To the Graduate Council:

I am submitting herewith a dissertation written by Douglas S. Carmichael entitled "DNA Regions Mediating Basal and Insulin-Responsive Tyrosine Aminotransferase Transcription." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

John W. Koontz

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Accepted for the Council:

Lew Minkel

Associate Vice Chancellor and Dean of The Graduate School

DNA REGIONS MEDIATING BASAL AND INSULIN RESPONSIVE TYROSINE AMINOTRANSFERASE TRANSCRIPTION

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Douglas S. Carmichael

May, 1993

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ABSTRACT

cis-acting DNA regions which mediate the transcriptional effects of insulin show little similarity among insulin-regulated genes. This study, using mutant promoter constructs transiently transfected into the KRC-7 rat hepatoma cell line, identified an insulin-responsive element (IRE) in the promoter of the gene encoding tyrosine aminotransferase (TAT). This IRE is required for insulin inhibition of both basal and cAMP-responsive transcription, and is located near a basal enhancer (BE) required for full basal expression and cAMP sensitivity. It is also distinct from a more proximal region required for insulin inhibition of glucocorticoid-induced transcription. In addition, the sufficiency of the BE for basal transcription was examined. A proximal TAT promoter region not essential for glucocorticoid-induced expression was found to be required for mediation of the BE's effect.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

The research described in this dissertation revealed the presence of an insulin-responsive element (IRE) in the promoter of a eukaryotic gene. In addition, it identified another region near the gene's start site specifically required for basal expression. This **Introduction** provides the context within which those results may be interpreted. The physiological role of the gene's product and its unique format of developmental, tissue-specific and hormonal regulation are outlined. A description of the multiple phenotypic states seen in liver cells is included to allow assessment of the validity of the experimental system employed here. Finally, the regulatory regions of the gene's promoter known prior to this study are described.

TAT: Metabolic Role of the Enzyme

Tyrosine aminotransferase (TAT; EC 2.6.1.5) is the first and rate-limiting enzyme in the eukaryotic degradation pathway of tyrosine. Complete tyrosine degradation involves four other enzymes, and ultimately yields fumarate and acetoacetate, energy-rich substrates for the citric acid cycle or gluconeogenesis (1). For this reason, and because TAT developmental and hormonal regulation is similar to other enzymes critical for gluconeogenesis (2), TAT is often described as a gluconeogenic enzyme as well. However, the

primary symptoms of its dysfunction do not include hypo- or hyperglycemia. Instead, TAT deficiency (tyrosinemia type II) manifests as neurological disorders (3), probably an indirect result of excess plasma tyrosine.

The enzyme itself serves to transfer the α-amino group from tyrosine to α-ketoglutarate (1). Pyridoxal phosphate is required as a cofactor, and serves as an integral part of the catalytic site, forming a transient covalent linkage with the ϵ-amino group of a lysine residue there (1).

TAT: Developmental, Tissue-Specific and Hormonal Regulation of Expression

In the fetus, TAT activities are low in liver tissue (4). Shortly before birth, however, constitutive or basal activity increases dramatically and permanently, reaching stable levels within 2 days after birth (5,6). This increase occurs only in the alveolar cells of the liver and the proximal tubule of the kidney; all other tissues so far examined retain their low prenatal levels of expression (7). The mechanism of this tissue-selective, temporally specific change is unknown, and has been variously associated with perinatal changes in plasma levels of glucagon (8), glucocorticoids (9) or insulin (10).

Not only is basal activity low before birth, it is also unresponsive to regulation by glucocorticoids (11) or insulin (12). The positive effect of glucocorticoids can

first be detected shortly after birth (5). Insulin has no apparent effect on expression until the immediate perinatal period, when it inhibits the rapidly increasing basal levels (13). Within 48 hours after birth, however, insulin exerts the stimulatory effect upon activity seen in adult liver (13), and presumably, kidney. Insulin also increases cAMP- and glucocorticoid-induced activities in adult liver (14,15).

cAMP can induce liver TAT activity in rats beginning at the 19th day of gestation (11), long before basal activity increases, and before glucocorticoids and insulin can exert their effects. Although glucocorticoids alone have no effect at this stage, they are able to enhance the cAMP-mediated effect (4).

Premature induction of TAT activity in prenatal rats by cAMP produces lasting results: basal levels of $mRNA^{TAT}$ remain elevated after treatment (4). This finding is strong support for the hypothesis that the increased glucagon level observed in neonates is involved at least in part in permanently altering the enzyme's basal expression upward to adult levels. (See further discussion below in this **Introduction** and **Discussion** on the relationship between cAMP-induced and basal levels of gene transcription.)

Several other factors regulate TAT activity, but the developmental changes in their effects have not been examined. Phorbol esters enhance glucocorticoid-induced

activity in adult rats (16), as does epidermal growth factor (EGF) in primary cultures of adult hepatocytes (17). Tyrosine, the enzyme's substrate, can act alone to increase its activity prenatally (18). TAT activities also show circadian (19), cell-cycle (100) and breeding-cycle rhythms (20); prolonged weightlessness has only a marginal effect (21).

TAT and Other Liver Phenotypes

In addition to the developmental states mentioned above, adult liver is capable of expressing specific, different phenotypes under other conditions. Regenerating liver displays a unique complement and format of liver-specific enzyme expression (22). Although similar to fetal liver in many ways, the regenerating liver phenotype shows increased TAT activities (23). TAT activities are also consistently high in the phenotypically unique and varied liver nuclei that are the loci of the liver's response to xenobiotics (24). (Some of those nuclei are the sites of chemically induced tumor growth; see further discussion below in this **Introduction.**)

In addition to these developmental and functional liver phenotypes, other phenotypes have been observed using liver cells in laboratory culture. Cells from fetal livers prematurely develop an adult-like phenotype when cultured (25). It remains uncertain if this change is

identical to the permanent one seen *in vivo*, since some of these adult characteristics fade as the culture ages (26). Even cells from livers of adult animals show this "dedifferentiation" in culture (27). These unstable phenotypes assumed in culture may be different from any developmental state observed *in vivo*.

Hepatoma cells, unlike the primary cultures described above, will grow and divide indefinitely *in vitro* if supplied with serum. At least one rat cell line has even been demonstrated to grow in a defined medium (28). The phenotypes expressed by these cell lines are extremely varied. A few seem to stably exhibit completely adult or completely fetal phenotypes for the characteristics examined so far (29), but even these do not show identical relative levels of those marker enzymes. Most cell lines cannot be easily classified as distinct developmental phenotypes.

All of the most commonly studied hepatoma cell lines have their origins in a few chemically induced tumors from rats, mice or humans. Nonetheless, each tumor is composed of a wide variety of cell types (30), and most cell lines derived from these have been further mutagenized *in vitro*. This, and each tumor's origin in the unique phenotypes of the mutagen-created liver nuclei, undermine the soundness of using any cell line as a strict model of any developmental state.

The KRC-7 rat hepatoma cells used in this study are derived from the Reuber hepatoma cells (31); like many hepatoma cell lines, they exhibit a complex phenotype. Since they are capable of growing in glucose-free medium (data not shown) they may be assumed to possess the fully expressed complement of gluconeogenic enzymes characteristic of adult liver. TAT transcription (32) and alanine aminotransferase activity (S. Sussmane, personal communication) are induced by glucocorticoids, another adult response. TAT activity and transcription are also induced by cAMP (33). However, insulin inhibits basal (33), cAMP- (33) and glucocorticoid-induced (32) transcription.

The KRC-7 cell line remains the only known example in which a hormone produces opposite effects on an enzyme's overall activity and transcription of its encoding gene (33). While insulin represses basal, cAMP- and glucocorticoid-induced TAT transcription in these cells, it enhances overall TAT activity by specifically increasing the half-life of the enzyme.

Insulin also induces an incomplete growth response in these cells (34), imperfectly mimicking its growth effects in regenerating liver (35).

TAT: Transcriptional Regulation and the *cis/trans* Model

In cell systems other than the KRC-7 cell line, a good correlation exists between TAT activity levels and the abundance of $mRNA^{TAT}$ (36), implying that gene transcription is the major point for regulation of overall expression and activity. The minor contribution of post-transcriptional control to TAT expression, with the exception noted above, has been defined by studies of transcription in isolated nuclei. They have so far revealed the primary role transcriptional control plays in all stages of developmental regulation (2), in tissue-specific expression (7), and the responses to several hormones, including insulin, cAMP (33) and glucocorticoids (32).

Logically ensuant to these findings is the search for regions of the TAT gene promoter which mediate those transcriptional effects. Such is the work described in this dissertation. Within the highly successful *cis/trans* model of transcriptional regulation (see (37) for review), those *cis* regions would serve as binding sites for protein factors encoded in *trans*. The abundance, binding affinity or activity of the *trans*-encoded protein would be altered, directly or indirectly, by the regulating agent; this change would, in turn, alter the *trans* factor's effect on transcription.

Unfortunately, the mechanism of any *trans* factor's effective interaction with a competent transcriptional

complex remains unknown. Presumably, that effect is a result of the physical interaction between bound *trans*-acting factors and the initiation complex and its associated factors (37). That physical interaction, which might require multiple "linker" or "mediator" proteins, could be stable or transient. In either case, if *trans* factors remain bound to their *cis* elements during or prior to this interaction, the intervening DNA must loop out between those critical *cis* regions. Although many of the assumptions that validate the *cis/trans* theory are untested, locating a *cis*-acting region still enables the researcher to isolate the *trans*-encoded factor that binds it, and begin to close the gap between the original signal and the modulated transcription that results.

The signals which regulate the function of the *trans* factor can be hormones, or any of the still largely unknown factors which regulate tissue specific expression. Developmental regulation of transcription seems, at first glance, a less comfortable fit for the *cis/trans* model, since epigenetic changes involving nucleosomes, base methylation or nuclear matrix interactions are often invoked to explain the developmental transcriptional changes observed. Nevertheless, such epigenetic changes, if gene specific, would require gene specific sequences to act upon. A protein capable of recognizing those sequences would also be required, if only to direct changes in nucleosomal

spacing, for example. Such a protein would necessarily be regulated by signals produced in a fixed temporal pattern for development to occur properly.

TAT: Regulatory Regions of the Gene

(See **Figure 1** for summary of TAT promoter structure.)

The Proximal Promoter: (See **Figure 2** for summary of proximal promoter structure.) The high basal TAT transcription observed in adult liver tissue is paralleled by DNase I hypersensitivity in the proximal promoter; this hypersensitivity is restricted to the first 220 base pairs upstream of the transcriptional start site and is not observed in non-liver cells (38).

Several segments in this region are DNase I footprinted by nuclear extracts from adult liver cells (39). This footprint is tissue-specific in that nuclear extracts from non-liver cells produce a different footprint (R. Peterson, personal communication). CCAAT sequences (40) are found centered at -42, -72, -167, -226, -285 and -297 (41). A TATTT sequence located at -29 is assumably the site for TATAA box-binding factor binding and assembly of the initiation complex (42).

This region alone (-351 to +62) is capable of driving low but significant basal transcription measurable by *in vitro* transcription (43) and transient transfection assays (data not shown), and at least two parts of the

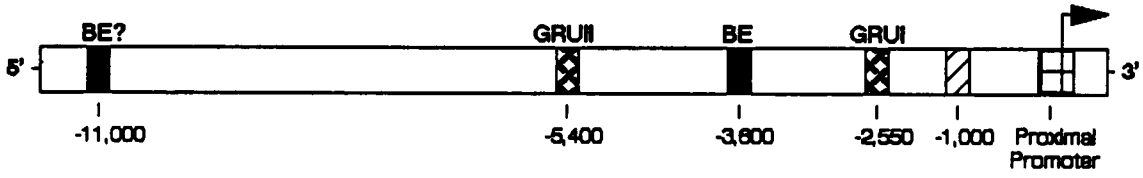


FIGURE 1 - *cis*-Acting Regions of the TAT Gene. Shown are regions from approximately -12,000 to +62. The arrow represents the start site of transcription. All of the indicated regions are DNase I hypersensitive. BE = basal enhancer (38, 57); GRU = glucocorticoid-responsive unit (38, 56).

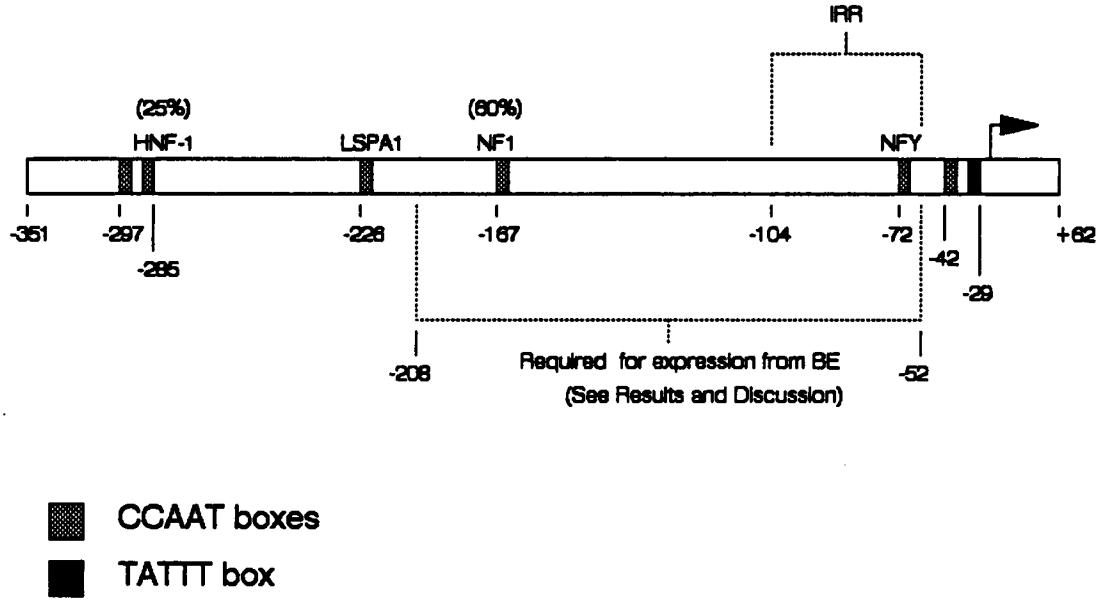


FIGURE 2 - Summary of TAT Proximal Promoter Structure. Shown are sequences from -351 to +62. Percentages above HNF-1 and NF1 sites indicate their respective portion of basal expression from this region alone. The arrow indicates the start site and direction of transcription. The role of the -208 to -52 region is described in **Results** and **Discussion.** HNF-1 = hepatic nuclear factor 1 (45); LSPA1 = liver specific protein A1 (46); NF1 = nuclear factor 1 (44); NFY = nuclear factor Y (48); IRR = insulin-responsive region (Li Pan, personal communication); BE = basal enhancer (38,57).

region have been demonstrated to be required for that activity (43). A region from -157 to -171, which contains a CCAAT box, is believed to bind nuclear factor 1 (NF1) (44), and confers 60% of the *in vitro* basal level (43). A region from -266 to -281 binds hepatic nuclear factor 1 (HNF-1) (45), accounting for a further 25% of the whole. A liver-specific protein (LSPA1) binds sequences from -215 to -231 (46), which contain both a CCAAT box and exhibit similarities to the concensus activator protein 1 (AP1) enhancer (47). However, that binding has not been correlated with any transcriptional function of the proximal promoter. Similarly, no functional relevance has been shown for the binding of nuclear factor Y (NFY) to the CCAAT box centered at -72 (43,48).

