non-hepatic cell-lines would also be examined: C_6, a rat glioma cell-line and PC12, a rat pheochromocytoma cell-line.

While most of the flanking region has been examined by this assay, it seemed prudent to concentrate on regions of the TAT 5′ flanking region that have previously been shown to be DNase I hypersensitive. This is important since regions that are hypersensitive have been shown to be involved in DNA-protein contact and mapped to regions of chromatin that are being actively transcribed (Weintraub and Groudine, 1976; Elgin, 1981). It is now believed that DNase I hypersensitivity is caused by local alterations in chromatin structure caused by DNA-protein interaction. The indirect end-labeling method (Wu, 1980) has been applied to examine DNase I hypersensitive sites in the 5′ flanking region of the TAT gene by Becker, et al. (1984) and by the same laboratory again in 1987 (Jantzen, et al.). It was found that several sites are constitutively hypersensitive in the TAT gene (indicated by arrows in Figure IV-3) located around 0, -200, -950, and -1050. Glucocorticoid treatment induces three hypersensitive sites in regions

now known to harbor glucocorticoid responsive elements (GRE). These sites have been designated GREs I, II, and III located at around -2600, -2500, and -2430, respectively. Although GRE I is not required for full glucocorticoid induction of TAT, it nonetheless becomes hypersensitive upon treatment with steroid (Jantzen, et al., 1987). The function of GRE I remains to be elucidated.

The nuclear extract preparation and the gel-shift assay itself should now be discussed. Nuclear extracts are prepared by a standard 0.42M NaCl extraction of nuclei. Thus the extract contains various soluble factors that are sufficient to support <u>in vitro</u> transcription (Wildeman, et al., 1984; Corthesy, et al., 1988), but by definition will not contain nuclear proteins that are more tightly associated with chromatin or nuclear matrix. It is possible that certain factors that are involved in regulation of transcription are missing in this extract. This is an obvious limitation that should be taken into consideration when interpreting data. It should also be mentioned that variation between extracts was sometimes very high, while gel-shifts with proteins from the same extract were extremely consistent. To a certain extent, this observation somewhat foiled the desire for consistent results and thus interpretation of data. As a consequence

a large number of extracts had to be screened before any conclusions could be made.

Initial experiments involved optimization of detection of shifted bands using a probe near the start site of transcription of the TAT gene by titrating the binding reaction components. The components that were held constant were HEPES, pH 7.9 (10mM) and glycerol (10%). The effects of spermidine, KCl, $MgCl_2$, NP-40, and the non-specific competitors pUC18 and poly [d(I-C)] were investigated. Poly [d(I-C)] was crucial to the success of the assay. It was found that 100μg/ml is sufficient to allow for reasonably sharp shifted bands. The presence of pUC18 in the binding reaction had a small but noticeable effect in the assay; 8μg/ml was used for this non-specific competitor. Of the remaining components, KCl had the greatest effect on detectable binding. Although low concentrations of KCl (25mM) gave a similar shift pattern as that of higher concentrations of KCl (100mM), the higher concentration caused the bands to be more intense; thus 100mM KCl was chosen. The remaining components, spermidine, $MgCl_2$, and NP-40 had very little effect on the binding. Rather than eliminate these components altogether, the working concentration was set at 5mM $MgCl_2$, 1mM spermidine, and 0.05% NP-40. The effect of DTT was also tested. It had virtually no effect on detection

of DNA-protein complexes with any fragment examined, so it was decided to eliminate this component. Lastly, the effects of time and temperature on the binding reaction were also tested. The binding appeared to be the same in samples incubated at 30° for 30 minutes (a condition used quite frequently as reported in the literature) as in those incubated for 15 minutes at room temperature (also widely used). For convenience the latter condition was chosen.

The results of the gel-shift experiments are organized such that the region from -2950 to -2561 (pTATBX400) will be discussed first, followed by regions further downstream in the flanking sequence. pTATBX400 contains GRE I which is only one-half of the palindromic sequence found in GRE's II and III. As mentioned earlier, GRE I does not seem to be required for the full glucocorticoid induced increase in TAT transcription. A gel-shift experiment performed with the insert of pTATBX400 is shown in Figure V-1. There is an obvious lack of observed DNA-protein complexes in this region regardless of the cell-line used or the hormonal treatment. Unless otherwise indicated, hormonal treatments were for one hour, which is a sufficient time to see an effect of these hormones on TAT transcription (Moore, 1986). There are several possible explanations for this result. First, it could be that

there are no DNA-protein interactions in this region, but seems unlikely since glucocorticoids induce hypersensitivity of GRE I. Second, the affinity or abundance of a protein that binds in this region could be extremely low making detection a problem. Third, the extract preparation may not contain a factor that binds to this region, since as mentioned earlier the extract will not contain all nuclear proteins. A final possibility is that a protein actually is binding, but due to the large size of this fragment (400 bp) the protein does not add enough additional mass to allow for the DNA-protein complex to be retarded compared to the free probe. Speculation about the function of this region will be considered later.

The next experiments to be discussed are those that involve the region that contains two DNase I hypersensitive sites at -1050 and -950. The initial probe used in these studies recognizes the region from -1225 to -945 (pTATSS.28). A typical gel-shift with protein extracted from two different cell-lines is shown in Figure V-2. Although several shifted bands are apparent, they appear to be unchanged as a result of hormonal treatment and are the same in the different cell-lines. As with other fragments, in the experiments presented there does

appear to be slight differences in the DNA-protein complexes. However, these differences were not reproducible from extract to extract. Thus, if a conclusion is drawn indicating that no differences were observed, it is because these differences were not consistently observed in all the extracts that were prepared under those conditions. For each DNA probe used, a minimum of three extracts were screened, and several gel-shifts were performed with each extract.

To further define the sites at which these interactions are occurring, a smaller probe was constructed, from -1144 to -945 (pTATSS199). Gel-shifts with pTATSS199 are shown in Figures V-3 and V-4. The gel-shift patterns obtained with SS199 and SS.28 appear to be similar, and it is concluded that the DNA-protein interactions occurring in the larger fragment are most likely the same as those in the smaller fragment (i.e., no proteins bind DNA in the region from -1225 to -1144). This correlates with the observation that two DNase I hypersensitive sites are detected at -1050 and -950 (Becker, et al., 1987). There also appears to be no difference in the binding pattern in cells in which insulin regulates TAT differently, that is, in Fao, KRC-7, or in cells that do not express TAT. As mentioned earlier, this region also contains a consensus APF binding site.

Experiments designed to test whether APF is one of the proteins responsible for the observed gel-shift complexes are discussed in the next chapter.

The region from -800 to -351 was examined by using two probes: PT181 (-800 to -619) and KT268 (-619 to -351). As seen in Figures V-5 and V-6, no shifted bands are detected. The conclusion that no distinct, specific DNA-protein interactions occur in this region is supported by the observation that increasing amounts of protein bind the probe, but give a smear rather than any distinct bands (Figures V-5 and V-6). Additionally it does not seem unusual that no detectable DNA-protein complexes are observed with these fragments since no DNase I hypersensitive sites are observed in or near this region from -800 to -351 (Jantzen, et al., 1987; Becker et al., 1984).

As mentioned earlier, initial experiments utilized a probe near the start site of transcription, from -2 to -351. However, these gel-shifts proved to be quite inconsistent, did not always reveal distinct DNA-protein complexes, and were therefore uninformative. To remedy this, two smaller probes were constructed: pTATHH104 (-312 to -208) and pTATHS206 (-208 to -2).

Using HH104 as a probe, three complexes are observed--

two rapidly migrating and another with much lower mobility (Figure V-7). It is also apparent that there are no observed differences as a result of hormonal treatment, and the complexes seem to be the same in hepatic and non-hepatic cell-lines. It is possible that the slowest migrating complex is more abundant in Fao cells than in KRC-7 or PC12 cells, but no function has been determined for this region.

The region from -312 to -208 has been used by another laboratory to study DNA-protein interactions (Lobanenkov, et al., 1988, 1989). They report that two distinct protein can be detected in liver vs. spleen extracts which they term $LspA_1$ and $LspA_2$. $LspA_1$ is present only in liver extracts, while $LspA_2$ is present exclusively in spleen extracts. Both $LspA_1$ and $LspA_2$ are observed as rapidly migrating complexes. No proteins are detected that bind to this region in embryonic liver extracts. The results obtained in these experiments are not in agreement with the aforementioned data.