Previous results from this laboratory suggest that the proximal promoter contains regions required for a hormonal effect on transcription (49). In the same hepatoma cell line used in this study, insulin can inhibit glucocorticoid-induced expression driven by a 250 base pair region containing the entire glucocorticoid-responsive unit I (GRUI, see below) ligated upstream of -351 of the TAT promoter. Insulin sensitivity is retained in constructs truncated to -208 and -104, but is lost when GRUI is ligated at -52 (Li Pan, personal communication) (See **Figure 3**). This delimits a proximal insulin-responsive region (proximal IRR) between -104 and -52.

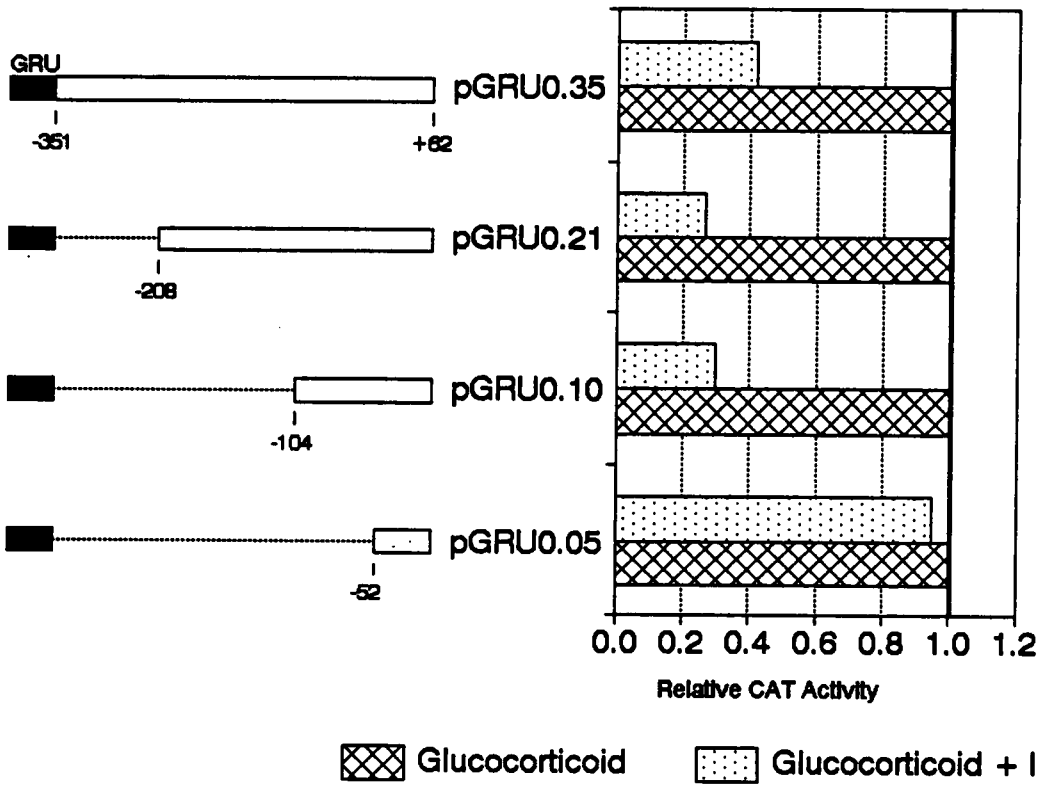


FIGURE 3 - An Insulin-Responsive Region Lies Between -104 and -52. Means of glucocorticoid-induced CAT expression from each plasmid were arbitrarily set at 1; expression after glucocorticoid and insulin (I) treatment is defined relative to those values. Values from glucocorticoid- and insulin-treated cells are significantly different from glucocorticoid-treated cells for all plasmids except pGRU0.05. n=9, 3 experiments for all constructs. GRU = glucocorticoid responsive unit. (After (49) and Li Pan, personal communication.)

The -1,000 Region: A second DNase I hypersensitive region is found between -1,090 and -1,008, and appears only in liver tissue (38). A protein (nuclear respiratory factor, NRF) originally isolated using a binding site from the cytochrome C gene can bind here *in vitro* (50); whether it does so *in vivo* is unknown. This TAT region is similar to the cytochrome C NRF site, and can replace its constitutive enhancing function in a truncated cytochrome C gene construct (50).

Glucocorticoid-Responsive Units I and II: A third DNase I hypersensitive region is found between -2,550 and -2,300, and appears only after treatment with glucocorticoids (38). This region, called glucocorticoid-responsive unit I (GRUI), contains two glucocorticoid-responsive elements (GRE's); both bind the glucocorticoid receptor (51). Only the more distal of the two can function alone in mutant constructs to mediate glucocorticoid induction; the more proximal one is inactive alone but synergistically enhances the effect mediated by the distal GRE (52).

These two GRE's are closely flanked (93) by demonstrated binding sites for CCAAT-enhancer binding protein (C/EBP) (53) and hepatic nuclear factors 5 (HNF-5) (54) and 3 (HNF-3) (94). Both are required for the full glucocorticoid effect conferred by GRUI, but become

incrementally less requisite as the entire region is moved closer to the start site (55).

Another GRU (GRUII) is located at -5,400, but it displays glucocorticoid-independent DNase I hypersensitivity (56). That region, too, contains two dissimilar GRE's which act similarly to those found in GRUI. However, the two GRU's are not functionally identical: promoters truncated to delete GRUII retain 30% of the original level of responsiveness driven by both GRU's. GRUI, but not GRUII, can drive glucocorticoid-induced transcription from a heterologous promoter (56). Thus GRUII can act to supplement GRUI's effect, but unlike GRUI, cannot act independently.

The Basal Enhancer (BE): (See **Figure 4** for summary of BE structure.) Another region of DNase I hypersensitivity occurs at -3,600 and is present only in liver tissue (38). This region mediates a basal level of transcription 20 to 50 fold greater than that seen with the proximal promoter alone ((57) and data not shown). Site-directed mutagenesis has indicated the presence of at least two discrete, mutation-sensitive elements within that region, designated BI and BIII (58). BI, the more distal element, contains an imperfect, asymmetric cAMP-responsive enhancer (CRE). Dimers of BI confer some basal expression and apparently complete cAMP responsiveness to a heterologous promoter. The BI sequences also show a transient footprint after cAMP

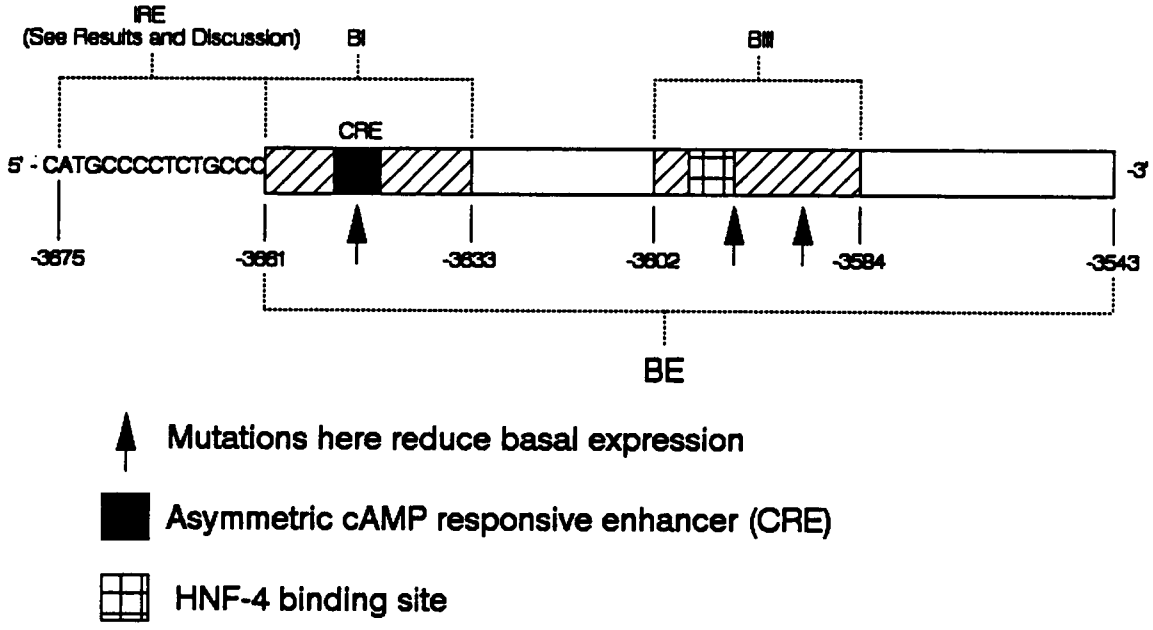


FIGURE 4 - Summary of the Basal Enhancer's (BE's) Structure. Shown are TAT sequences from -3,675 to -3,543. The BE spans -3,661 to -3,543. The role of the IRE (insulin-responsive element) is described in **Results** and **Discussion.** HNF-4 = hepatic nuclear factor 4 (96).

treatment, and can bind a variety of CRE-binding proteins (CREB's) (58,59). CREB binding to this "weak" CRE is enhanced after phosphorylation by cAMP-dependent protein kinase (95). Appropriate 3 base pair substitutions in the CRE within BI can abolish the cAMP response mediated by the entire region in its native promoter context, as well as reduce basal levels of transcription (58).

BIII also is important in basal expression. Dimers of BIII drive greater basal expression from a heterologous promoter than do dimers of BI, but fail to mediate any cAMP response (58). Appropriate 3 base pair substitutions in BIII virtually abolish basal transcription mediated by the entire region. In vivo, BI and BIII may act together for full basal expression from the BE.

The BIII region binds HNF-4 (96), a member of the steroid receptor superfamily (97). It also shows similarities to the hepatitis B viral enhancer (58), and has been reported to bind a single-stranded DNA-binding protein (60,61). No transcriptional effect has been associated with that binding, however.

Although BIII appears dispensable for cAMP responsiveness in constructs containing dimers of BI, BI itself may be insufficient for full sensitivity, in view of a report of significant cAMP induction mediated by a TAT promoter truncated at -3,000 (62).

In addition to its role in basal and cAMP-sensitive transcription, BI also appears to be the target for a mechanism that confers part of the tissue-selective expression of TAT. A *trans*-encoded protein, tissue-specific extinguisher 1 (TSE1) can decrease binding of CREB to BI and repress basal transcription (58). TSE1 has been shown to be an inhibitory regulatory subunit of cAMP-dependent protein kinase (RIα) and is most abundant in cells which do not express TAT (63). It has also been suggested that TSE1/RIα displays a temporal pattern of expression which might allow it a role in developmental regulation of TAT as well (63).

The effect of TSE1/RIα can be overcome by cAMP (58), a phenomenon not observed in fully extinguished non-liver cells. Thus TSE1/RIα may not be sufficient for full tissue-selective regulation of TAT. This latter hypothesis has been supported by studies of chromosomal mutants in which the repressed transcription seen in TSE1/RIα-expressing cells is not overcome by cAMP (64).

Non-liver cells do not express HNF-4 (97). Thus it appears that although its *cis* binding site within BIII is dispensable for cAMP sensitivity and imparts only a portion of basal expression from the entire BE, it may be integrally involved in extinction of the BE's activity in non-liver tissue.

The BE has been further implicated in a unique form of transcriptional control (65,66). Termed a glucocorticoid

modulatory element (GME) in this context, the region has been shown to be required for recreation of *in vivo* ratios of sensitivity to glucocorticoids versus their inhibitory analogs. A mechanism for such a function is not intuitively accessible, nor would the function itself appear to impart any selective advantage. Thus, further dissection and explanation of this phenomenon is required before it may be integrated into our current understanding of this complex region.

Mutations in the the regions immediately flanking the BE upstream and downstream do not affect basal expression (58). However, the results of this study show the importance of the immediate upstream region for insulin responsiveness. (See **Results** and **Discussion**, below.)

Deletion analysis suggests that another basal enhancer exists beyond -11,000. If that more distal enhancer is deleted, basal levels are reduced 60% (57). If the BE at -3,600 is deleted, however, the distal enhancer alone imparts only barely detectable basal transcription. Thus, in a manner analagous to the two GRU's described previously, the activity of the more proximal BE can be supplemented by the -11,000 region, but that enhancer cannot act independently. This distal basal enhancer binds three different members of the HNF-3 family (93); two of those are absent in non-liver tissue. The region exhibits liver-

specific DNase I hypersensitivity and activity (93), and those binding events may regulate this effect.

Summary

In summary, with respect to basal transcription of TAT, three separate regions have been shown to be involved. The proximal promoter (-351 to +62) is capable of driving minimal but significant basal expression; that level is only about 2% of that seen when the BE at -3,600 is present. Another enhancer beyond -11,000 confers further basal activity.

The low level of basal transcription driven by the proximal promoter depends upon the binding of two proteins, NF1 and HNF-1, but the nature of their interaction with the initiation complex has not been determined. The BI region in the BE at -3,600 appears critical for higher basal levels, cAMP inducibility and tissue-specific expression, but may not be sufficient for any. BIII is required for full basal expression from the BE, and may be required for full tissue-specific regulation of its activity. The requirements for BE function within the TAT promoter have not been previously examined. The work described in this dissertation revealed the dependence of BE function on a proximal promoter region.

Previous results from this laboratory comprise the sole source of information on promoter regions required for

insulin-responsive TAT transcription. They have demonstrated the importance of the -104 to -52 region (IRR) in insulin modulation of the glucocorticoid effect on transcription. Results of this study indicate the location of a more distal insulin-responsive element (distal IRE) distinct from the IRR. In the KRC-7 cell line, this new IRE mediates insulin inhibition of both basal and cAMP-induced transcription.

METHODS

Cell Culture

KRC-7 cells were grown in 100 mm x 20 mm Falcon Primaria dishes in Dulbecco's Modified Eagle's Medium (DME) (1 mg glucose/ml) plus 10% iron-supplemented calf serum (HyClone) at 37°C with 10% CO_2 in the air. Penicillin was added to 100,000 units/L; streptomycin sulfate to 100 mg/L. Stocks were maintained in liquid nitrogen; no more than 10 passages were used before restarting working cultures from stock. (Cells were frozen slowly over a 3 hour period in DME + 5% fetal calf serum, 5% iron-supplemented calf serum, 5% dimethyl sulfoxide (DMSO). Cells were thawed quickly at 37°C and plated into DME + 10% serum.) When confluent, cells were removed from dishes with 0.05% trypsin in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.3) and replated at less than 10% confluency.

Hormone Treatment

Porcine insulin (Elanco, lot # 7RH87D, 26.2 USP units/mg) was dissolved in 100 mM HCl at a concentration of 1 mg/ml and stored at 4°C. For addition to cell cultures, 6 ul of this solution were dissolved in 994 ul of 1 mM HCl and then added to 10% in medium in the dish. Final insulin concentration was therefore 10 nM, assuming a molecular

weight of about 6000 for insulin. 8-bromo cAMP was generously provided by W.D. Wicks and was used at a final concentration of 2 mM.

Protein Assay

Standard samples (0 to 20 ug protein) were prepared in 800 ul H_2O from a 1 mg/ml bovine serum albumin stock. Test samples of constant volumes of cell extract (eg. 10 ul) were also brought to 800 ul with H_2O. To each, 200 ul of Bradford's concentrated dye reagent (67) were added. (For 100 ml of Bradford's reagent: 100 mg Coomassie Blue G, 50 ml methanol, 100 ul 85% H_3PO_4, 50 ml H_2O.) Samples were mixed vigorously and allowed to stand at room temperature for 5 minutes before measuring the optical absorbance at 595 nm on a Shimadzu UV-160 spectrophotometer. Test absorbances were interpolated into the standard curve to determine protein concentrations.

Plasmids

Schütz Constructs: The following plasmids, generously provided by Günther Schütz, were used in transfection experiments and in construction of other plasmids used in this study.

pBLCAT3 (68) contains the coding region of the bacterial chloramphenicol acetyltransferase (CAT) gene ligated downstream of a multiple cloning region and upstream of a

simian virus (SV40) polyadenylation region. It also contains a constitutively expressed bacterial gene encoding a transmembrane protein that imparts ampicillin resistance.

pBLCAT2 (68) is identical to pBLCAT3 except that it contains the proximal promoter (-105 to +51) of the *Herpes simplex* virus thymidine kinase (HSV TK) gene upstream of the CAT gene.

pTC3.92 contains TAT sequences from -3,922 to +62 in pBLCAT3 and is identical to pTATCAT-3922/+62 as described in (58).

pTC2.95 contains TAT sequences from -2,950 to +62 in pBLCAT3 and is identical to pTATCAT3 as described in (52).

TTC124 (58) contains TAT sequences from -3,661 to -3,543 (the basal enhancer (BE)) inserted upstream of the viral promoter in pBLCAT2.

Cheatham and Moore Constructs: The following plasmids, generously provided by Bentley Cheatham and Pat Moore, were employed in construction of some of the plasmids used in this study.

pTC2.55 (49) contains TAT sequences from -2,561 to +62 in pBLCAT3 and was built by XbaI digestion of pTC2.95 and religation.

pGRU0.35 contains the TAT sequences from -2,561 to -2,308, which contain GRUI, ligated at -351 of the TAT promoter in pBLCAT3, and is identical to pTC2.55Δ-2,308/-351 as described in (49). Built by insertion of the SmaI/KpnI

fragment from pTC2.55 into KpnI/blunt EcoRI-digested pTATXT250.

pTATXT250 contains TAT sequences from -2,561 to -2,308 in pGEM3 (Promega) and was built by BamHI/TthI digestion of pTATBX1.3, blunt-ending and religation.

pTATBX1.3 contains the TAT seqences from -2,550 to -1,300 in pGEM3 and was built by insertion of the BamHI/XbaI fragment from pTC2.95 into BamHI/XbaI-digested pGEM3.

pTATHS206 contains TAT sequences from -208 to -2 in pGEM3 and was built by HpaI/SmaI digestion of pTATKS.35 and religation.

pTATKS.35 contains TAT sequences from -351 to -2 in pGEM3 and was built by insertion of the SstI/KpnI fragment from pTC2.95 into pGEM3.

Pan Constructs: These plasmids were built and tested by Li Pan. Those results are cited in this work.

pGRU0.21 contains the TAT sequences from -2,561 to -2,308, which include GRUI, ligated at -208 of the TAT promoter in pBLCAT3.

pGRU0.10 contains the TAT sequences from -2,561 to -2,308 ligated at -104 of the TAT promoter in pBLCAT3.

pGRU0.05 contains the TAT sequences from -2,561 to -2,308 ligated at -52 of the TAT promoter in pBLCAT3.

Carmichael Constructs: The following plasmids were built by standard cloning methods (69) using available restriction sites, and were used in transfection experiments

or in construction of other plasmids. The expected structures of pBE0.35a and pBE0.35b were confirmed by DNA sequencing. All other plasmids were analyzed after maxiprep by restriction enzyme digestion. Specifically, a combination of enzymes was chosen that would free fragments of pre-determined size only from the expected structures.

pTC3.67 contains TAT sequences from -3,675 to +62 in pBLCAT3 and was built by religation of SphI-digested pTC3.92.

pTC3.66 contains TAT sequences from -3,661 to +62 in pBLCAT3 and was built by insertion of the PstI fragment from pTC3.67 into PstI-digested pTC3.92.

pBE2.55 contains the BE ligated at -2,550 in pBLCAT3 and was built by insertion of the XbaI/HindIII fragment from TTC124 into XbaI/HindIII-digested pTC2.95.

pBE1.33 contains the BE ligated at -1,300 in pBLCAT3 and was built by insertion of the BamHI/HindIII fragment from TTC124 into BamHI/HindIII-digested pTC3.92.

pBE0.80 contains the BE ligated at -800 in pBLCAT3 and was built by insertion of the SphI/BamHI fragment from TTC124 into SphI/BglII-digested pTC3.92Δ-2,950/-800.

pTC3.92Δ-2,950/-800 is identical to pTC3.92 except for the indicated deletion and was built by religation of BglII-digested pTC3.92.

pBE0.35a contains the BE ligated at -351 in pBLCAT3 and was built by insertion of the XbaI/HindIII fragment from TTC124 into XbaI/HindIII-digested pTC0.35.

pTC0.35 contains TAT sequences from -351 to +62 in pBLCAT3 and was built by XbaI digestion of pGRE0.35 and religation.

pGRE0.35 contains two copies of the more distal GRE (GREII) from the TAT GRUI (See **Introduction**) inserted at -351 of the TAT promoter in pBLCAT3. Built by insertion of a synthetic oligonucleotide containing the GRE's into blunt XmaI/SalI-digested pTC2.55$_{\Delta}$-2,308/-351.

pBE0.35b is similar to pBE0.35a except it was built by insertion of the blunt XbaI/HindIII fragment from TTC124 into SmaI/HindIII-digested pTC2.55$_{\Delta}$-2,308/-351.

pBE0.35c is similar to pBE0.35a except it was built by insertion of the blunt BamHI/HindIII fragment from TTC124 into SmaI/HindIII-digested pTC2.55$_{\Delta}$-2,308/-351.

pBE0.21 contains the BE ligated at -208 in pBLCAT3 and was built by insertion of the XbaI/HindIII fragment from TTC124 into XbaI/HindIII-digested pTC0.21.

pTC0.21 contains TAT sequences from -208 to +62 in pBLCAT3 and was built by insertion of the ApaI/HindIII fragment from TATHS206 into ApaI/HindIII-digested pTC2.95.

pBE0.05 contains the BE ligated at -52 in pBLCAT3 and was built by insertion of the XbaI/HindIII fragment from TTC124 into XbaI/HindIII-digested pTC0.05.

pTC0.05 contains TAT sequences from -52 to +62 in pBLCAT3 and was built by religation of the blunt BamHI/blunt ApaI-digested pTC0.21.

pTC3.67Δ-3,543/-208 is identical to pTC3.67 except for the indicated deletion and was built by insertion of the SphI/XbaI fragment from pTC3.92 into SphI/XbaI-digested pTC0.21.

pTC3.66ΔΔ is identical to pTC3.66 except for two internal deletions from -3,543 to -2,550 and from -2,308 to -351. Built by insertion of the XbaI/HindIII fragment from TTC124 into XbaI/HindIII-digested pTC2.55Δ-2,308/-351.

Other: pSV2PAP (70) was generously supplied by T. Kadesch and contains the placental alkaline phosphatase (PAP) coding region under constitutive control of the Sendai virus promoter.

Preparation of Competent *Escherichia coli*

Strain JM109 commercial stock (Promega) was inoculated into 25 ml Luria broth (LB) (For 1 L: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 ml 1 M NaOH) and incubated overnight at 37°C with vigorous agitation. This culture was added to 400 ml LB in a 4 L flask and incubated at 37°C to an optical absorbance at 600 nm of 0.4. The culture was chilled and pelleted at 1000 x G for 10 minutes at 4°C, then resuspended in 80 ml cold $CaCl_2$ solution (60 mM $CaCl_2$, 15% glycerol, 10 mM 1,4-piperazinediethanesulfonic acid, pH 7.0) (71). This centrifugation and resuspension were repeated twice, resuspending the pellet in 8 ml $CaCl_2$ solution after the third centrifugation. The suspension was

stored on ice for 2 days, then aliquotted and stored at -80°C.

Transformation of Competent Bacteria with Plasmid DNA

1 ug of plasmid DNA (containing a constitutively expressed ampicillin resistance gene) was mixed with a thawed 250 ul aliquot of $CaCl_2$-prepared JM109 and allowed to stand on ice for 30 minutes. The suspensions were warmed to 37°C for 5 minutes, then 1 ml of LB was added to each and they were allowed to incubate 1 hour at 37°C. 2 ml of LB top agar (LB + 0.7% agar) warmed to 45°C were added, mixed quickly and spread on LB plate agar (LB + 1.5% agar) containing 50 ug/ml ampicillin. Plates were incubated upside down at 37°C overnight.

Miniprep of Plasmid DNA

Isolated colonies from incubated LB/ampicillin plates were inoculated into 10 ml LB containing 50 ug/ml ampicillin and incubated overnight. 5 ml of each culture were centrifuged (12,000 x G) for 1 minute and resuspended in 100 ul cold solution A (50 mM sucrose, 10 mM ethylene-diaminetetraacetic acid (EDTA), 25 mM Tris[hydroxymethyl]-aminomethane (Tris) pH 8.0, 4 mg/ml lysozyme). After 5 minutes of incubation at room temperature, 200 ul of solution B (0.2 N NaOH, 1% sodium dodecyl sulfate (SDS)) were added, mixed gently by inversion and the samples placed

on ice for 5 minutes. 150 ul of cold solution C (3 M $KC_2H_3O_2$) were added, the tubes gently vortexed 10 seconds and placed on ice for 5 minutes. After 5 minutes of centrifugation (12,000 x G) at 4°C the supernatants were transferred to new tubes and extracted once each with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). 2 volumes of ethanol were added and the samples precipitated on dry ice for 15 minutes. The 12,000 x G x 15 minute pellets were washed once with cold 70% ethanol, briefly vacuum dried and dissolved in 20 ul TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) containing 100 ug/ml DNAse-free RNAse. Suitable clones were selected for maxiprep by electrophoretic resolution of their restriction enzyme digestion products in agarose/TBE gels (TBE is 0.089 M Tris, 0.089 M boric acid, 0.2 mM EDTA.).

Maxiprep of Plasmid DNA

1 L of LB containing 50 ug/ml ampicillin in a 4 L flask was inoculated with transformed bacteria from the remainder of the appropriate miniprep culture and incubated 24 hours at 37°C with vigorous shaking. The culture was centrifuged at 4°C for 10 minutes (3,000 x G) and the pellet resuspended in 10 ml cold solution A (See **Miniprep of Plasmid DNA**) and incubated at room temperature for 10 minutes. 10 ml cold solution B were added, mixed by inversion, and the samples left on ice for 10 minutes. 15 ml cold solution C were

added, mixed and incubated in the same manner. The 47,000 x G x 20 minute supernatant was precipitated with 0.6 volumes of isopropanol at room temperature for 20 minutes, then centrifuged 30 minutes at room temperature (12,000 x G). The pellet was washed once with cold 70% ethanol, vacuum dried, dissolved in 5 ml TE containing 100 ug/ml DNAse-free RNAse and incubated 3 hours at 37°C. The solution was extracted once each with phenol:chloroform: isoamyl alcohol and chloroform:isoamyl alcohol and brought to 2 M $NH_4C_2H_3O_2$. Two volumes of ethanol were added and the solution precipitated at -20°C for 2 hours. The 12,000 x G x 30 minute pellet was washed and dried as described above, dissolved in 1.8 ml solution D (10 mM Tris pH 8.0, 1 mM EDTA, 1 M NaCl) and spun through a pZ523 column (5'→3', Inc.) at 1,100 x G for 12 minutes. The eluate was precipitated with 0.6 volumes of isopropanol at room temperature for 20 minutes and centrifuged at 12,000 x G for 30 minutes at room temperature. The pellet was washed, dried and dissolved in 500 ul H_2O. DNA was quantitated by measuring the optical absorbance at 260 nm (A_{260} = 1.0 ≡ 50 ug DNA/ml).

Transfection of KRC-7 Cells with Plasmid DNA

250 ul of 10 mg/ml diethylaminoethyl dextran (DEAE-dextran) (72) were added to 750 ul Tris-buffered saline (TBS) (25 mM Tris pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$) and incubated 10 minutes at 37°C. 500 ul of TBS containing 100 ug plasmid DNA were added, the solution incubated another 15 minutes at 37°C and brought to 5 ml with 37°C TBS. (For placental alkaline phosphatase (PAP) experiments, 100 ug test plasmid and 25 ug pSV2PAP were used.)

For each plasmid test group, 70% confluent cells were removed from 4 150 mm x 25 mm dishes with PBS + 0.05% trypsin, pelleted briefly and resuspended in the 5 ml TBS/DNA/DEAE-dextran solution. After 25 minutes of incubation at room temperature with gentle agitation, 500 ul aliquots of this suspension were added to 9 100 mm x 20 mm dishes already holding 5 ml DME + 5% serum and the cells allowed to attach for 6 hours at 37°C. The medium was removed and replaced with DME + 20% DMSO at room temperature for 5 minutes, and the plates were washed once with PBS before 10 ml DME + 10% serum were added. 24 hours later, this medium was changed to DME only; 24 hours later 3 dishes were treated with 10 nM insulin and 3 were harvested as described below. After 12 hours more the remaining 3 insulin treated and 3 untreated dishes were harvested.

Cells were harvested for chloramphenicol acetyl-transferase (CAT) assays by scraping into 1 ml TBS with a rubber policeman, pelleting briefly and resuspending in 100 ul of harvest buffer (250 mM Tris pH 7.8, 5 mM EDTA, 0.5% Triton X-100). The samples were heated to 55°C for 10 minutes to inactivate endogenous acetyl coenzyme A - requiring activities (73) and centrifuged at 12,000 x G for 5 minutes. The supernatants were transferred to new tubes and stored at -80°C until assay.

For PAP assays, 200 ul aliquots of the initial TBS harvest suspensions were stored at -80°C until assay.

Chloramphenicol Acetyltransferase Assay

10 ul of each cell extract were assayed for protein content (67) and identical masses (usually 25 - 75 ug) of each sample were brought to 140 ul with harvest buffer (See **Transfection of KRC-7 Cells with Plasmid DNA**). To each were added 107.5 ul of reaction mix (100 ul 2.5 mM chloramphenicol in 100 mM Tris pH 7.8, 5 ul 5 mM acetyl coenzyme A in 10 mM $NaC_2H_3O_2$ pH 5.0, 2.5 ul ^{14}C-acetyl coenzyme A in 10 mM $NaC_2H_3O_2$ pH 5.0 at 50 mCi/mM and 0.020 mCi/ml). The samples were mixed and incubated 3 hours at 37°C. (The reaction rate is linear within that time for the activity ranges assayed; data not shown.) To stop the reaction, 300 ul cold ethyl acetate were added, the samples mixed well and centrifuged briefly to separate the phases,

and 200 ul of each upper, organic layer removed to a scintillation vial. 2 ml scintillation fluid were added and the decay rate measured on a Beckman LS 3801 liquid scintillation counter, using an upper limit of 670. (For 4 L scintillation fluid: 1,332 ml Triton X-100, 400 ml 10X TTF, toluene to 4 L. For 4 L 10X TTF: 112 g 2,5-diphenyloxazole, 1.4 g 1,4-bis[5-phenyl-2-oxazolyl]-benzene, toluene to 4 L.)

Placental Alkaline Phosphatase Assay

Each 200 ul cell suspension in TBS was centrifuged briefly and the pellets resuspended in 50 ul TBS, heated to 65°C for 30 minutes and chilled. (Heat treatment inactivates endogenous phosphatase activities (70).) 20 ul of each suspension were added to 400 ul of reaction mix (see below) and incubated 30 minutes at 37°C with vigorous shaking. The optical absorbances of the suspensions at 405 nm were measured and normalized to the protein concentration in each sample as determined by Bradford's assay (67).

Reaction mix (70) is 19 parts DEA buffer:1 part PNPP buffer. DEA buffer is 1 M diethanolamine, 0.28 M NaCl and 0.5 M $MgCl_2$. PNPP is 0.1 M p-nitro-phenyl phosphate in DEA buffer. Stored at -20°C shielded from light.