The reason for the discrepancy between these results and those reported by Lobanenkov, et al. is unknown, but it is suggested here that proteases are responsible for the lack of detection of the slowest migrating band in the experiments performed by Lobanenkov, et al. (1988, 1989). While both those extracts and the ones used in these

experiments were prepared in the presence of PMSF, the extracts used in these experiments also contained benzamidine; those used by Lobanenkov, et al. did not. Indeed, if benzamidine is not included in the extraction buffers, the slowest migrating band is not detected and the faster moving complexes are not as clearly observed (Figure V-8).

pTATHS206 was used to probe the DNA-protein interactions in the region from -2 to -206. This region has been footprinted (Becker, et al., 1987) and several protected areas are observed. Figure V-9 shows the gel-shift results using this probe. Again, several DNA-protein complexes are detected but none appear to be altered as a result of hormonal treatment. The aforementioned footprinting of this region detected no observable changes when using extracts from a different cell-line. The extract from PC12 cells was compared with extract from KRC-7 and Fao cells (Figure V-9). An initial experiment suggested (at least to this investigator) that the slowest migrating complex could have a slightly different migration than the analogous complex in hepatic cells. Attempts to separate these two complexes proved to be futile (data not shown). It is also possible that the factor is less abundant in hepatic cells, and this

difference in abundance is responsible for the initial apparent difference in mobility. If there were a different factor that binds in this region in hepatic vs. non-hepatic cells, it would most likely be related to tissue-specific expression of the TAT gene. However, other laboratories have been able to express chimeric constructs containing this region in non-hepatic cells (Jantzen, et al., 1987). Similar experiments in this laboratory proved to be inconclusive (data not shown). Based on this information it was concluded that the proteins binding in this region are most likely the same in hepatic and non-hepatic cells.

CHAPTER VI

ALBUMIN PROXIMAL FACTOR

Much progress has recently been made in the identification of liver-specific trans-acting factors involved in the expression of liver-specific genes, in particular, albumin, α-1-antitrypsin, and β-fibrinogen. Several laboratories have characterized proteins that have been given a variety of names such as hepatocyte nuclear factor I (hNF-I) (Baumhueter, et al., 1988), APF (Cereghini, et al., 1988), AFP-1 (Sawadaishi, et al., 1988), and HP1 (Ryffel, et al., 1989; Kugler, et al., 1988). From the basis of analysis of the consensus sequence to which these factors bind, it would seem likely that all these laboratories are characterizing the same factor. One NF-1 like factor has been purified and cloned (Paonesse, et al., 1988). Sequence comparison has shown that it has homology with C/EBP (CCAAT box enhancer binding protein) another liver-enriched trans-acting factor (Landschulz, et al., 1988). It would appear though that multiple trans-acting factors are required for conferring liver-specific expression (Costa, et al., 1989; Li, et al., 1988).

Two groups have reported that hNF-I or APF varies

between differentiated and dedifferentiated cells (Cereghini, et al., 1988; Baumhueter, et al., 1988). The variant form has been termed vHNF-I or vAPF. In particular, the form found in differentiated hepatoma cells and liver is 92 kD, while the variant form found in dedifferentiated hepatoma cells is 72 kD. It has not been determined whether or not the two proteins arise from the same gene or not, although both of these proteins recognize the same consensus sequence. As mentioned earlier, there are two consensus APF binding sequences in the 5′ flanking region of the TAT gene (Figure VI-1). Since TAT is expressed exclusively in the liver, it would be of interest to determine if these sites bind APF, and therefore would putatively be important in the liver-specific expression of TAT.

Toward this goal two oligonucleotides were synthesized and their sequence is shown in Figure VI-1. One of these is directed against the endogenous sequence in TAT, called DHE, while the other is against the binding site in the albumin gene, called PE56. The oligonucleotides were synthesized as single-stranded DNA's and then annealed for use in experiments. After annealing a BamHI site is present at the 5′ end, while a Xba I site is present at the 3′ end. These sites would be convenient for labeling and cloning.

An initial experiment involved competition with unlabeled DHE and PE56 in a gel-shift experiment with radiolabeled SS199 (-1144 to -945) to determine if the oligonucleotides could compete for binding with any of the observed shifted bands. Figure VI-2 shows the competition experiment performed with PE56. It is apparent that PE56 will not compete for binding to any any of the complexes at 500 fold molar excess. The analogous experiment performed with DHE (the endogenous TAT homologous sequence) is shown in Figure VI-3. While it is possible that DHE may compete for binding to the fastest migrating complex, any competition is only observed at the higher molar excesses of competitor. The region that DHE represents has been footprinted and part of this sequence is protected (Becker, et al., 1987). It is possible that DHE may contain only part of the sequence to which the protein binds in this region in TAT. This would necessarily be accompanied by a change in the affinity of the DNA-protein complex. If this were occurring it could explain why slight competition is observed at only the highest concentrations of competitor. This possibility is supported by the fact that the full-length endogenous DNA abolishes observed complexes at 20 fold molar excess (Figure VI-4). Thus one would expect that 500 fold molar excess of PE56 would compete for one of the complexes, if

indeed one of the proteins responsible for the shift was APF.

A similar competition experiment was performed to determine if the consensus APF binding sequence present in the fragment HH104 (-312 to -208) was binding APF. Figure VI-5 shows that no competition is observed at 50 fold molar excess of PE56.

As a direct test that the oligonucleotide was capable of binding protein, PE56 was used as a labeled probe in the gel-shift assay. Figure VI-6 shows the results with Fao, KRC-7, and PC12 extracts. One major band is detected in the hepatoma cell-lines, and no complexes are observed in PC12 cells. This correlates with previous observations that APF is only found in liver (Cereghini, et al., 1988).

From these observations it is concluded that neither of the consensus APF binding sites in TAT bind APF, since PE56 fails to compete for binding to any of the observed shifted bands. Additionally, the gel-shift patterns from the fragments containing the consensus sequences are identical in hepatic and non-hepatic cells, but no complexes are formed with PE56 and extract from non-hepatic cells.

Earlier experiments by another member of the laboratory suggested that KRC-7 cells produced the variant form of APF. Additionally it was suggested that

glucocorticoids cause KRC-7 cells to produce the adult or non-variant form of APF. It did not seem unreasonable that KRC-7 cells produce vAPF since it has been previously shown that these cells do not make albumin (P. Moore, unpublished observation). A direct correlation between APF and albumin expression and vAPF and the lack of albumin expression has been observed (Cereghini, et al., 1988). It has also been shown that glucocorticoid will induce albumin expression (Nawa, et al., 1986). Thus, it might be possible that glucocorticoid induces APF, which in turn could induce albumin expression. To carry out these experiments it was necessary to have controls for the variant and adult forms of APF. Fao cells and adult rat liver have been shown to have the adult form of APF and produce albumin, while C2 cells (a dedifferentiated rat hepatoma cell-line) produce vAPF and do not make albumin (Cereghini, et al., 1988).

Initial experiments showed only very faint shifted bands, so several adjustments were made in the extract isolation and binding buffers. Most importantly, the additional protease inhibitors pepstatin A, leupeptin, and α_2 macroglobulin were included in the extraction buffers. The binding buffer was also changed so that sharper bands were detected. The glycerol concentration was lowered to

6%, DTT was added to a final concentration of 0.5mM, and the $MgCl_2$ concentration was increased to 7mM. While Cereghini, et al. (1988) use a KCl concentration of 30mM, it was found that 100mM KCl provided much more intense bands (data not shown) so the higher concentration was used.

Again, since it was suggested that glucocorticoid caused KRC-7 cells to produce APF, a time course was performed with extracts prepared from KRC-7 cells exposed to glucocorticoid for different lengths of time. As shown in Figure VI-7, it is apparent that the KRC-7 cells produce adult APF. As controls, KRC-7 extract was compared to extract from rat liver (kindly provided by D. Schlichter) and C2 cells. Liver and KRC-7 extract produce major bands of the same R_f, while that from C2 cells produces a band of higher mobility (vAPF). This is expected based on previous studies (Cereghini, et al., 1988). Glucocorticoid treatment did not have any effect on the production of APF vs. vAPF.

While Fao and KRC-7 nuclear extract produce a minor band of similar mobility to a band in C2 cells, it is believed that this band is not vAPF in Fao and KRC-7 cells. It has been reported that APF and vAPF do not coexist in the cell at any particular time. Competition

experiments would only indirectly address this discrepancy. More direct determination would require UV cross-linking of the excised bands for a determination of the molecular weight of the proteins that are responsible for the observed shift. Multiple bands have been seen with PE56 by other investigators (Cereghini, et al., 1988). It might also be possible that this band is seen because of differences in abundance (due to the procedure used for the extract preparation) or because of differences in autoradiographic exposure. Multiple bands are also seen using rat liver extract (Figures VI-7 and VI-8). At any rate, the major observed shifted bands in liver, Fao, KRC-7, and C2 cells are in correlation with that reported in the literature in that the C2 cells produce a band with a smaller Rf than that seen in liver, Fao, and KRC-7 cells.

Figure VI-8 compares the extracts isolated from rat liver, and from Fao, KRC-7, and C2 cells that were either left on serum, or changed to serum-free medium plus or minus dexamethasone for 2 days. Fao and KRC-7 cells produce APF as does liver. Glucocorticoid treatment does not influence this. C2 cells produce vAPF and the aforementioned conditions do not influence its production. As further evidence that KRC-7 cells and Fao cells produce the same factor that binds to PE56, a competition experiment was performed comparing the two cell-lines.

Both Fao and KRC-7 nuclear extract show similar displacement, and the binding of protein to PE56 in both cell-lines is displaced 50% by 1 to 2 fold molar excess of cold PE56 (Figure VI-9).

From these data it is concluded that none of the treatments tested--serum, glucocorticoids, or serum withdrawal--cause any change in the expression of APF or vAPF in Fao, KRC-7, or C2 cells. Thus, while it is an interesting hypothesis that glucocorticoids induce or maintain the non-variant form of APF, this theory cannot be supported experimentally at the present time. In these cells it would seem that there is not a correlation between albumin expression and the adult form of APF. It could be that the KRC-7 cells are deficient in some other factor that is required for albumin expression, assuming there are no mutations in the albumin gene, since as mentioned earlier, multiple trans-acting factors are required for expression of the albumin gene (Costa, et al., 1989; Li, et al., 1988).

CHAPTER VII

DISCUSSION

To the author's knowledge, insulin effects on TAT remain the only example of a hormone increasing an enzymes activity while decreasing the transcription of its mRNA. This paradoxical phenomena has only been observed in KRC-7 cells. Fao cells show an increase in TAT transcription and activity after insulin treatment (Crettaz, et al., 1988) similar to what is observed in adult rat liver (Lee, et al., 1988). Our current model is that the KRC-7 clone represents a neonatal rat liver phenotype. This is based on reports that suggest insulin is the agent that is responsible for suppressing TAT activity until after birth (Cake, 1986) and that insulin will inhibit TAT transcription in the neonatal liver (Johnson, et al., 1988), a time when TAT activity begins to appear. With regards to TAT alone the cells fit this model. Further characterization of the KRC-7 clone will be required to determine if KRC-7 cells can still be considered neonatal with regards to other markers of differentiation.

Several possible mechanisms by which insulin might regulate TAT have been investigated. The half-life of TAT mRNA was measured in the presence and absence of insulin.

Since there were no detectable difference in the half-life of TAT mRNA under these two conditions it was concluded that stabilization of TAT mRNA cannot contribute to the observed increase in TAT activity after insulin treatment. While it has been reported that other mRNAs can be stabilized by estrogen (Brock and Shapiro, 1983) and by mitogens (Levin, et al, 1986), no such stabilization of a specific mRNA has been reported to occur as a result of insulin treatment. Insulin is known to have several general effects that include an increase in RNA synthesis (Hill, et al., 1981; Miller, 1988), an increase in protein synthesis (Miller, 1988; Hansson and Ingelman-Sundberg, 1985), and a decrease in the rate of protein degradation (Draznin and Trowbridge, 1982; Moore and Koontz, manuscript submitted). It is possible that the observed increase in TAT activity is non-specific, since the half-life of TAT, which is about 2 hours, is much shorter than the half-life of total protein, which is about 80 hours (Schimke, 1970). Thus, if TAT is stabilized as is long-lived protein, its activity would appear to increase specifically due to its rapid turnover (for review see Schimke, 1970).

The TAT 5′ flanking region obtained from the chimeric gene has been subcloned and partially sequenced. These

subclones and sequence information provide an essential base which can be utilized in future experimentation. Very little is known about the regions of DNase I hypersensitivity which have been shown to interact with protein. It is natural to assume, however, that they do play some role in the transcription of the TAT gene.

The function of the region from -2950 to -2561 still remains to be demonstrated. At the present time two possibilities seem reasonable. These are based on the observation that CAT activity in Fao cells transfected with TAT-CAT chimeric genes that either contain or are missing this region is different. That is, both dexamethasone induced and uninduced CAT activities are greater in cells transfected with the chimeric gene that lacks the region from -2950 to -2561 (data not shown).

Due to the recent models about steroid receptor binding cooperatively to the GRE as a dimer (Tsai, et al, 1988), one possibility is that the half-site GRE I acts by competing with the two full-site GREs II and III to reduce the induction by glucocorticoid. This seems unlikely, however, since transfections in the absence of glucocorticoid also show reduced CAT activity using the plasmid that contains this region. Recent evidence has shown that three GREs fused in tandem to a heterologous promoter show no difference in induction of expression by

glucocorticoid from chimeric genes containing two or four GREs fused in tandem (Tsai, et al., 1989). Thus this region may contain some sort of negative enhancer function. Negative enhancers as well as negative GREs (Sakai, et al., 1988) have been reported in other genes (Powers, et al., 1989).

Several DNA-protein complexes were observed with the TAT gene fragment from -1144 to -945. Transient transfection analysis using mutants missing this region have demonstrated that glucocorticoid treatment will cause a greater activity of the reporter gene product (Cheatham and Koontz, 1989). However, this increased activity may be merely a consequence of moving the GREs closer to the start site of transcription, which has been reported by another laboratory (Jantzen, et al., 1987). This region does not appear to be involved in the inhibition of TAT transcription caused by insulin (Cheatham and Koontz, 1989). As mentioned earlier, this region has been footprinted (in vitro) and has also been analyzed by in vivo DMS footprinting (Becker, et al., 1987) in both TAT expressing and non-expressing cells. It was found that in vitro footprinting revealed identical protected regions in expressing and non-expressing cells. In vivo footprints, however, were dissimilar in TAT expressing and non-expressing cells. It was also demonstrated that

methylation prevents the interaction of one of these factors with the DNA.

The aforementioned results correlate with those reported here in that the factors binding to this region are found in non-hepatic cells. It is possible that the inability of one of these factors to bind to this region (caused by methylation) allows for the suppression of TAT expression, but this remains to be demonstrated. At any rate, the sites found to be hypersensitive in this region are only hypersensitive in TAT expressing cells (Becker, et al., 1984), and this region is not necessary for insulin inhibition of transcription (Cheatham and Koontz, 1989).

It has been reported that a liver-specific nuclear factor interacts with the region from -312 to -208 (Lobanenkov, et al., 1988, 1989). Those results differ from those presented here. Furthermore, it is suggested that these differences lie in the ability to inactivate proteases, which would seem to be crucial for the analysis of DNA-protein interactions <u>in vitro</u>. By gel-shift analysis the factors that are interacting with this region are also found in non-hepatic cells. Like other regions of the TAT gene, no function has been ascertained for the region from -312 to -208.

Although Lobanenkov, et al. (1989) report that an AP-1

site exists (from -226 to -218), and that this site is protected from exonuclease III digestion, the homology to AP-1 is quite weak (4 out of 8 bases). That same group claims that phorbol ester induces TAT expression in Morris hepatoma 7777 cells. Those results do not correlate with results found in this laboratory. We find that TAT transcription is inhibited by phorbol ester in KRC-7 cells (P. Moore, unpublished observation). Additionally, it has been found that the expression of the chimeric gene is increased by phorbol ester in KRC-7 cells, but only in the presence of glucocorticoid (P. Moore, unpublished observation). The differences between these results cannot be explained at the present time. It could be possible that DNA methylation and/or the cell-type used is responsible for this discrepancy.

The last region of the TAT 5′ flanking region in which DNA-protein interactions were studied was from -208 to -2. Again it is evident that several binding sites are present. This region has also been footprinted and much of the sequence was found to be protected (Becker, et al., 1987). There are three areas of CCAAT box homology, but these are only weakly protected <u>in vitro</u>. Another region of weak homology is the TATTT sequence located at -30. This region shows strong protection <u>in vitro</u>, but it is

not known whether or not this is due to the TATA box binding protein (Wu, et al., 1987). For the purposes of this study, none of the DNA-protein interactions are altered as a result of hormonal treatment, and furthermore, these nuclear proteins were found in all cell-types examined.

The two consensus APF sites located in the TAT 5′ flanking region were examined to determine if APF might play some role in the tissue-specific expression of TAT. Since an oligonucleotide containing a sequence known to bind APF failed to compete for the binding of any factor in the regions that contain the consensus sequences, it was concluded that APF does not bind to these homologous regions. This is supported by the observation that the factors binding in the 5′ flanking region of TAT appear to be ubiquitous, while APF is found only in hepatic cells.

Lastly, the role of glucocorticoid in APF expression was examined. A time course of glucocorticoid treatment in KRC-7 cells comparing APF and vAPF showed that KRC-7 cells produce APF independently of steroid. Fao cells also produce APF independently of glucocorticoid while C2 cells make vAPF as expected, but again this does not appear to be affected by the presence of the hormone. Competition experiments in Fao and KRC-7 cells showed similar displacement of binding activity, further suggesting that

they produce the same factor that binds to the oligonucleotide PE56.

In retrospect it does not seem unusual that insulin does not cause alterations in DNA-protein interactions in the fragments from -1144 to -945 and -312 to -208 since it has been shown that these regions are not required for the insulin inhibition of TAT transcription (Cheatham and Koontz, 1989). Excluding the GREs, any putative insulin regulatory element must lie between -111 to +62 (Cheatham and Koontz, 1989). While it is possible that insulin does alter DNA-protein interactions in the examined region from -111 to -2, none can be detected at the present time.

How might insulin regulate TAT transcription? Currently there is one favored hypothesis. Insulin might alter protein-protein interactions. This type of interaction has been shown to occur in the c-fos/c-jun complex only recently (Gentz, et al., 1989). Both of these factors are oncogenes and while c-fos is not a DNA-binding protein, it is found complexed with c-jun, which is a DNA-binding protein. Both proteins must be extremely important by virtue of how they were first discovered (i.e. they are oncogenes). The ability to detect protein-protein interactions obviously depends on the conditions used for the assay, and it is possible that the conditions used in

these experiments are not optimal to observe these higher-order complexes.

Another possibility is that insulin changes the conformation (e.g. phosphorylation state) of a protein that is always bound to DNA. As a consequence the transcription rate of TAT is changed by altering the ability of RNA polymerase to make the necessary contacts with recognition sites in the DNA, or with other proteins. Both of these possibilities are related. Finally, it is also possible that whatever factor(s) is affected by insulin treatment is not present in the nuclear extract preparation or is not detected under the conditions used in the assay.