Analysis and Presentation of CAT Assay Results

CAT activities were determined as the change in activity seen over the 12 hour treatment period. Specifically, the mean CAT activity measured in cells harvested at the beginning of the treatment period was subtracted from each value measured in the treated and untreated cells harvested at the end of the 12 hour treatment period. These relationships can be represented formulaically as follows:

If:

A_0 = mean CAT activity at start of treatment period

A_{12} = replicate CAT activities from untreated cells at end of treatment period

AT_{12} = replicate CAT activities from hormone-treated cells at end of treatment period

Then:

Basal CAT activity = $A_{12} - A_0$

Hormone-modulated CAT activity = $AT_{12} - A_0$

Mean mock values measured with extracts from untransfected cells in each experiment were subtracted from all other values. Extracts from untransfected cells and cells transfected with the promoterless vector pBLCAT3 showed similar, low activities (data not shown).

For comparison of basal activities of the various mutant promoters, each transfection experiment included the full-length promoter construct pTC3.92, as well as mutant promoter constructs in separate suspension batches. Each test basal replicate's value was calculated relative to the mean basal expression from pTC3.92 in that experiment (Relative basal value = $^{\text{test basal}}/_{\text{pTC3.92 basal mean}}$). Means, population standard deviations and population variances were computed from those relative values.

For comparison of insulin-treated with untreated cells, each replicate's value was calculated relative to the mean basal expression from that same plasmid in that experiment (Relative insulin effect = $^{\text{test insulin-treated}}/_{\text{test basal mean}}$). Means, population standard deviations and population variances were computed from those relative values.

Statistically significant differences between variances and means were determined at the 0.05 alpha level of confidence by F and T tests, respectively.

Mean specific CAT activity accumulated at the end of the 12 hour treatment period after transfection with pTC3.92 was ≅ 150 pM/mg/minute; the range was 10 to 330 pM/mg/minute. The mean accumulation during the treatment period was 45 pM/mg/minute.

DNA Sequencing

Preparation of Labelled DNA Probe: 2 ug of plasmid DNA were digested with an appropriate restriction enzyme, generating a 5' overhang at a site near the region of interest. In the same buffer, the overhang was filled in using Klenow fragment (4 units), dGTP, dCTP and dTTP at 0.25 mM, 20 uCi of [α-^{32}P] dATP (10 uCi/ul, 3000 Ci/mM) and incubation for 1 hour at 30°C. The reaction mix was extracted with phenol:chloroform:isoamyl alcohol and chloroform:isoamyl alcohol, precipitated in 0.1 M NaCl and 2 volumes of ethanol, redissolved and cut with a second restriction enzyme to generate a shorter double DNA strand labelled on only one end on one strand. This digestion reaction was electrophoresed in a 2% agarose/TBE gel with TBE buffer (See **Miniprep of Plasmid DNA**) for an appropriate period and the gel placed on Kodak XRP-1 film for 5 minutes. The autoradiogram was used to locate and excise the radioactive fragment. The labelled DNA was eluted from the agarose slice with a Model UEA unidirectional electroeluter (International Biotechnologies, Inc.) following manufacturer's recommendations, then precipitated in 10 M NH_4Cl with 2 volumes of ethanol at -20°C. The centrifuged pellet was washed and dried, then dissolved in H_2O to a specific activity of 400,000 cpm/10 ul as measured by Cerenkov radiation with a Beckman LS3801 liquid scintillation counter using an upper limit of 1,000.

The G Reaction: (74) To 10 ul of labelled probe solution were added 200 ul of dimethyl sulfate reaction buffer (For 100 ml: 0.8 g sodium cacodylate in 99 ml H_2O pH 8.0, 1 ml 100 mM EDTA pH 8.0.) and 1 ul dimethyl sulfate. The reactions were mixed, incubated 3 minutes at room temperature and stopped by addition of 50 ul dimethyl sulfate stop buffer (For 100 ml: 20.4 g $NaC_2H_3O_2$ in 70 ml H_2O pH 7.8, 7.8 g mercaptoethanol, 100 ul transfer RNA at 100 mg/ml, H_2O to 100 ml.) and 1 ml ethanol. The solutions were allowed to precipitate on dry ice for 10 minutes.

The G+A Reaction: (74) To 10 ul of labelled probe solution were added 50 ul formic acid. The reactions were incubated 5 minutes at room temperature and stopped with 400 ul hydrazine stop buffer (For 200 ml: 20 ml 3 M $NaC_2H_3O_2$ pH 6.0, 200 ul 100 mM EDTA, 50 ul tRNA at 100 mg/ml, 179.8 ml H_2O.) and 1 ml ethanol. The solutions were allowed to precipitate on dry ice for 10 minutes.

Cleavage of Modified DNA: (74) Precipitated samples were centrifuged (12,000 x G x 20 minutes) at 4°C and the supernatants discarded into 5 M NaOH. The pellets were washed twice in cold 70% ethanol and 70 ul of 10% piperidine added. The reactions were mixed and incubated at 90°C for 30 minutes. The piperidine was vacuum evaporated, replaced with 100 ul H_2O and re-evaporated, and this rinse was repeated. The dry pellets were dissolved in 10 ul formamide

loading dye (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue).

Gel Electrophoresis: An 8% denaturing polyacrylamide gel (40 g urea, 8 ml 10X TBE, 16 ml 40% acrylamide: 2% bisacrylamide, H_2O to 80 ml. 10X TBE is a 10-fold concentrate of TBE.) was polymerized by addition of 500 ul 10% $(NH_4)_2(SO_4)_2$ and 53 ul N,N,N',N'-tetramethyl-ethylenediamine and poured between silanized glass plates with 0.4 mm spacers. After complete polymerization, the gel was prerun for 1 hour at 90 watts. Aliquots of the G and G+A reactions containing 40,000 dpm's were heated to 90°C for 3 minutes, placed on ice immediately and loaded in the gel. To obtain good resolution at all regions of the probe, multiple loadings were performed during the 70 watt run. Total run length varied with the size of the probe used. After electrophoresis the gel was wrapped in plastic wrap and placed on Kodak XRP-1 film overnight at -80°C with an intensifying screen.

Hazardous Waste Management

All cultures containing transformed *Escherichia coli* or other antibiotic-resistant bacteria were autoclaved prior to disposal. Phenol, chloroform and other hazardous non-radioactive materials were disposed of in accordance with the Tennessee State Hazardous Waste Management Act under Environmental Protection Agency Waste Generator license

TND003387891 as authorized by Title 10 of the Code of Federal Regulations. All radioactive materials were disposed of in accordance with Tennessee State Regulations for Protection against Radiation under Tennessee license # R-47005-I97, as authorized by the Nuclear Regulatory Commisssion. Personal radiation exposure was monitored by semi-annual thyroid counts and monthly counts of lapel-worn exposure badges. Permanent deep radiation exposure over the entire research period totalled 180 milliRems.

RESULTS

Optimization of Transient Transfection Protocol

The work described in this chapter began with the acquisition of a 4 kilobase pair TAT promoter (-3,922 to +62) ligated upstream of the chloramphenicol acetyl-transferase (CAT) promoterless gene (58). This construct enabled the search for *cis*-acting DNA regions which mediate insulin's effect on basal transcription. Transfection of KRC-7 cells with the full-length promoter and related constructs using a DEAE-dextran technique and cell harvest protocol previously established in our laboratory initially yielded unsatisfactory results, however. Specifically:

1) Basal levels from a given plasmid varied within a single experiment, and this variation was even larger when different experiments were compared (data not shown).

2) That variation suggested that comparison of basal expression from different plasmids, even within the same experiment, might not be valid.

3) The observable insulin inhibition of basal expression was statistically significant, but small enough to be intuitively questionable (35%; data not shown).

It appeared likely that the protocol that yielded these results might be altered to optimize the observed insulin effect and reduce variability between and within experiments. Thus, experiments tracing the time course of

the accumulation of basal and insulin-modulated CAT expression were performed. In addition, KRC-7 cells were transfected with a second plasmid (pSV2PAP) as an index of plasmid uptake efficiency.

Time-Course Experiments: KRC-7 cells were transfected with pTC3.92 and cells were harvested and stored identically at regular intervals over a 7 day period (See **Figure 5**). Basal CAT expression remained low until approximately 36 hours after the DMSO shock. After 36 hours, basal expression rose rapidly for the next 4 days, reaching a plateau on the 6th day after DMSO shock.

A similar experiment included insulin treatments, and cells were harvested at short intervals spanning the previously used treatment period (See **Figure 6**). No effect of insulin on CAT activity was observed at the earliest time point, 4 hours after treatment (52 hours after DMSO shock). By 8 hours, however, insulin treatment caused a decrease in CAT expression. The rate of accumulation of insulin-modulated CAT activity remained less than the rate of basal accumulation through the 12th hour of treatment. Beyond that time, CAT expression in treated and untreated cells accumulated at the same rate.

The accumulation of basal activity appeared roughly linear during the 16 hour treatment period in this experiment (See **Figure 6**); thus the time at which insulin treatment was started (48 hours after DMSO shock) was

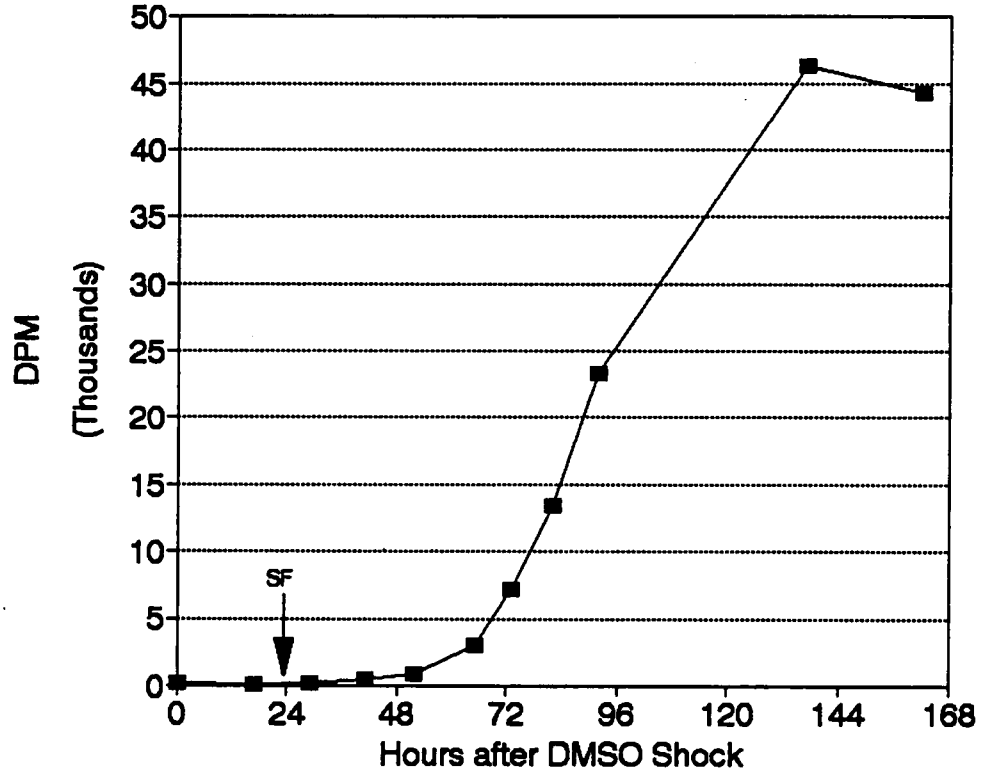


FIGURE 5 - Time Course of the Accumulation of Basal CAT Activity. The plasmid pTC3.92 was used. Shown are decays per minute (DPM's) from acetylated chloramphenicol versus time following the DMSO shock. Means are shown; n=3 for each, 1 experiment. SF = serum free; DMSO = dimethyl sulfoxide.

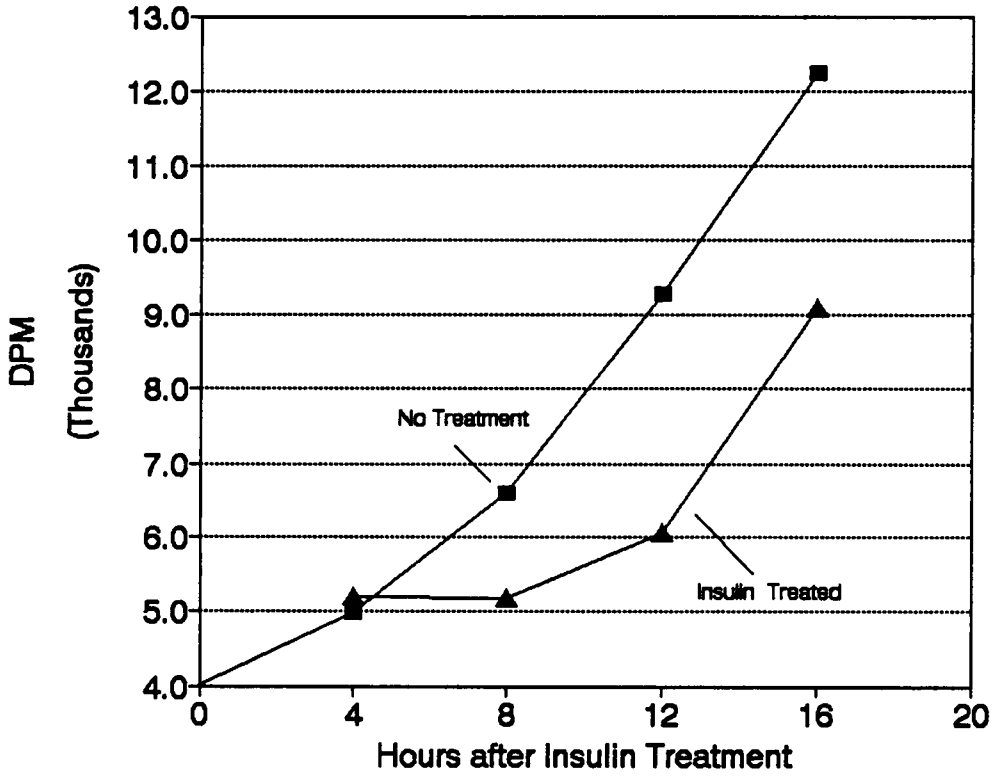


FIGURE 6 - Time Course of the Insulin Effect. The plasmid pTC3.92 was used. Shown are decays per minute (DPM's) from acetylated chloramphenicol versus time following insulin treatment. The 0 hour value was extrapolated from the measured curve. Means are shown; n=3 for each, 1 experiment.

retained from the old protocol. However, it was apparent that the insulin effect on accumulation of CAT activity was nonexistant after 12 hours. Therefore, the 16 hour treatment period was shortened to 12 hours.

Examination of the ordinate scale in **Figure 6** reveals another problem with the earlier measurements: Significant basal CAT activity had already accumulated prior to the hormone treatment. When CAT activity was measured only at the end of the treatment period, as in the old protocol, hormone-altered activities were added to the activity level unmodulated by hormone that had already accumulated in the pretreatment period, thus minimizing the hormone effect. To correct this in the experiments described below, cells were harvested at the beginning of the treatment period, and treated and untreated cells harvested at the end. The mean of the activities at the start of treatment was then subtracted from all other values (See **Methods**).

Co-transfection with pSV2PAP: To assess and normalize for plasmid uptake differences, a plasmid encoding a constitutively expressed second reporter enzyme (placental alkaline phosphatase, PAP) was exposed to cells along with each test CAT plasmid. The validating assumption for this technique is that reporter plasmids of different types in a common solution are taken up in fixed proportions by each cell (See more in **Discussion**).