Although the inability of insulin to cause a change in the half-life of TAT mRNA does not seem surprising at present, it eliminated one possibility by which insulin could increase TAT activity. Subsequently, investigations were undertaken to examine insulin regulation at the molecular level. It is now known that proteins that interact with the 5′ flanking region appear to be ubiquitous. In addition although no insulin inducible changes in DNA-protein interactions have been detected, these results provide a basis for further experimentation. It is suggested that future research be performed with a region that is known to confer insulin responsiveness.

Ideally, the smallest fragment that can confer insulin responsiveness to a normally unresponsive promoter would be used. Smaller fragments generally give a more reproducible and a more sensitive gel-shift (P. Moore, personal observation). Although gel-shift analysis has been carried out with a fragment that may contain insulin responsive sequences (HS206), the fragment also contains the region from -208 to -111, which is not necessary for the insulin response. It is further suggested that the sequences around the GREs be tested for insulin responsiveness by transfection of an oligonucleotide (in front of a heterologous promoter) that contains only the GREs. It can then be known with more certainty that the region from -111 to +62 contains insulin responsive elements. Smaller probes could then be constructed to simultaneously test for insulin inducible alterations in DNA-protein interactions and for functionality in a transient transfection assay. Thus the examination of DNA-protein interactions would have more meaning, in addition to providing the investigator with a higher probability of obtaining "positive" results.

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APPENDIX

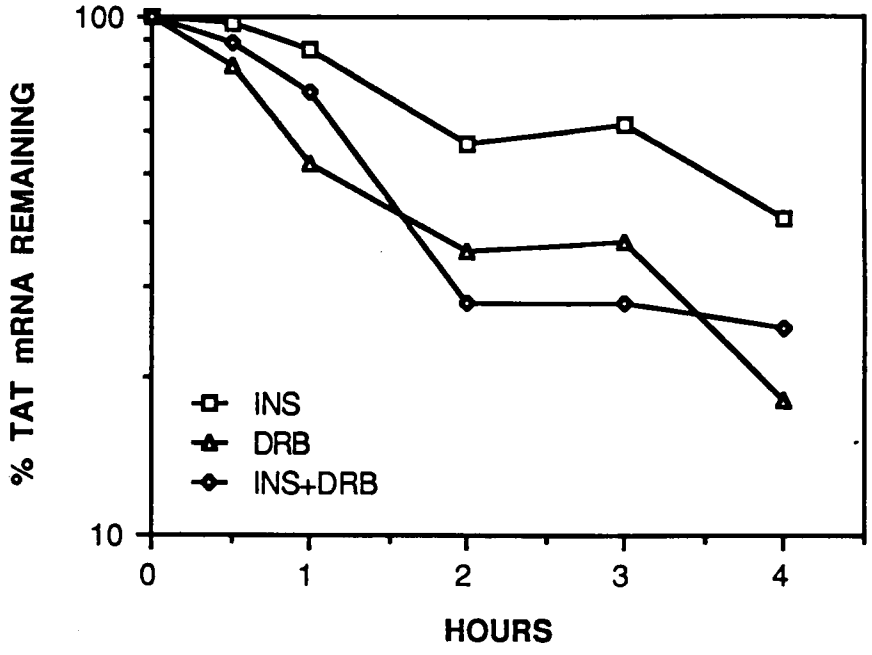

FIGURE III-1. Effect of Insulin on the Half-Life of TAT mRNA. RNA was prepared and analyzed as described in Materials and Methods. Legend is indicated in the figure.

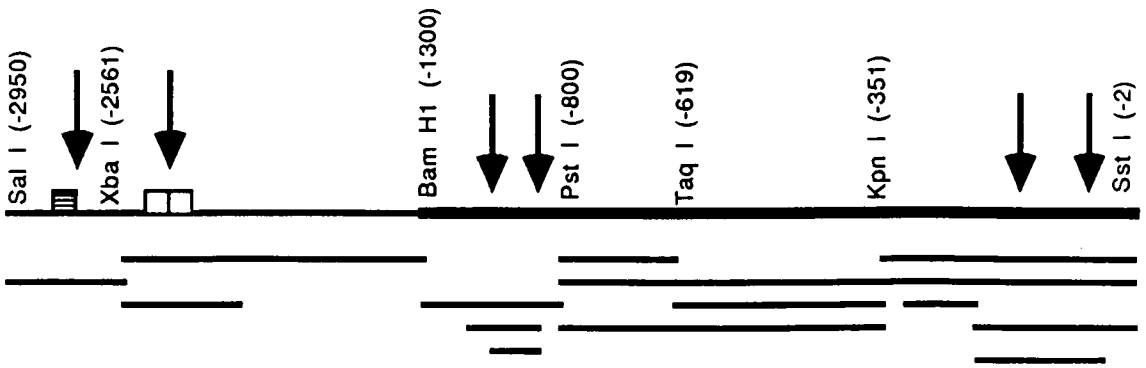

FIGURE IV-1. Map of the 5′ Flanking Region of TAT and Clones Constructed. Arrows indicate regions of DNase I hypersensitvity as reported by Becker, et al. (1984). GRE's are indicated by open boxes (full-site GRE's) and by the striped box (half-site GRE). GRE's are hypersensitive only after glucocorticoid treatment. Thick line indicates area sequenced. Lines below the flanking region indicate clones constructed.

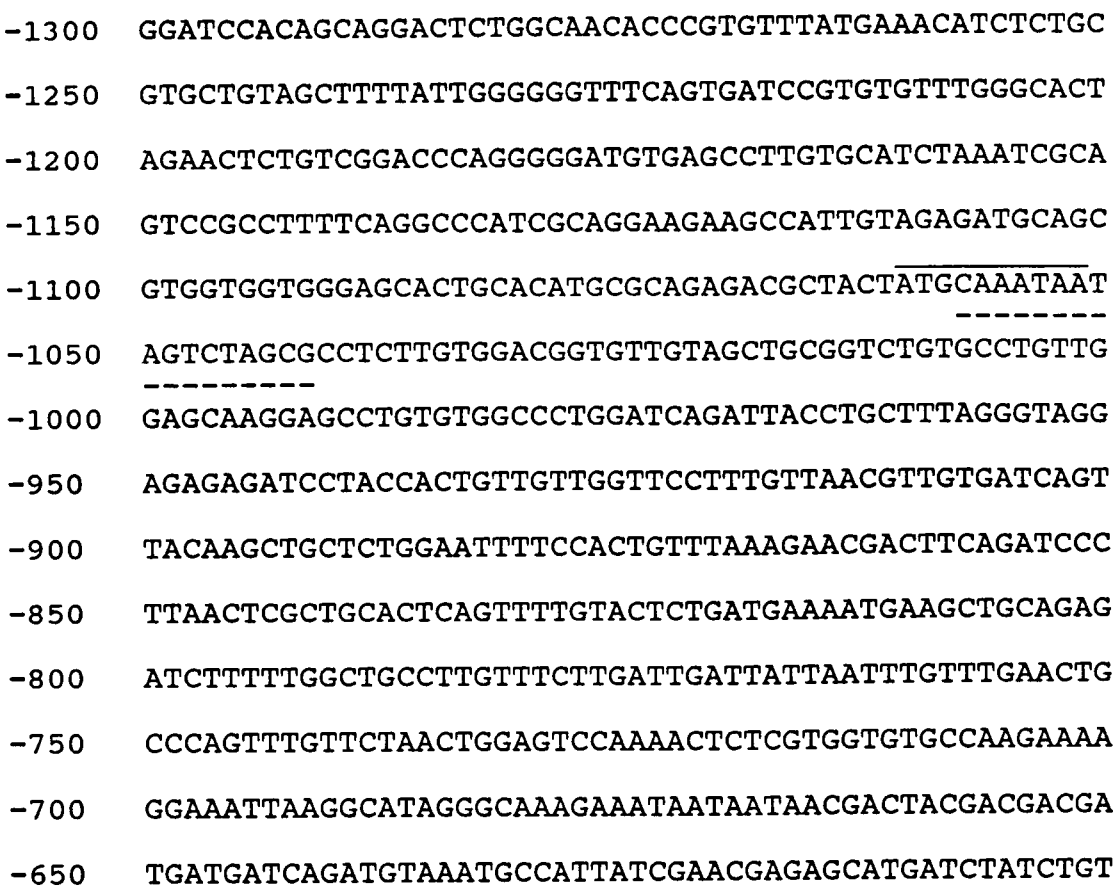

FIGURE IV-2. Sequence of the 5′ Flanking Region of TAT From -1300 to -600. Homology to DNA Topoisomerase recognition site is indicated by a solid line above the sequence. Homology to the APF binding site is indicated by a dashed line below the sequence. The non-coding strand is shown.