The measurable confirmation of that assumption should be the appearance of a consistent correlation between constitutive (basal) CAT expression from a test plasmid and constitutive expression from the second plasmid. As seen in **Figure 7**, such a correlation was not found for pTC3.92 and pSV2PAP. Thus, the validating assumption of normalization by co-transfection appears weak, and this technique was not used further (See **Discussion**).

An alternative was chosen for the experiments described below: Each transfection experiment included pTC3.92 (in a separate batch from each test plasmid batch) and all basal replicate values were expressed relative to the mean basal expression from pTC3.92 in that experiment. This allowed "interplasmid" comparison of those relative basal values between experiments. However, this method, just as co-transfection normalization techniques, requires multiple assumptions which should be considered when such comparisons are made (See **Discussion**).

A computative, "intraplasmid" method of normalization was chosen for assessment of hormone effects. Each replicate's value was expressed relative to the mean basal expression from that test plasmid in that experiment. The relative treatment effects and variations around the means thus calculated were compared between test plasmids and experiments. (This method of evaluation has limitations that may produce descriptive artifacts (See **Discussion**).)

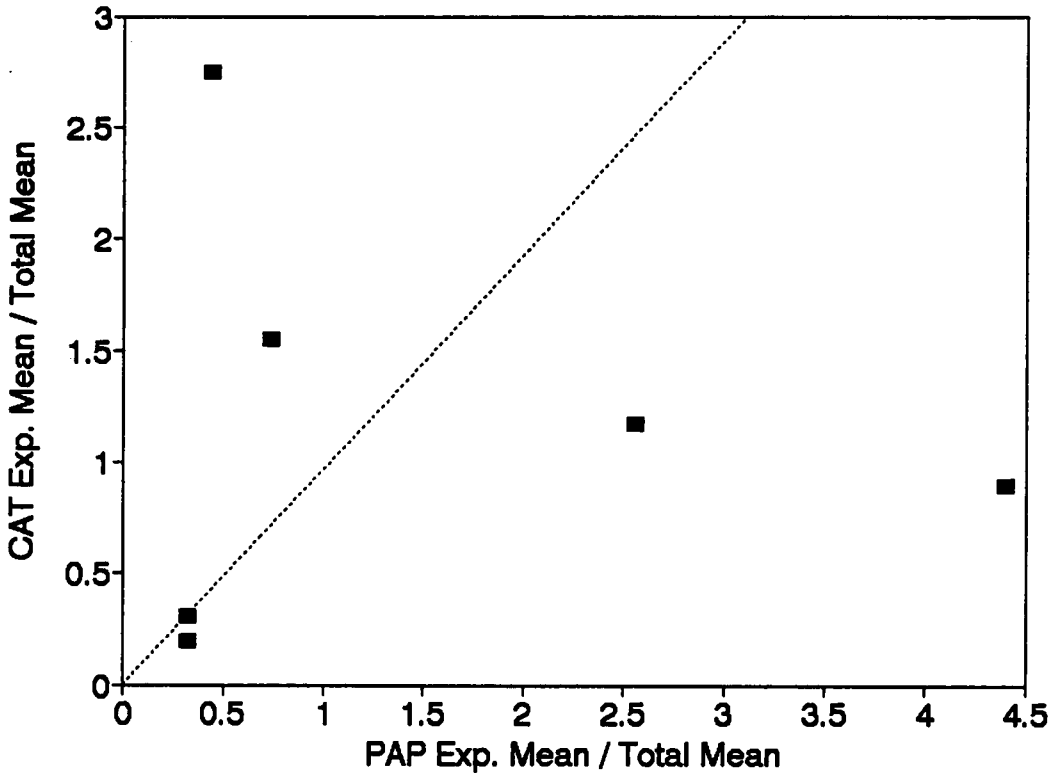


FIGURE 7 - No Correlation Between CAT and PAP Expression. Each point represents a separate experiment; 6 experiments, n=3 in each. CAT and PAP values were calculated as experimental means relative to their means from all 6 experiments. The dashed line represents the expected correlation of those values if CAT and PAP expression were proportional in each experiment. CAT = chloramphenicol acetyltransferase; PAP = placental alkaline phosphatase.

Insulin-Modulated Expression from Mutant TAT Promoters

Previous results from this laboratory suggest that an insulin-responsive region (IRR) is located in the proximal promoter (-351 to +62) of the TAT gene ((49) and **Figure 3**, page 13). This proximal IRR mediates an insulin inhibition of glucocorticoid-induced transcription in KRC-7 cells, but its ability to do the same for basal or cAMP-induced expression was uncertain.

The acquisition of pTC3.92 allowed the investigation of insulin's effect on basal expression, since pTC3.92 contains the basal enhancer (BE; -3,661 to -3,543). As described in the **Introduction**, this region can mediate levels of basal transcription 20 to 50 fold greater than from the proximal promoter alone. In transient transfections using KRC-7 cells, the full-length TAT promoter (pTC3.92; -3,922 to +62) drives high basal expression and insulin inhibits that expression by 65% (See **Figure 8**).

Acting from the assumption that the proximal IRR might be the mediator of this effect, the BE was ligated upstream of truncated TAT promoters of different lengths:

-800 to +62; (pBE0.80)

-208 to +62; (pBE0.21)

There was no significant insulin inhibition of basal expression from either of these plasmids (See **Figure 8**), but both responded to insulin with significantly increased expression.

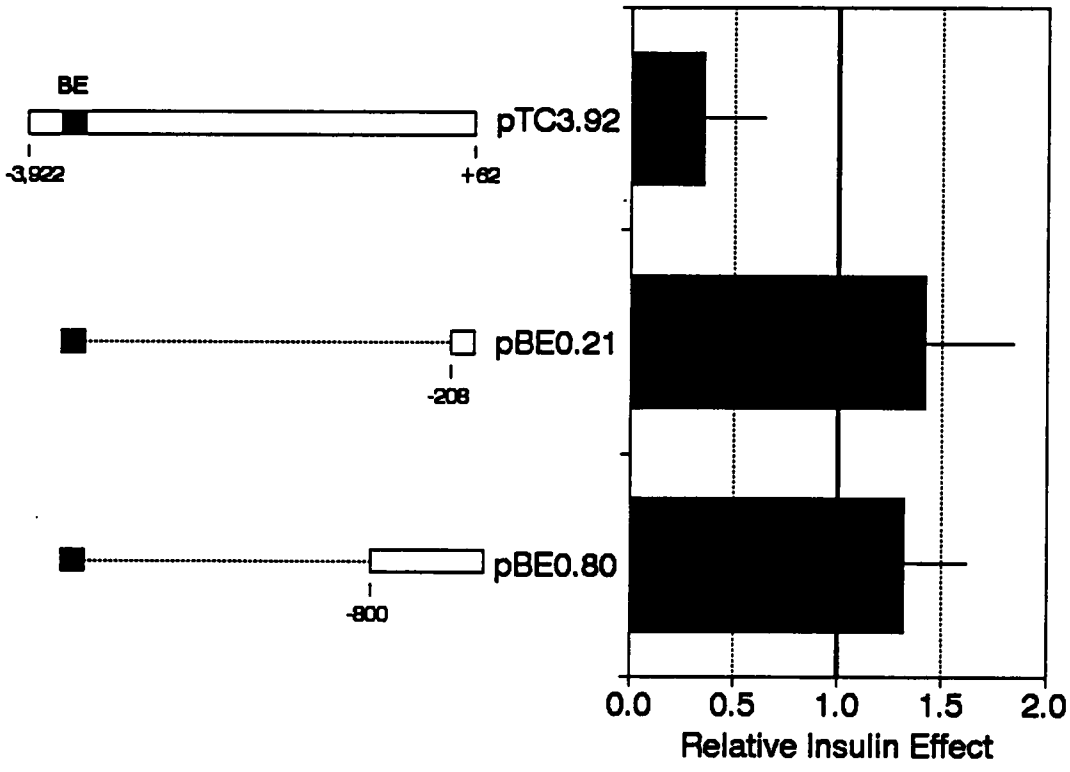


FIGURE 8 - The Proximal IRR Cannot Mediate Insulin Inhibition of Basal Expression. Mean basal expression from each plasmid was arbitrarily set at 1; insulin effects are shown relative to those values. Values from insulin-treated cells are significantly different from basal for all 3 plasmids. For pTC3.92, n=48, 16 experiments; for pBE0.21 and pBE0.80, n=9, 3 experiments. The thin lines represent population standard deviations. IRR = insulin responsive region; BE = basal enhancer.

Since none of these constructs recreated the insulin inhibition seen with the endogenous gene and pTC3.92, the BE was progressively moved farther from the start site by ligating it to longer promoters. pBE1.30 and pBE2.55 both failed to show any significant insulin inhibition; insulin increased CAT expression from pBE2.55 (See **Figure 9**).

The short region of the 3,922 base pair promoter 5' to the BE was then targeted. A truncation from -3,922 to -3,675 (pTC3.67) still allowed a significant insulin inhibition of basal expression, although it was less than that seen with pTC3.92 (See **Figure 10**). A further 15 base pair deletion to -3,661 eliminated any significant insulin effect.

To investigate further the sufficiency of this distal insulin-responsive element (IRE; -3,675 to -3,661) delimited by these results, pTC3.67Δ-3,543/-208 was constructed. This plasmid is identical to the hormone-refractory pBE0.21 except for the presence of the 15 base pair distal IRE. (pBE0.21 is the shortest promoter construct that retained strong basal activity; see **Basal Expression from Mutant TAT Promoters**, this chapter.) Insulin significantly inhibited CAT expression from this new construct (See **Figure 11**).

cAMP-Modulated Expression from Mutant TAT Promoters

Previous results from this laboratory (33) show that cAMP increases $mRNA^{TAT}$ abundance 3.5 fold within 3 hours in

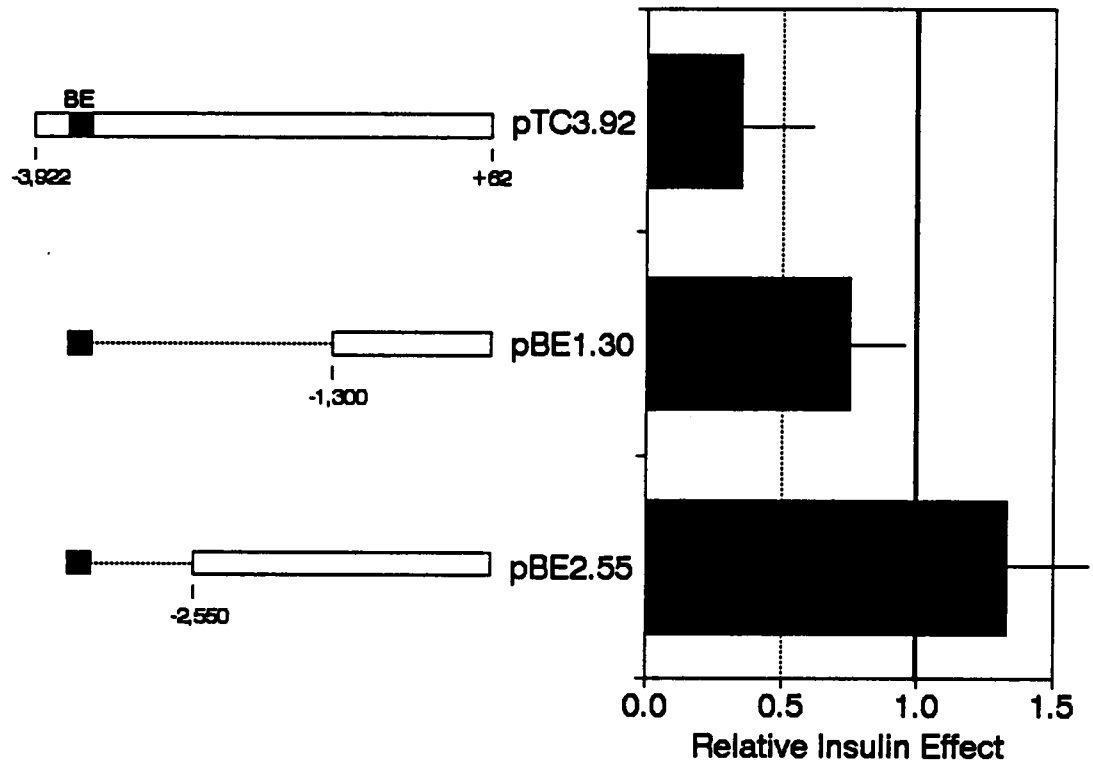


FIGURE 9 - Regions Required for the Insulin Inhibition of Basal Expression Lie 5' to -2,550. Mean basal expression from each plasmid was arbitrarily set at 1; insulin effects are shown relative to those values. Values from insulin-treated cells are significantly different from basal for pTC3.92 and pBE2.55, but not for pBE1.30. For pTC3.92, n=48, 16 experiments; for pBE1.30 and pBE2.55, n=9, 3 experiments. The thin lines represent population standard deviations. BE = basal enhancer.

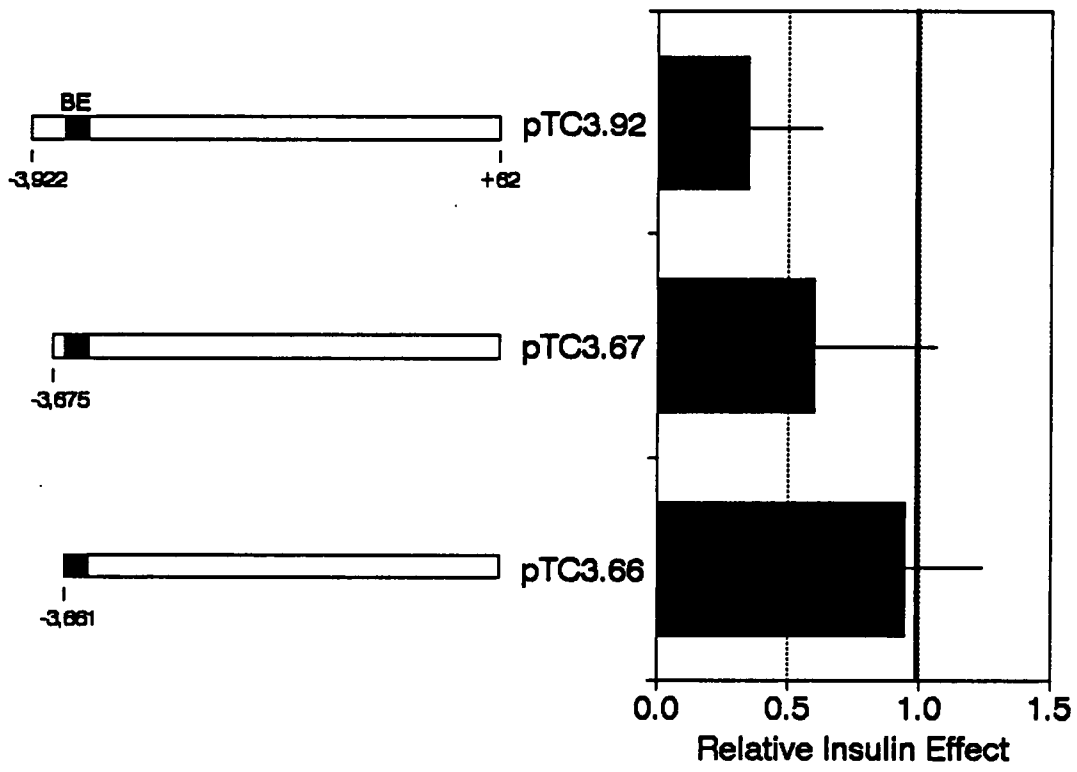


FIGURE 10 - The Insulin-Responsive Element (IRE) Lies Between -3,675 and -3,661. Mean basal expression from each plasmid was arbitrarily set at 1; insulin effects are shown relative to those values. Values from insulin-treated cells are significantly different from basal for pTC3.92 and pTC3.67, but not pTC3.66. For pTC3.92, n=48, 16 experiments; for pTC3.67, n=21, 7 experiments; for pTC3.66, n=9, 3 experiments. The thin lines represent population standard deviations. BE = basal enhancer.