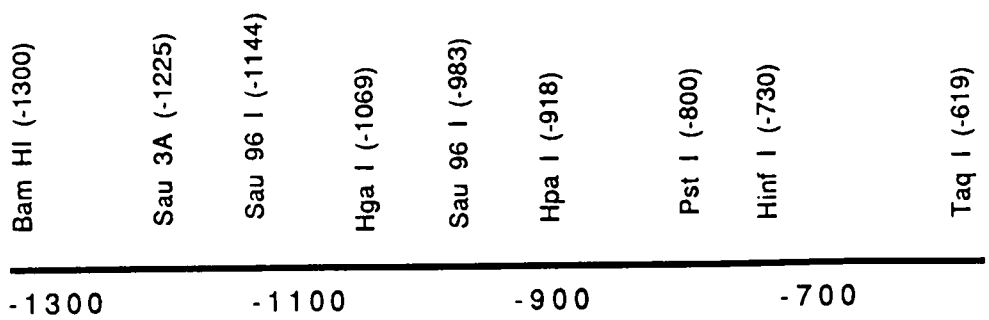

FIGURE IV-3. Restriction Map of the Region From -1300 to -600. Restriction sites are as indicated. Not all restriction sites are shown.

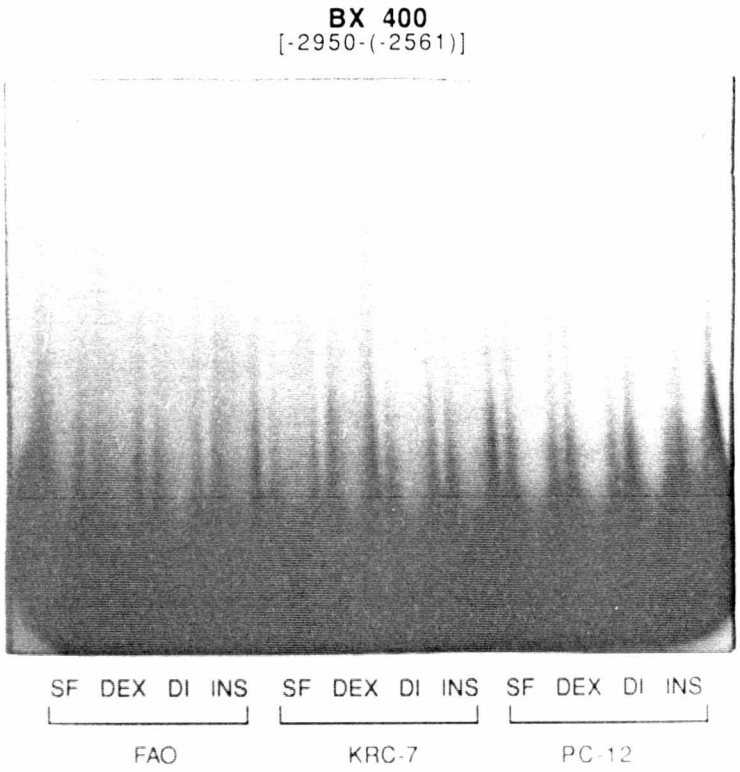

FIGURE V-1. Gel-Shift Analysis of BX400. Cell lines and treatments are indicated below each lane. SF, serum-free media; DEX, dexamethasone (1μM) for one hour; DEX + INS, Dexamethasone plus insulin (10 nM) for one hour; INS, insulin for one hour. Ten μg of nuclear protein extract and 25,000 cpm of labeled probe was used for the assay.

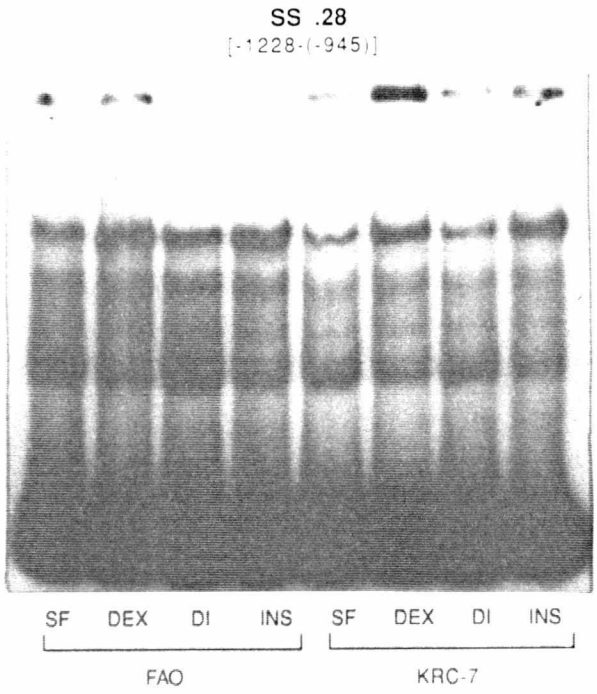

FIGURE V-2. Gel-Shift Analysis of SS.28. Cells and treatments are as indicated in Figure V-1. Five μg of nuclear protein extract and 35,000 cpm of labeled probe was used for the assay.

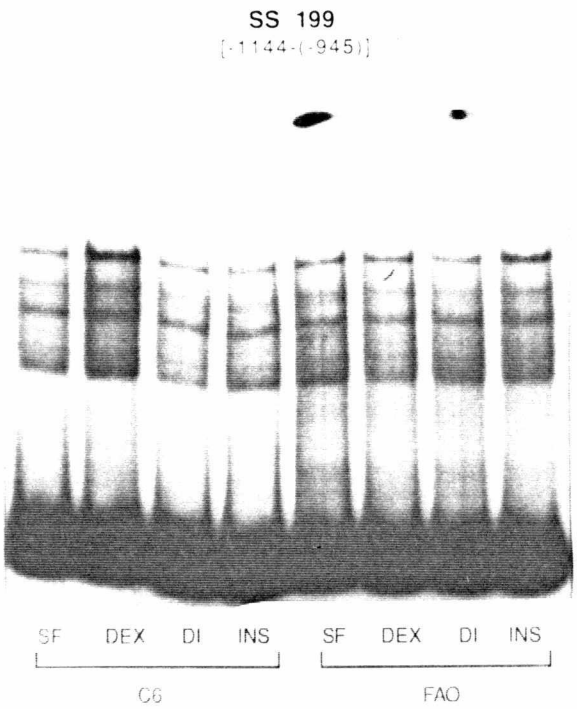

FIGURE V-3. Gel-Shift Analysis of SS199 in C_6 and Fao Cells. Treatments are as indicated in Figure V-1. Five μg of nuclear protein extract and 25,000 cpm of labeled probe was used for the assay.

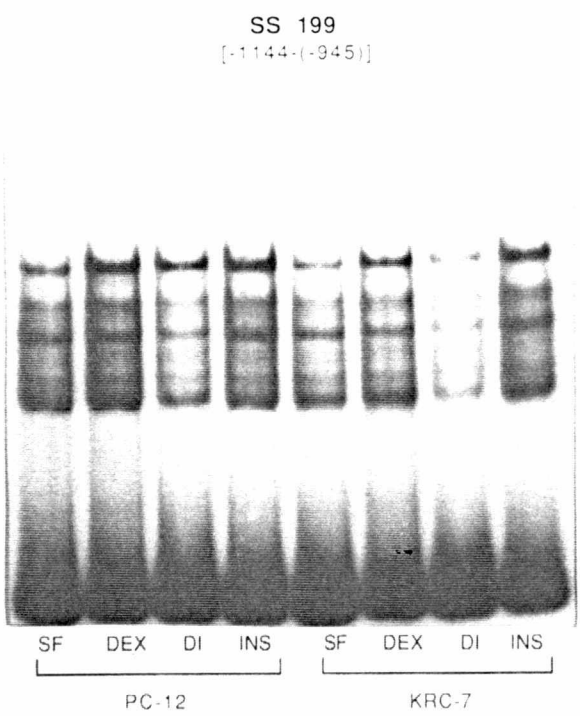

FIGURE V-4. Gel-Shift Analysis of SS199 in PC12 and KRC-7 Cells. Treatments are as indicated in Figure V-1. Five μg of nuclear protein extract and 36,000 cpm of labeled probe was used for the assay.

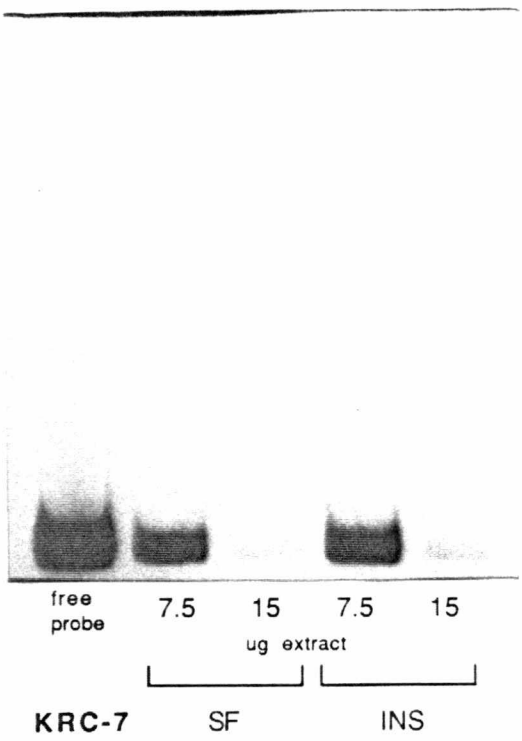

FIGURE V-5. Gel-Shift Analysis of PT181. Treatments are as indicated in Figure V-1. Experiment presented is from KRC-7 cells. 2000 cpm of labeled probe was used for the assay.

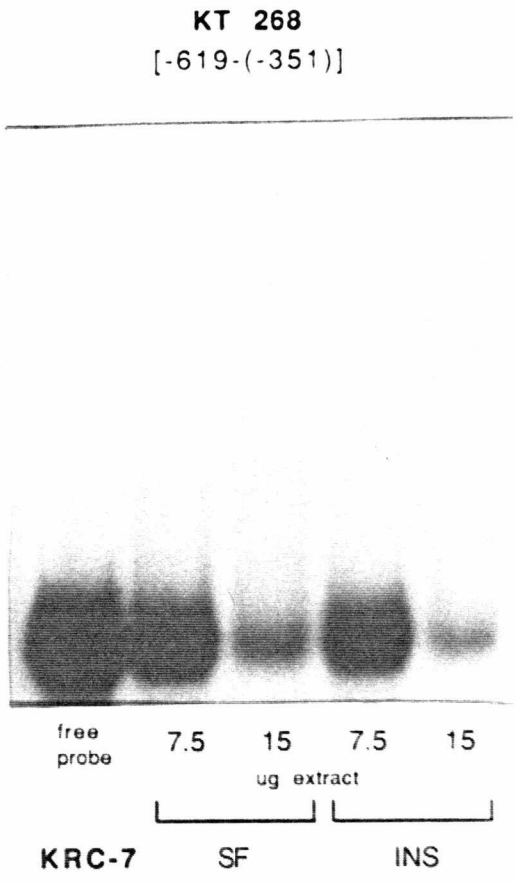

FIGURE V-6. Gel-Shift Analysis of KT268. Treatments are as indicated in Figure V-1. Experiment presented is from KRC-7 cells. 20,000 cpm of labeled probe was used for the assay.

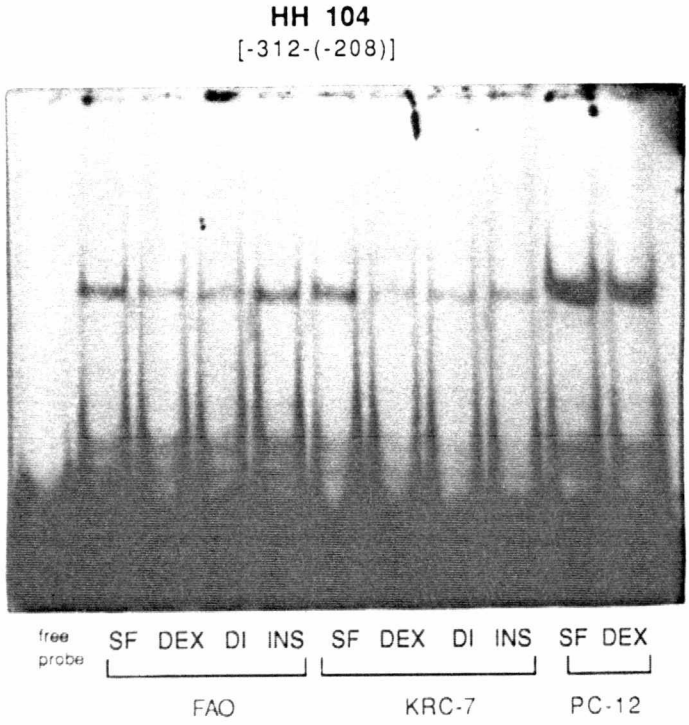

FIGURE V-7. Gel-Shift Analysis of HH104. Cells and treatments are as indicated in Figure V-1. Five μg of KRC-7 and Fao nuclear protein extract and 10 μg of PC12 nuclear protein extract was used for the assay. 24,000 cpm of labeled probe was used.

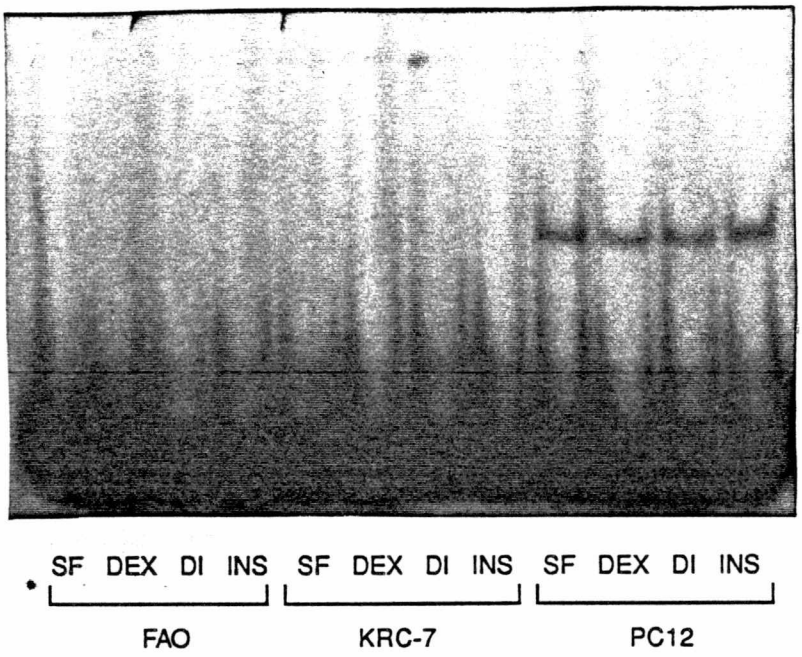

FIGURE V-8. Effect of Benzamidine on the DNA-Protein Complexes With HH104. Cells and treatments are as indicated in Figure V-1. 10 μg of nuclear protein extract and 28,000 cpm of labeled probe was used for the assay. Fao and KRC-7 extracts were prepared in the absence of benzamidine. PC12 extract was prepared in the presence of benzamidine.

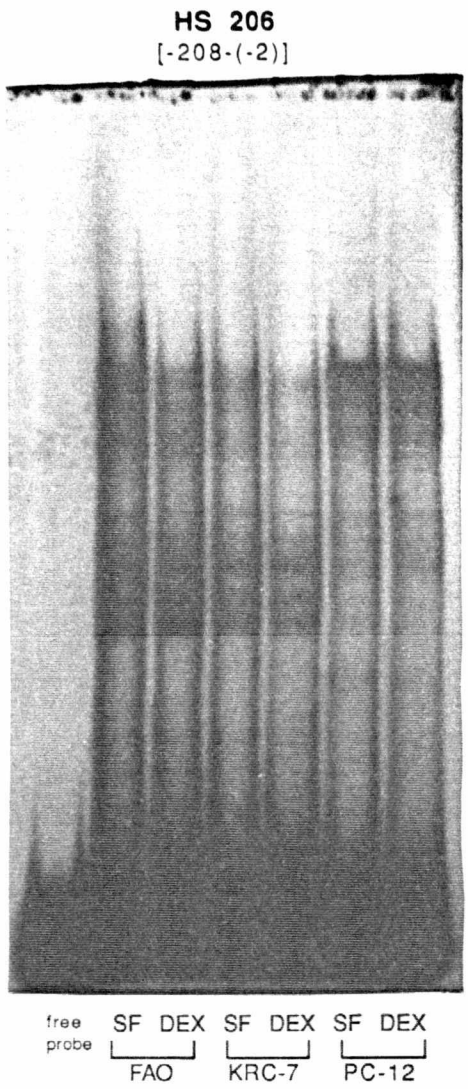

Figure V-9. Gel-Shift Analysis of HS206. Cells and treatments are as indicated in Figure V-1. Five μg of nuclear protein extract and 29,000 cpm of labeled probe was used for the assay.

A.

```
ALBUMIN  -60   TGGTTAATGATCTACAG   -45
                  **          *
  TAT   -1060  CAAATAATAGTCTAGCG    -1045

CONSENSUS    G/AAGTTAATNNTCTACA/C
                       **   * **
  TAT    -275  AAGTTATCACTGTCACC   -260
```

B. PE56

```
5' GATCCGTGGTTAATGATCTACAGTTAT  3'
    3' GCACCAATTACTAGATGTCAATAGATC  5'
```

DHE

```
5'  GATCCTGCAAATAATAGTCTAGCGCCT  3'
    3'  GACGTTTATTATCAGATCGCGGAGATC  5'
```

FIGURE VI-1. Consensus APF Sites and Oligonucleotides Synthesized. A. Consensus sequences found in TAT compared to the binding site in albumin and the consensus binding site. B. Oligos synthesized. Strands are depicted after annealing. Astericks above TAT indicate mismatches with consensus sequence.