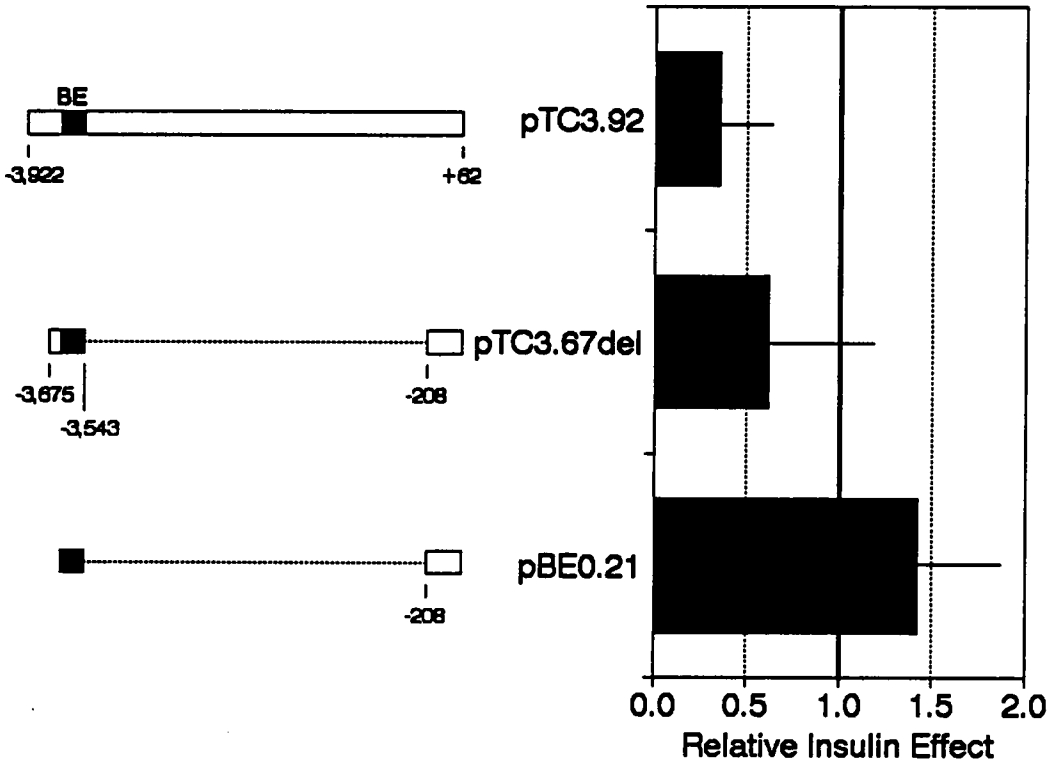


FIGURE 11 - A 15 Base Pair Sequence Mediates Insulin Inhibition of Basal Expression from a Truncated Promoter. Mean basal expression from each plasmid was arbitrarily set at 1; insulin effects are shown relative to those values. Values from insulin-treated cells are significantly different from basal for all 3 plasmids. For pTC3.92, n=48, 16 experiments; for pTC3.67del (same as pTC3.67Δ-3,543/-208) and pBE0.21, n=9, 3 experiments. The thin lines represent population standard deviations. BE = basal enhancer.

KRC-7 cells. Similarly, cAMP-modulated TAT transcription in isolated nuclei increases 2.2 fold within 30 minutes (33). In this study, only a modest cAMP effect was observed with pTC3.66, but pTC3.67 responded with a 3.5 fold increase in CAT activity (See **Figure 12**. Also see further discussion of this fold change in **Discussion.**). Insulin, when administered simultaneously with cAMP, significantly inhibited that cAMP effect in pTC3.67, but failed to do the same with pTC3.66. (As described above, insulin also inhibited basal CAT expression from pTC3.67 but not pTC3.66.) These experiments again delimit the distal IRE, but for an apparently different role (See **Discussion**).

Basal Expression from Mutant TAT Promoters

As each plasmid was tested, its basal level of expression was measured as a standard for assessment of the insulin effect on that plasmid. Yet, as mentioned above in this chapter (**Optimization of Transient Transfection Protocol**, and described further in **Discussion**), several assumptions are required for comparison of basal levels from different plasmids. Nevertheless, such comparisons may be instructive, if not conclusive.

The relative basal activities from pTC3.92 (relative value = 1) and other mutant promoters are shown in **Figure 13**. pTC3.67 showed basal expression almost identical to pTC3.92, but truncation to -3,661 (pTC3.66; removal of

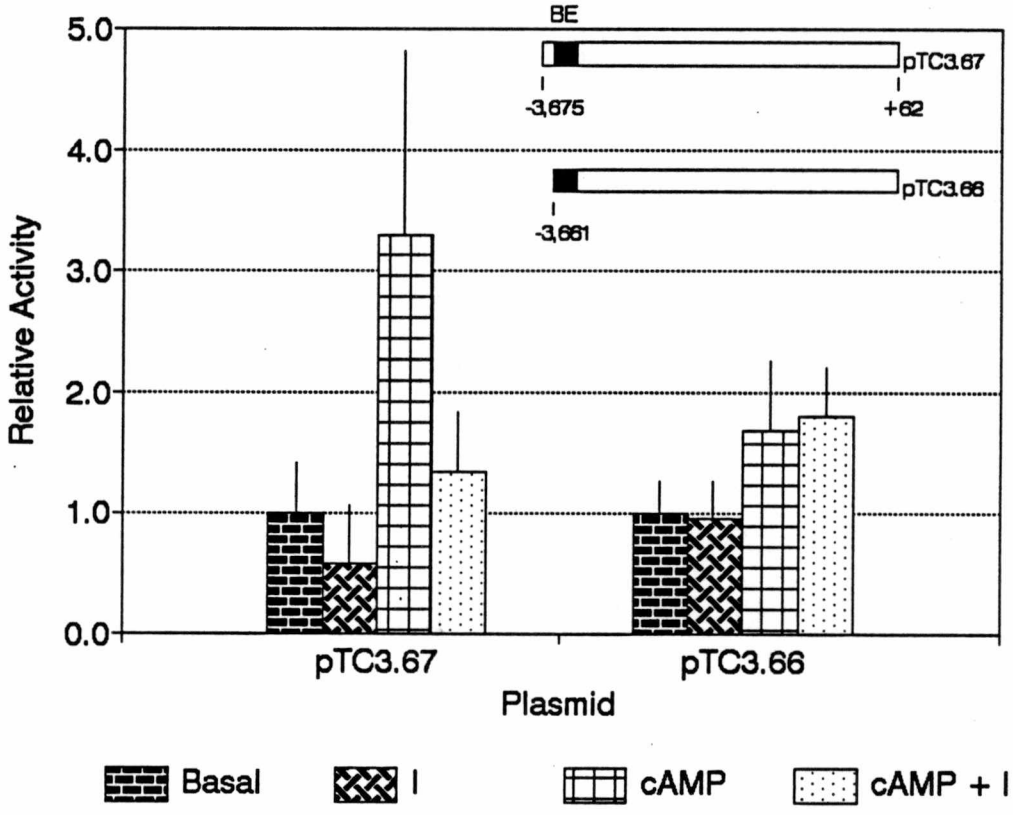


FIGURE 12 - The IRE Also Mediates Insulin Inhibition of the cAMP Response. Mean basal expression from each plasmid was arbitrarily set at 1; hormone effects are shown relative to those values. For pTC3.67, values from insulin-treated (I) and cAMP-treated cells are significantly different from basal; the cAMP + I mean is different from the cAMP mean. For pTC3.66, the cAMP effect is significant. For pTC3.67 and pTC3.66, n=6, 2 experiments. The thin lines represent population standard deviations. BE = basal enhancer.

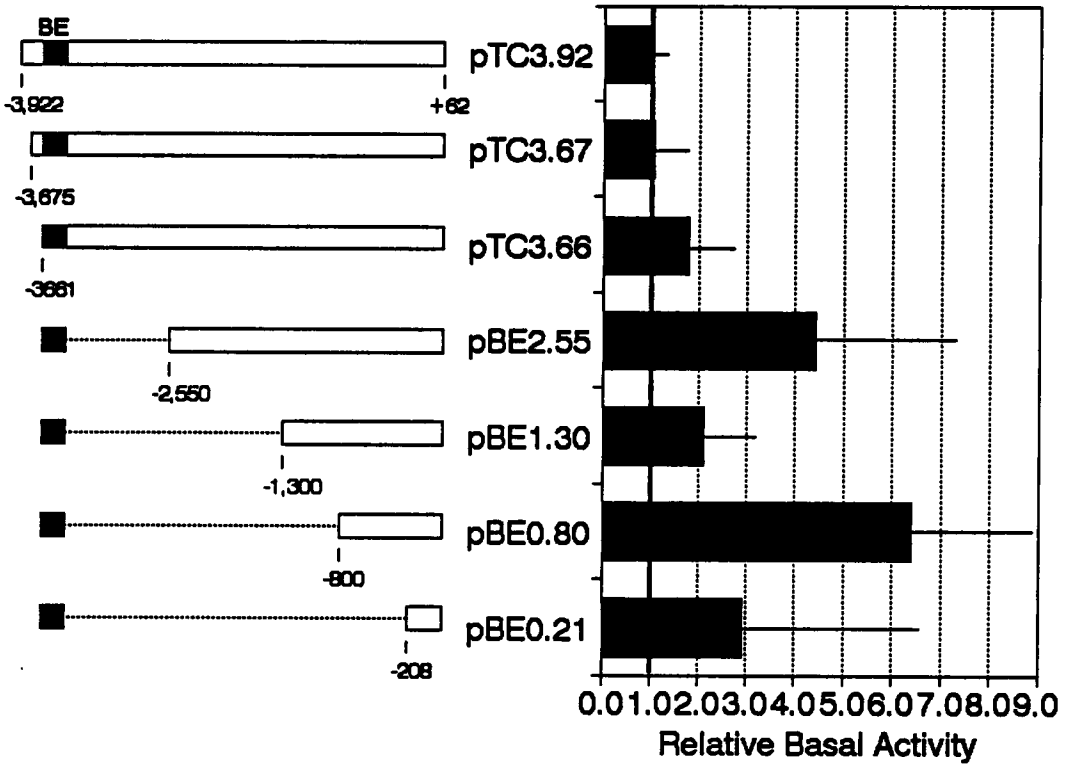


FIGURE 13 - The BE Is Sufficient for Strong Basal Expression When Linked to a Truncated Promoter. Mean basal expression from each plasmid is shown relative to mean basal expression from pTC3.92 in the same experiment. Values for pTC3.66, pBE2.55 and pBE0.80 are significantly different from pTC3.92. For pTC3.92, n=48, 16 experiments; for pTC3.67, n=21, 7 experiments; for all other constructs, n=9, 3 experiments. The thin lines represent population standard deviations. BE = basal enhancer.

the IRE) allowed a significantly greater level. This significantly elevated expression was retained in pBE2.55, which is identical to pTC3.66 except for a large internal deletion from -3,543 to -2,550.

Expanding this internal deletion toward the start site to -1,300 (pBE1.30) restored basal levels not significantly different from those from pTC3.92. Further expansion of the deletion to -800 (pBE0.80) again produced significantly greater basal levels; this was lost again in deletion to -208 (pBE0.21).

None of these plasmids showed mean basal levels less than those from pTC3.92, indicating that none lacked regions required for the levels of basal expression mediated by the BE within the full-length promoter context (pTC3.92). A further expansion of this internal deletion to -52 (pBE0.05), however, drastically reduced basal expression (See **Figure 14**). This suggested that the -208 to -52 region is required for basal expression in addition to the BE. The specificity of this additional requirement for basal activity is shown by previous results from this laboratory using plasmids containing glucocorticoid-responsive elements ligated at -52, which exhibited uncomprimised transcriptional activity (Li Pan, personal communication) (See **Introduction** and **Discussion**, following). (See **Figure 15** for a summary of these results.)

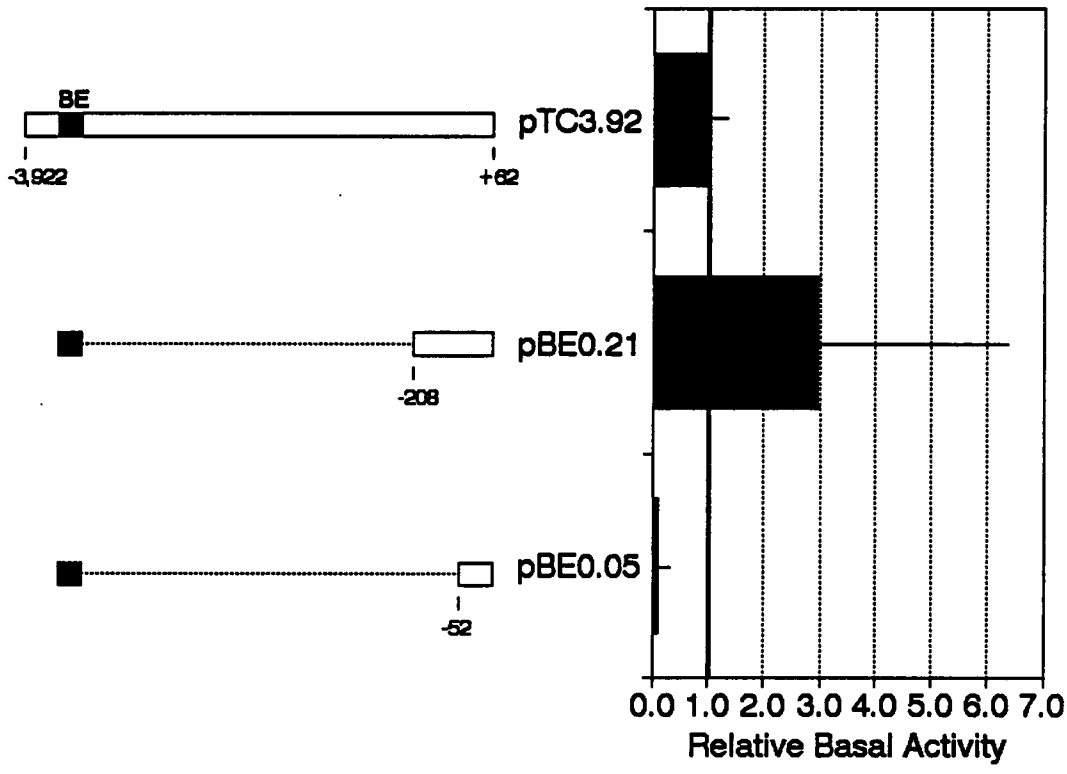


FIGURE 14 - BE Function Requires a Proximal Promoter Region Between -208 and -52. Mean basal expression from each plasmid is shown relative to mean basal expression from pTC3.92 in the same experiment. Values from pTC3.92 and pBE0.05 are significantly different. For pTC3.92, n=48, 16 experiments; for pBE0.21 and pBE0.05, n=9, 3 experiments. The thin lines represent population standard deviations. BE = basal enhancer.