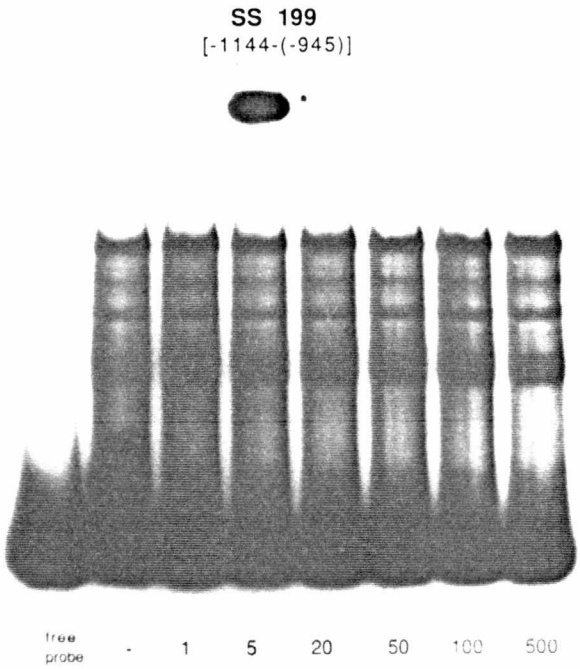

FIGURE VI-2. Competition of PE56 for Binding in SS199. Five μg of nuclear protein extract from Fao cells on SF medium for two days was used for the assay. 23,000 cpm (11 ng, 0.083 pmol) of labeled probe was used.

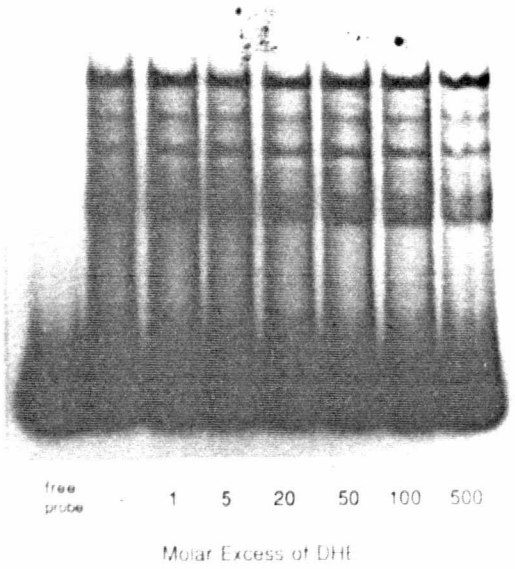

FIGURE VI-3. Competition of DHE for Binding in SS199. Five μg of nuclear protein extract from Fao cells on SF medium for two days was used for the assay. 23,000 cpm (11 ng, 0.083 pmol) of labeled probe was used.

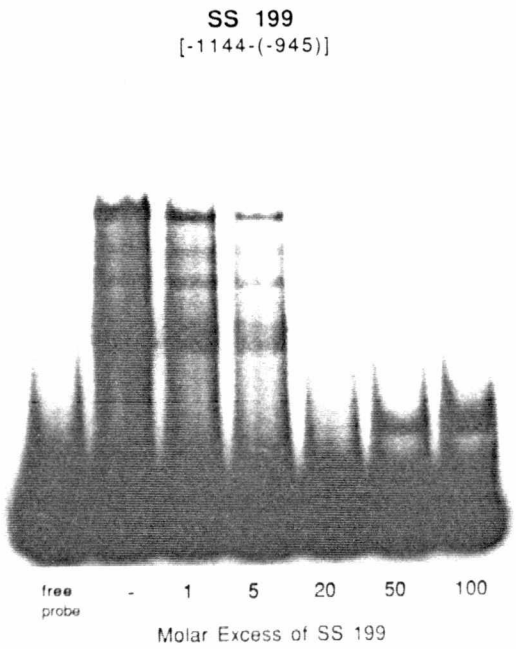

FIGURE VI-4. Competition of SS199 for Binding With Itself. Five μg of nuclear protein extract from Fao cells on SF medium for two days was used for the assay. 23,000 cpm (11ng, 0.083 pmol) of labeled probe was used.

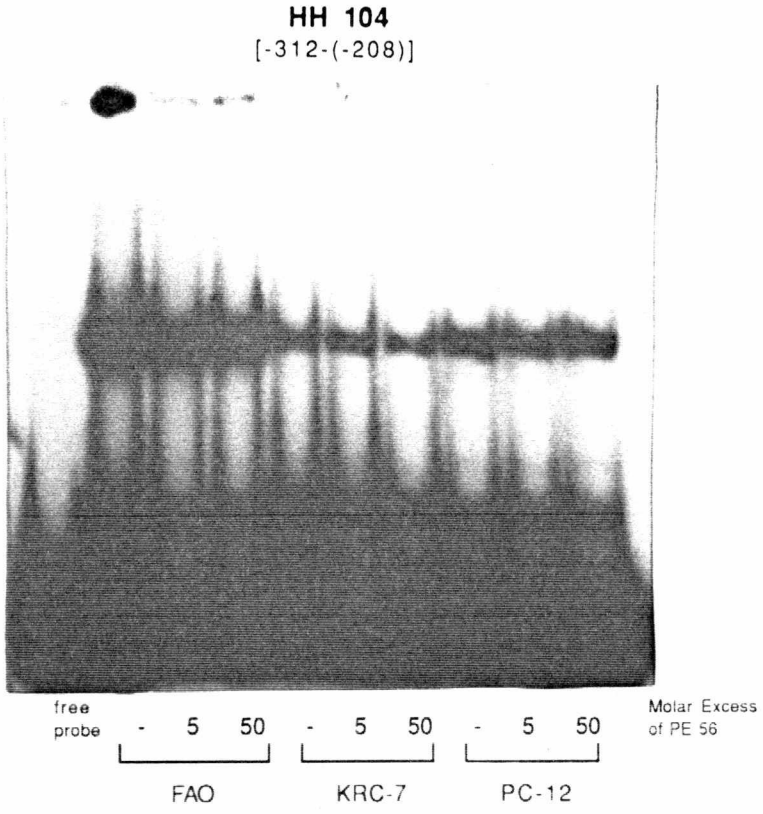

FIGURE VI-5. Competition of PE56 With Binding in HH104. Five μg of nuclear extract protein from the indicated cell-lines that had been on SF medium for two days was used for the assay. 30,000 cpm (8.7 x 10^7 cpm/μg) of labeled probe was used for the assay.

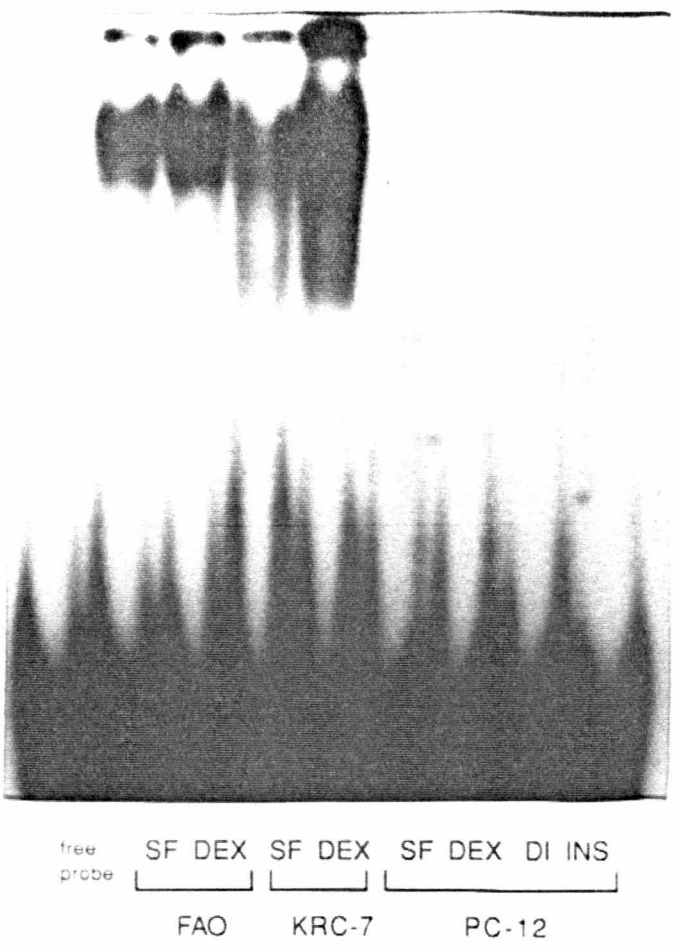

FIGURE VI-6. Gel-Shift Analysis Using PE56 in Fao, KRC-7, and PC12 Cells. Treatments are as indicated in Figure V-1. Ten μg of KRC-7 and Fao extract was used; 20 μg of PC12 extract was used.