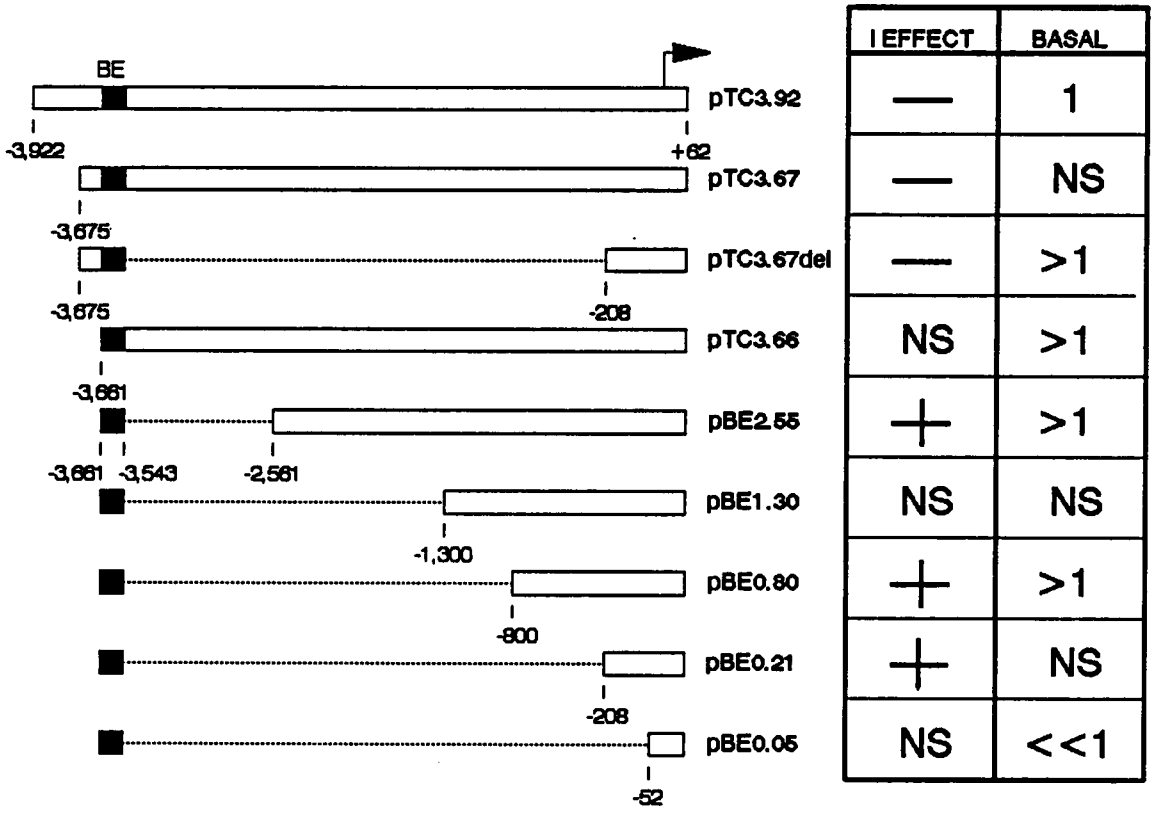


Figure 15 - Summary of Results. Insulin effects for each plasmid are shown relative to that plasmid's mean basal expression. Basal values are shown relative to pTC3.92's mean basal expression. - = inhibition; + = induction; NS = no significant change.

Two other plasmids constructed during this study showed no basal activity, even though the BE was present. When the BE was placed at -3,337 (pBE3.34) or -351 (pBE0.35a) basal activities never exceeded those from mock-transfected cells (data not shown). This suggested the presence of regions in the promoter that can mediate strongly suppressive effects on basal activity when freed from their endogenous promoter context (See **Discussion**). Alternatively, the interactions between *cis* elements (BE and proximal promoter) might depend on specific relative orientations on the DNA helix (See **Discussion**). This latter possibility was selected for further investigation.

Two other forms of pBE0.35a were built, pBE0.35b and pBE0.35c. Each contained the BE, but each at a slightly different distance from the base pair at -351 (See **Figure 16**). These three constructs together encompassed a range including almost a full turn of the DNA helix (10.5 bp) (1), yet no significant basal activity was detected from any. DNA sequencing confirmed the expected structure of pBE0.35a and pBE0.35b; pBE0.35c was confirmed by restriction enzyme analysis.

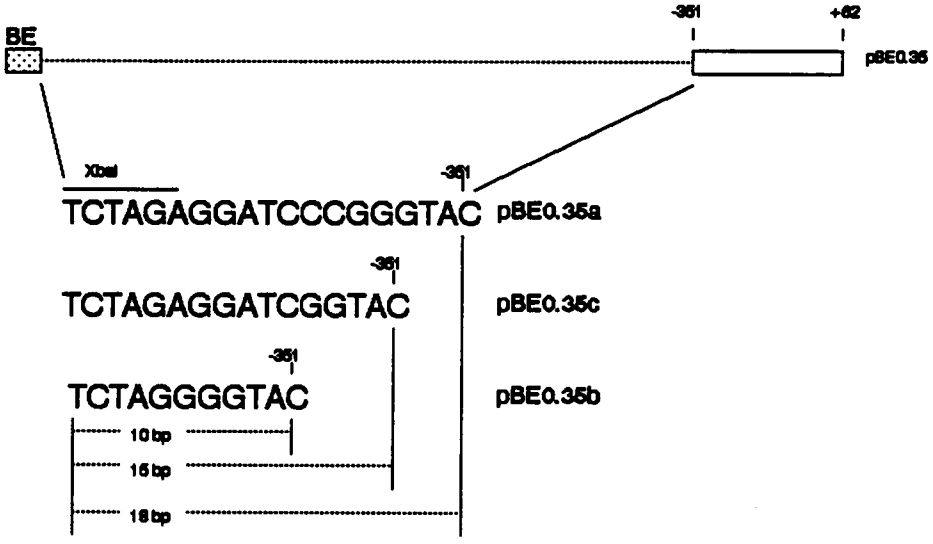


FIGURE 16 - Three Different Forms of pBE0.35 Fail to Mediate Basal Expression. Shown are the regions between the BE and TAT -351 in the three constructs. The XbaI site is at the 3' end of the BE (basal enhancer). The sequences between the XbaI restriction enzyme site and TAT -351 are derived from the polylinker of pGEM3 (Promega).

DISCUSSION

Insulin-Responsive TAT Expression in KRC-7 Cells

The KRC-7 cells used in this study exhibit high basal levels of TAT transcription, and cAMP effects a 3 fold increase of that level. Since insulin reduces both those levels by >50%, these cells provide an excellent system in which to study insulin's inhibitory effects.

The work described here shows that the distal IRE mediates these inhibitory insulin effects in KRC-7 cells. It is possible that this region also mediates insulin's positive effects on TAT, seen in other cell lines and developmental states. Two different proteins might bind the same DNA sequence but have opposite effects on transcription by virtue of different activation domains. A permanent switch in expression of these two forms could impose the permanent developmental switch.

Alternatively, the IRE might not mediate insulin's positive effects. If a separate insulin-responsive enhancer exists in the TAT promoter, developmental changes in TAT expression might be the outcome of competing influences from both positive and negative *cis* elements. The relative activities of their respective DNA-binding proteins would determine that outcome.

The prediction that a separate insulin-responsive enhancer exists in the TAT gene is not unreasonable in view

of the complexity already described. The fact that insulin exerts its different effects through different *cis* elements in TAT (glucocorticoids and the IRR; basal, cAMP and the IRE) appears unique among all hormones. In addition, insulin effects appear to be mediated by different mechanisms in different genes (see further discussion below).

Several constructs which lack the IRE mediated significant positive insulin effects (See **Figure 8**, page 49 and **Figure 9**, page 51), suggesting the presence of one or more *cis*-acting insulin-responsive enhancers. The IRE, when present, negatively dominates the function of those enhancers in KRC-7 cells. Further study of the function of the TAT IRE in a cell line (e.g., Fao rat hepatoma cells (29)) in which the positive insulin effect dominates could begin dissection of this regulatory switch.

pTC3.92 as the "Wild-Type"

Transient transfection of KRC-7 cells with pTC3.92 (TAT sequences from -3,922 to +62) recreated several critical parameters seen *in vivo* with transcriptional run-off assays (33). Specifically:

- significant levels of basal transcription
- ~3 fold cAMP induction of basal level
- >50% inhibition of basal transcription by insulin

- >50% inhibition of cAMP-induced transcription by insulin

Transient transfection allows no direct comparison to absolute transcription rates seen in run-off assays. Such comparisons might be accomplished by assaying transcription of both TAT and CAT genes in nuclei isolated after transfection. In the absence of such data, many researchers assume that *in vivo* conditions are recreated in transfection if hormone-modulated levels assume the same relative (fold) magnitude, with respect to basal, as is seen *in vivo*. That assumption validates the findings of this study, which used a TAT promoter known not to contain all of its functional *cis* elements. Specifically, the role of the additional basal enhancer at -11,000 was not examined. However, its apparent dependence on the BE at -3,600 (See **Introduction**) strengthens the assumption that modulation of expression from the more proximal BE is the critical regulation point in the endogenous gene, and further validates the relevance of these current findings.

Mathematical Descriptions of Transcriptional Effects

As stated above, relative transcriptional changes in transfection similar to those seen *in vivo* are assumed to be an indication that an endogenous regulatory context has been recreated. In reality, however, there is little basis for believing that multiplication of basal expression (fold

change) describes what actually occurs when enhancers modulate transcription. For example, in the absence of hormone treatment, a basal level of transcription results from the sequential action of a series of initiation complexes, each with modifications imposed (in unknown fashion) by all basal *cis*- and *trans*-acting factors. Hormone treatment may have two different results:

1) Hormone-responsive *cis* and *trans* factors may modulate the existing basal mechanism to increase its rate of initiation, "multiplying" its effect.

2) Hormone-responsive *cis* and *trans* factors may facilitate assembly of core initiation complexes by an independent mechanism, "adding" initiation events to the pre-existing (and continuing) basal level.

More specifically, with respect to this latter possibility, initiation events driven by hormone effects may occur without disrupting basal events, and effectively add to them. Although such a result can be described by a multiplicative function (fold change), that description may not be accurate. For example, identical "additions" to basal expression may result from identical hormone induction mechanisms in two different constructs. These, however, would be reported as different if basal expression differed from the two plasmids. Alternatively, if two different promoters exhibit identical "ceilings" of maximal hormone-induced levels, those hormone effects would again be

reported as different if basal levels differed, even though the hormone produced maximal effects on both constructs.

The possible independence of initiation mechanisms induced by different enhancers (basal versus hormone-dependent) would not be realistically reflected in multiplicative descriptions of such changes, since they impose an arbitrary, perhaps nonexistant functional relationship between basal and hormone-induced transcription. In the absence of complete understanding of how enhancers functionally interact with initiation complexes, multiplicative descriptions can unconsciously confirm unfounded assumptions about the nature of those interactions, as well as impose descriptive artifacts upon experimental results. (See further discussion below concerning the cAMP effect on TAT.)

Transient Transfection and Transcriptional Regulation

Transient transfections have several advantages when compared to stable transfection (75) or transgenic experiments. The most obvious is the rapidity and ease with which many different promoter constructs may be tested. Stably transfected cell lines and transgenic animals are expensive and time-consuming to create, and in the case of transgenics, involves the destruction of animals. This latter point is increasingly relevant as animal care costs, federal regulations and public opposition mount.

However, stable transfection and transgenic experiments have one outstanding attribute that transient transfections cannot match: basal levels of expression from the transgene are steady state. Thus, studies of modulation of basal levels of transcription in transient transfections have an added layer of relativity and ambiguity not present in those other methods. (See further discussion below in **Accumulation of CAT Activity.**)

Transgenic experiments also have the added advantage of an *in vivo* milieu of nuclear proteins which may differ from that found in isolated cells *in vitro*. However, further claims for the advantages of stable transfection or transgenic experiments may not be well founded. Specifically, proponents of those methods argue that the transgene, after integration into the chromosomal genome (76), reflects a more natural context for transcriptional regulation than does the transgene found in minichromosomes after transient transfection (77). These claims are made despite the observation that those transgene minichromosomes are organized in regularly spaced nucleosomes, are methylated, and can replicate (77).

Except for a few systems in which site-specific integration has been accomplished, the integration site of the transgene after stable transfection or transgenic manipulations appears variable (76). This is reflected in the highly variable expression from different stable cell

lines or transgenic animal lines transfected with the same gene. Those different levels of expression presumably reflect the impact of different integration contexts. Most researchers who use these techniques simply select the cell or animal line that exhibits the desired characteristics.

Such arbitrary selection is not available to the experimenter using transient transfections. The transgene there could conceivably be integrated into a different nuclear context in each cell it enters, and thus would be regulated differently in each cell. Transient transfection results therefore reflect the summation or average of all those resultant effects. Whenever insertion cannot be controlled specifically, such averaging is more statistically reasonable than arbitrary selection of cell or animal lines that may only coincidentally exhibit an apparent *in vivo* phenotype.

Accumulation of CAT Activity

The actual movement of DNA during transient transfection manipulations has not been determined. That movement and integration into a transcriptional context would affect the initial rates of transcription. The protocol used in this study is designed from several assumptions about those events:

1) DEAE-dextran forms a positively charged linker between the negatively charged transfectant DNA and

negatively charged cell surface. (Viral infection and naked DNA transfection rates are increased in DEAE-dextran treated cells (98).)

2) DMSO initiates the movement of DNA across the cell membrane. This could occur by altering or damaging the phospholipid bilayer (99), during which, or during the repair of which, DNA would be non-specifically transported.

3) The 24 hour serum exposure (immediately following the DMSO shock) initiates a round of cell division, during which nuclear envelope breakdown allows movement of the transgene into the nucleus.

Since the KRC-7 cells exhibit a 24 hour doubling time and an 8 hour lag after arrest in G_0 (34), these assumptions allow the prediction that cells might allow entry of DNA into their nuclei over a period spanning the 32 hours after DMSO treatment, depending on their stage in the growth cycle. Thus, the non-linear increase seen at the earliest time points of the curve in **Figure 5** (page 43) may reflect transcription from an increasing number of transgenes in different cells.

Some of the variation seen in basal expression from the same promoter in different experiments could be due to differences in the distribution of growth states in the cultures used, since TAT expression shows cell cycle changes (100). All cultures were growing rapidly at the beginning of the transfection protocol, and presumably were

randomly distributed throughout the cell cycle. If the distribution of those states is changed by the experimental manipulations, different initial rates of CAT accumulation could result in different experiments. Measurements made at fixed times after DMSO shock would intersect those different rate curves at much different activity values.

Initiation of Hormone Treatment

In transient transfection assays, the next best thing to a steady state basal level is a steady rate of accumulation of basal activity. Comparison of hormonal effects to a changing rate of basal accumulation would be fraught with interpretive difficulties; even comparison to a steady increase imposes a relative value on all observations not directly comparable to values from steady state basal systems.

In the experiment described in **Figure 6** (page 44), the initial changes in rate of CAT expression (change of slope of the curve) stabilized to a relatively steady rate of increase within 48 hours after DMSO shock (start of treatment). Thus, the treatment period employed in these experiments starts at the very beginning of that steady rate. Future experiments should explore the possibility of initiating treatment at a later time, to ensure that the early hours of hormone treatment are not compared to a changing rate of basal accumulation.

Duration of Hormone Treatment

As seen in **Figure 6**, no effect of insulin upon accumulation of CAT activity was observed until 8 hours after treatment began. After the 12th hour of treatment, CAT activity in insulin-treated and untreated cells accumulated at identical rates.

Although a treatment period between 8 and 12 hours long might have enhanced the apparent insulin inhibition to a greater degree than that seen with the 12 hour treatment, a 12 hour duration was chosen to allow greater overall CAT accumulation. Even at 12 hours, values from some insulin-treated replicates showed >100% inhibition (i.e., values from insulin-treated cells were less than those from the untreated cells harvested at the beginning of the treatment period).