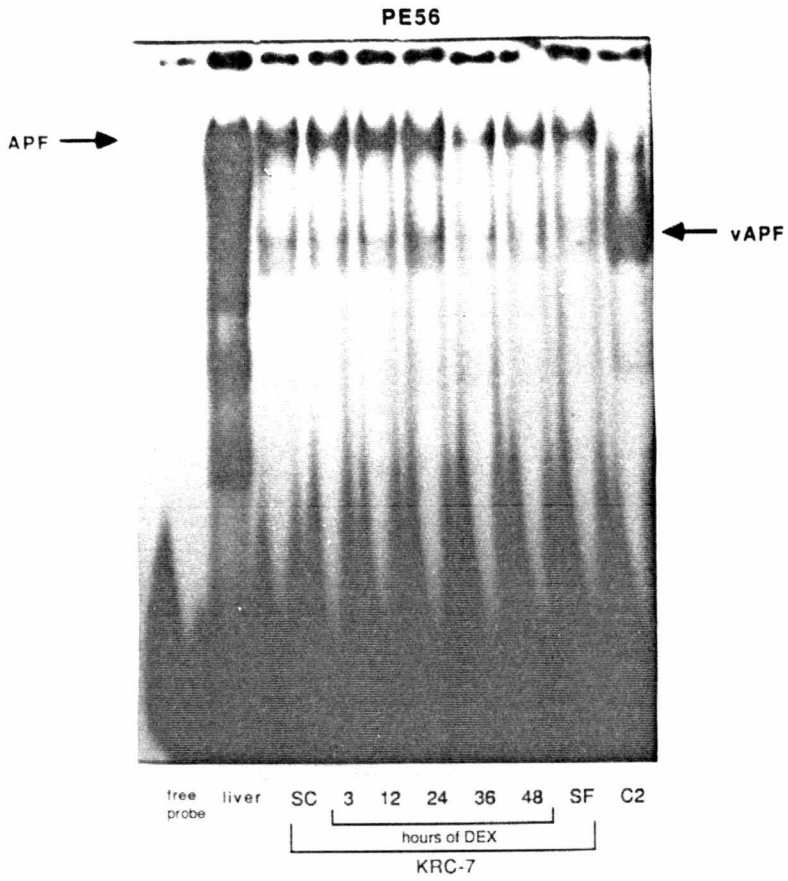

FIGURE VI-7. Effect of Dexamethasone on the Production of APF in KRC-7 Cells. Times are indicated in hours as well as the source of the extract. SC, serum-containing medium. Amounts of extract used for the assay was: 2.5 μg liver, 10 μg KRC-7, and 15 μg C2. Nuclear protein extract from C2 cells that were on serum-containing medium. 30,000 cpm of labeled probe was used for the assay.

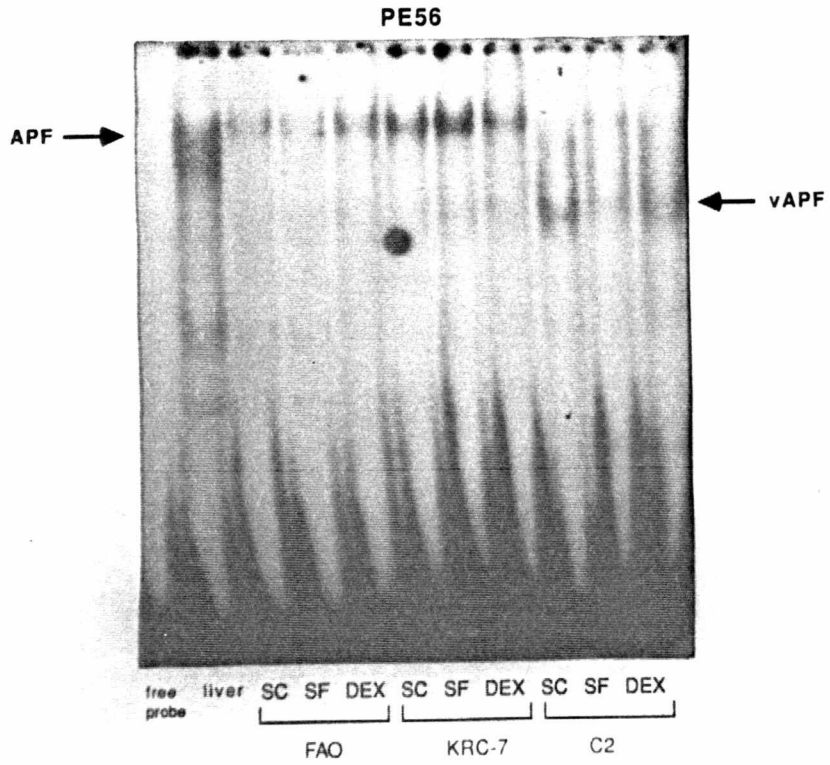

FIGURE VI-8. Effect of Dexamethasone on the Production of APF and vAPF in Fao, KRC-7, and C2 Cells. Treatments were for two days. Amounts of protein used was: 2.5 μg liver, 10 μg Fao and KRC-7, 15 μg C2. 20,000 cpm of labeled probe was used for the assay.

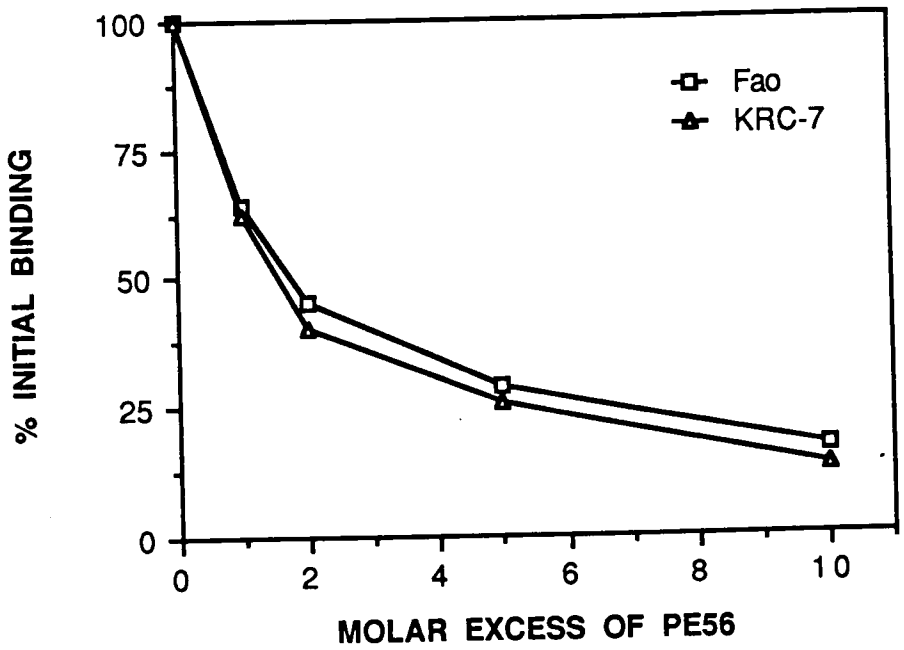

FIGURE VI-9. Comparison of Displacement of Binding of APF in Fao and KRC-7 Cells. Legend is indicated in the figure. % Initial binding was determined by laser-scanning densitometry and normalized to samples with no cold competitor. 5 ng of labeled probe was used.

TABLE III-1

EFFECT OF INSULIN ON THE HALF-LIFE OF TAT mRNA

Experiment #	TAT mRNA Half-Life	
	DRB	DRB + Insulin
1	1.78 (.97)*	1.64 (.98)
2	1.55 (.89)	1.43 (.94)
3	2.02 (.83)	1.79 (.90)
average	1.78	1.62
std. dev.	.24	.18

*Units are in hours. Numbers in parenthesis indicate the correlation coefficient. Details on experimental procedure are given in Materials and Methods and in Chapter III.

TABLE III-2

EFFECT OF DEXAMETHASONE, cAMP, OR PREINDUCTION ON THE HALF-LIFE OF TAT mRNA

Condition	TAT mRNA Half-Life
Dexamethasone	1.82 (.99)*
Dibutyryl cAMP	1.70 (.99)
Preinduction	
DRB	2.05 (.90)
DRB + Insulin	2.14 (.96)

*Units are in hours. Numbers in parenthesis indicate the correlation coeffecient. Details on experimental procedure are given in the Materials and Methods and in Chapter III.

TABLE IV-1

PLASMIDS CONSTRUCTED AND THEIR USES

Plasmid	Region	Restriction Sites	Uses*
pTATKS.35	-351 to -2	Kpn I/ Sst I	A,B,C
pTATKP.45	-800 to -351	Pst I/ Kpn I	A,B
pTATSP.8	-800 to -2	Pst I/ Sst I	D
pTATBX1.3	-2561 to -1300	Xba I/ Bam HI	C
pTATPB500	-1300 to -800	Bam HI/ Pst I	A,C
pTATBX400	-2950 to -2561	Bam HI/ Xba I	B
pTATSS.28	-1225 to -945	Sau3A/ Sau3A	B,C,E
pTATSS199	-1144 to -945	Sau96 I/ Sau3A	B,E
pTATHH104	-312 to -208	Hpa I/ Hpa I	B,E
pTATXT250	-2561 to -2308	Xba I/ Tth111 I	B,F
pTATHS206	-208 to -2	Hpa I/ Sst I	B,C
pTATHA156	-208 to -52	Hpa I/ Apa I	B

*A, sequencing
B, gel-shift
C, further subcloning
D, as a cassette for Bal31 deletion mutants
E, orientation confirmed by sequencing
F, construction of GRE-tk plasmids

VITA

Patrick Shawn Moore was born in Livonia, MI on October 3, 1962. He graduated from The Baylor School in June, 1980 and entered The University of Tennessee in September of the same year. He received a B.A. in March 1984 with a double major in Biology and Philosophy. Loving The University of Tennessee he entered graduate school in the department of Biochemistry in September 1984 and shortly thereafter began research in the laboratory of John Koontz. A Master's degree was awarded to him in December 1986. He continued his work in the same laboratory and received his PhD in Biochemistry in June of 1989. After nine long years at UT, he began postdoctoral research at the *Institut National de la Sante et de la Recherche Medicale* in Strasbourg, France. Patrick is currently still living after failing to attain immortality through the gel-shift assay.