Co-Transfection with pSV2PAP

The failure to discover a correlation between constitutive expression from pTC3.92 and pSV2PAP was surprising, in light of the wide acceptance of such methods for normalization of plasmid uptake. Some research has revealed the appearance of different co-transfected DNA's in the nucleus as concatamers (78,79). Those concatamers retain within their structure the ratio of the two DNA's seen in the transfecting solution. Final expression of those reporters assumably reflects that ratio as well.

Few researchers report the validation of their normalization methods. This study produced results which challenge the critical assumption of that technique. The failure of that assumption in this system may be unique to the KRC-7 cells or to pSV2PAP. This latter seems unlikely, since it is a vector widely used for this purpose. It appears certain that this technique should be validated whenever it is employed.

The assumption underlying the technique finally employed here for comparison of basal levels from different plasmids may be equally unfounded. In comparing all basal values to the mean basal expression from pTC3.92 in that experiment, one must assume:

1) All plasmids have identical uptake per unit mass.

2) Variation in uptake exists solely between experiments, and not between batches within experiments.

Neither of these assumptions may be sound. Indeed, with regard to the latter, expression may vary 3 fold between separate batches of the same plasmid within the same experiment (data not shown). Nonetheless, in the face of an invalidated co-transfection normalization technique, such a method allows otherwise inaccessible comparisons.

The Basal Enhancer and the Proximal Promoter

The BE has been previously demonstrated to be required for basal expression from TAT promoters (58,80). Its

sufficiency to drive significant basal expression from a heterologous promoter (HSV TK) has also been shown (58,80), but its sufficiency within the TAT promoter context had not been previously investigated.

The results of this study, together with other unpublished data from this laboratory, clearly delimit a region in the proximal promoter required for basal expression in addition to the BE. More specifically, the minimal expression from pBE0.05 indicates that a required region has been deleted (See **Figure 14**, page 58). Significant basal expression from pBE0.21 is the result of restoration of that region (-208 to -52).

Another construct previously tested in our laboratory (pGRU0.05; Li Pan, personal communication) demonstrates that truncation to -52 does not compromise the core promoter regions required for assembly of a competent initiation complex. pGRU0.05 contains the TAT region from -2,561 to -2,308, including the entire GRUI, ligated at -52. This construct produces significant CAT activity after glucocorticoid treatment. Conversely, pBE0.05 mediates barely measurable transcription. Thus, these two distal enhancers, one hormone-dependent and one not, have different proximal promoter requirements for their enhancing activities. Presumably, whatever DNA/protein complex which might assemble at the BE must interact with the -208 to -52

region, and possibly with the polymerase complex in addition.

Two candidates for the target(s) within that region are the proteins that bind CCAAT boxes at -167 and -72 (See **Introduction**). NF1 (44) appears to bind the more distal site (43), and is critical for most of the low basal expression seen from the proximal promoter alone in *in vitro* transcription assays. Thus, it might seem the more likely candidate, especially since NFY (48), binding at -72, has no demonstrated functional importance. Although no NF1:protein interactions have yet been described, the BE complex might be able to influence polymerase activity through contact with NF1.

Such a postulated mechanism appears distinct from that of some enhancers, which exhibit more direct functional relationships with their initiation complexes. For example, GRE's can drive significant expression when ligated to HSV TK promoters truncated to delete all but the TATAA box (Li Pan, personal communication); BE function requires additional sequences. Whatever those critical sequences are in the -208 to -52 region, they are present as functional analogues (at least with respect to basal transcription) in the longer HSV TK promoter (-105 to +51) normally used in heterologous promoter experiments. That promoter contains CCAAT boxes which might bind NF1 in liver nuclei.

The -208 to -52 region includes the IRR (-104 to -52), required for insulin modulation of glucocorticoids' transcriptional effects (See **Introduction**). Thus it is possible that the *cis* regions required for these two different functions (mediation of basal enhancement and insulin modulation of glucocorticoid effects) coincide.

The Role of the CRE in the BE

Mutations within the CRE in BI of the BE severely reduce, but do not abolish basal transcription (58), and mutations within BIII can reduce basal expression even more. Thus the CRE appears an integral part of the basal enhancer, but not sufficient, and perhaps not the most important part. cAMP responsiveness is mediated exclusively by the CRE in BI, however; BIII is dispensable if BI is dimerized (58). This confusing structural separation of basal and cAMP-dependent responses undermine any assumption that cAMP responsiveness has the same proximal promoter requirements as does BE function. Although the -208 to +62 promoter might be sufficient for mediation of both cAMP-induced and basal transcription, the CRE's requirements within that region may not be identical to the BE's requirements, and it might require additional sequences outside that region. Future experiments might employ existing constructs to investigate the proximal promoter requirements for cAMP responsiveness.

In some systems, hormone-responsive enhancers appear to drive basal expression as well. Multiple GRE's can increase basal expression from the HSV TK promoter, even in the absence of glucocorticoids (55). In yeast, pheromone-responsive elements are sufficient for basal expression of the FUS1 gene (81). CRE's drive basal expression in genes encoding plasminogen activator inhibitor type 2 (82) and transforming growth factor β3 (83).

Such mechanisms of basal expression might rely on a background or basal level of hormone (or hormone-modulated factors) to maintain the basal level of expression. Basal levels of activated cAMP-dependent protein kinase are detectable in hepatoma cells (84). In the case of the TAT gene, those levels, acting through the CRE, may account for a part of basal expression.

Repressors and De-Repressors?

The complete absence of basal activity in two constructs studied (pBE0.35 and pBE3.34), despite the presence of the BE and the required proximal promoter regions, remains an enigma. The three isoforms of pBE0.35 (pBE0.35a, b and c) demonstrate that if the BE/proximal promoter interaction is helix-face dependent, then that dependence is extremely specific. If the -351 to -208 region is regarded as a spacer, some torsional flexibility might be expected over those 143 base pairs that would

minimize the requirement for any such specificity. Since the three constructs together included BE/proximal promoter rotational positions spanning 288° (360° x $^{8}/_{10.5}$), some activity might be expected from one. The significant basal expression from pBE0.21 and other plasmids (See **Figure 13**, page 56) further suggests that distance is not a critical consideration.

An alternative hypothesis (with respect to pBE0.35) would view the -351 to -208 region as a strong repressor of basal transcription (cAMP effects on this construct have not been tested). Obviously, its effects are not manifested in the endogenous gene, nor are they in several mutant promoters. The -800 to -351 region can completely "de-repress" the function of its proximal neighbor, as can the -2,561 to -2,308 region (See **Figure 17**).

The repressing function of the -351 to -208 region also appears specific for the BE. A construct containing synthetic GRE's ligated at -351 (pGRE0.35) exhibits significant expression after glucocorticoid treatment in these same cells (49). This suggests that this repressor region specifically obstructs interaction of the BE with the critical -208 to -52 region.

The apparent independence of the BE/proximal promoter interaction from distance and helix-face restraints, together with the specificity of the function of the -351 to -208 region, support the postulation of *cis*-acting

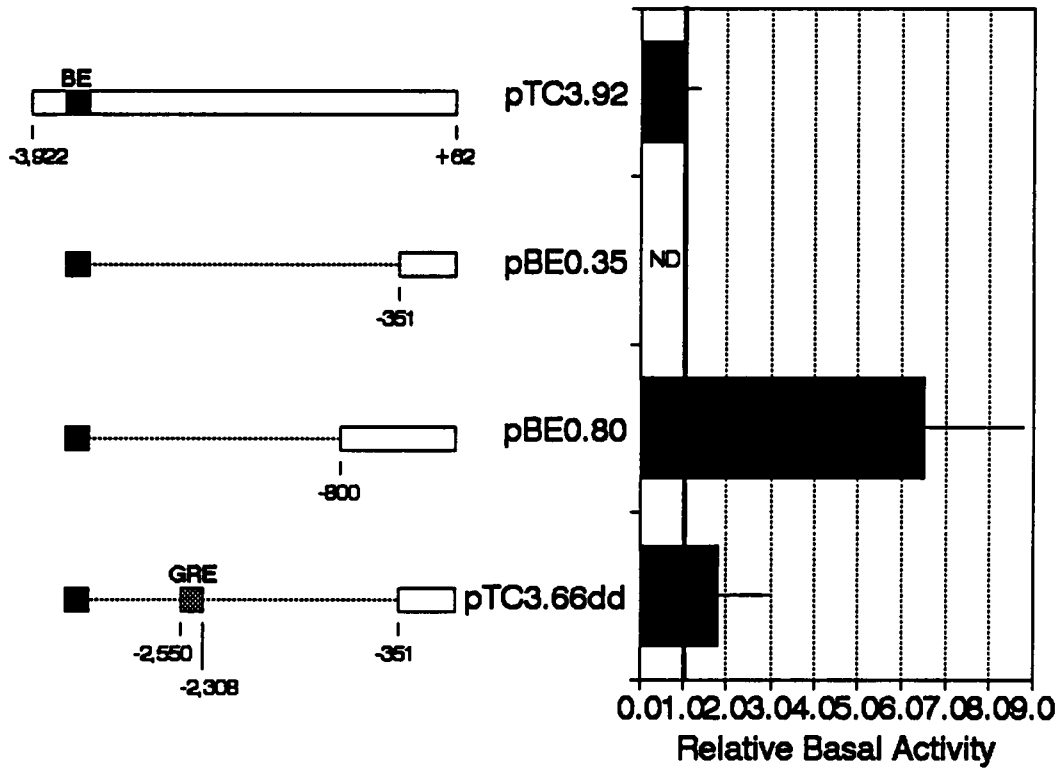


FIGURE 17 - A Proximal Repressor Region Is Inactive in Longer Constructs. Mean basal expression from each plasmid is shown relative to mean basal expression from pTC3.92 in the same experiment. pTC3.66dd is the same as pTC3.66ΔΔ described in **Methods**; values for it and pBE0.80 are significantly different from those for pTC3.92. pBE0.35 represents the results from all 3 forms of this plasmid; expression was not detectable (ND) from any. For pTC3.92, n=48, 16 experiments; for pBE0.35a, b and c, n=6, 2 experiments; for pBE0.80 and pTC3.66ΔΔ, n=9, 3 experiments. The thin lines represent population standard deviations. BE = basal enhancer; GRE = glucocorticoid-responsive element.

elements within that region that exert strong and specific suppressive effects. Such a repressor might interact with more proximal sequences, with the BE, or both. Since the relative position of the proximal promoter and -351 to -208 repressor is identical in many other constructs which show strong BE function (See **Figure 13**, page 56) it appears likely that the repressor interacts with the BE complex.

Deletion of either of the two de-repressing regions (-2,561 to -2,308; -800 to -351) has no significant effect on basal activity, as long as the repressor region is deleted as well (See pBE0.21, **Figure 13**, page 56). This suggests a model of the TAT promoter which contains basal repressor elements that are constitutively de-repressed; that de-repression might actually be the result of the repressor's position in the TAT native context, and not a specific interaction with another *cis* sequence.

Such a repressor system might be critically involved in forms of TAT regulation not yet recognized. That regulation might be based on alteration of the BE's activity by alteration of its interactions with the proximal promoter. Alternatively, this repressor/de-repressor system could represent a set of dormant switches that were once active, or may eventually become active as evolution proceeds.

The IRE and the BE

In the KRC-7 cell line, study of the inhibitory effects of insulin on TAT expression have required the constant presence of auxiliary *cis*-acting enhancers. Previous studies in this laboratory employed GRE's and glucocorticoid treatment to produce the effect upon which insulin's effects were studied. As described previously, those studies demonstrated the sufficiency of the proximal promoter for mediation of insulin inhibition of glucocorticoid induction.

Since basal expression from TAT is critically dependent upon the BE at -3,600, this current study of insulin's effects upon that basal expression required the BE's presence. The critical and specific function of the -208 to -52 region suggests that pBE0.21 is the "core" basal promoter available for this study: pBE0.21 provides all *cis*-acting regions required for basal expression comparable to that from the -3,922 to +62 promoter (pTC3.92), and is thus sufficient in its entirety.

The failure of insulin to inhibit expression from pBE0.21 is therefore significant: it indicates that insulin cannot act solely by modification of the apparatus assembled on pBE0.21 during basal expression. Although such modifications cannot be ruled out as part of insulin's overall effect, they cannot be sufficient. Instead, the failure of pBE0.21 to recreate the insulin effect seen with

pTC3.92 indicated the existence of one or more *cis*-acting regions which mediate insulin's effect.

The failure of insulin to inhibit basal expression from pBE0.21 also revealed a unique feature of insulin's mechanisms in this gene: The *cis* regions required for different insulin effects (inhibition of glucocorticoid-induced versus basal expression) are distinct. Promoter mutants which targeted the area around the BE eventually revealed a new insulin-responsive element (IRE), distinct from the IRR required for the insulin effect on glucocorticoid induction.

The clearest delimitation of that region is found in the comparison of pBE0.21 and pTC3.67$_{\Delta}$-3,543/-208 (See **Figure 11**, page 53). These two plasmids are identical except for the presence of the 15 base pair IRE (-3,675 to -3,661). While pBE0.21 is hormone-refractive, pTC3.67$_{\Delta}$-3,543/-208 mediates a significant insulin inhibition of basal transcription.

The 15 base pair IRE may actually contain sequences sufficient for its function. Alternatively, it may contain as little as a single base pair at its 3' end required to complete a necessary sequence that lies largely within the BE, immediately downstream. In either case, the critical sequences appear to be distinct from the important CRE, which lies 10 base pairs downstream from the 3' end of the IRE in its native position. Those 10 base pairs are

refractory to point mutations that abolish basal expression and cAMP sensitivity when targeted to the CRE. However, they may contain sequences required for insulin responsiveness.

The mechanism by which the IRE might modulate expression from the BE could target the CRE, and its portion of the total basal expression. Although BIII, further downstream, appears more important for full basal transcription, crippled CRE function could more than account for the >50% inhibition observed.

The IRE, and presumably its bound protein(s), might cripple the CRE's function by sterically hindering the binding of CREB. If the critical insulin-responsive sequences lie largely within the BE, as described above, they might be close enough to the CRE to produce this effect. Alternatively, the IRE complex might modulate the CRE's activity only after CREB has bound.

The model described above, in which the IRE function targets the CRE to modulate basal expression, can also serve to explain its role in insulin inhibition of cAMP induction. The same steric effects or protein:protein interactions could obstruct the hormone-induced response mediated by the CRE.

Paradoxically, the IRE's presence (without insulin treatment) significantly inhibits basal expression (relative to pBE3.66, see **Figure 13**, page 56) but potentiates the

apparent cAMP response (See **Figure 12**, page 55). The former effect supports the previously described model: background levels of any IRE-binding protein might exert background levels of inhibition, mediated by the IRE in the absence of added hormone.

The IRE's apparent potentiation of the cAMP response deserves closer attention, since it may be an artifact of the mathematical method with which these transcriptional effects have been described. As mentioned above, the presence of the IRE decreases basal expression. Since hormonal effects are reported in **Figure 12** (page 55) as fold changes relative to basal, decreased basal levels might reflect as increased cAMP induction, even if the absolute ceiling of induction was unchanged in the presence and absence of the IRE.

As seen in **Figure 18**, cAMP increased expression from pTC3.67 and pTC3.66 to comparable absolute levels, which may reflect a maximal cAMP-induced transcription rate in this system. (This level is not a limitation of the CAT assay; see **Methods**. The 8-bromo cAMP concentration chosen produces maximal effects; data not shown.) Since both plasmids achieved a cAMP-induced "expression ceiling", one cannot be accurately said to exhibit a greater cAMP response than the other. Therefore the IRE cannot be said to potentiate the cAMP response.

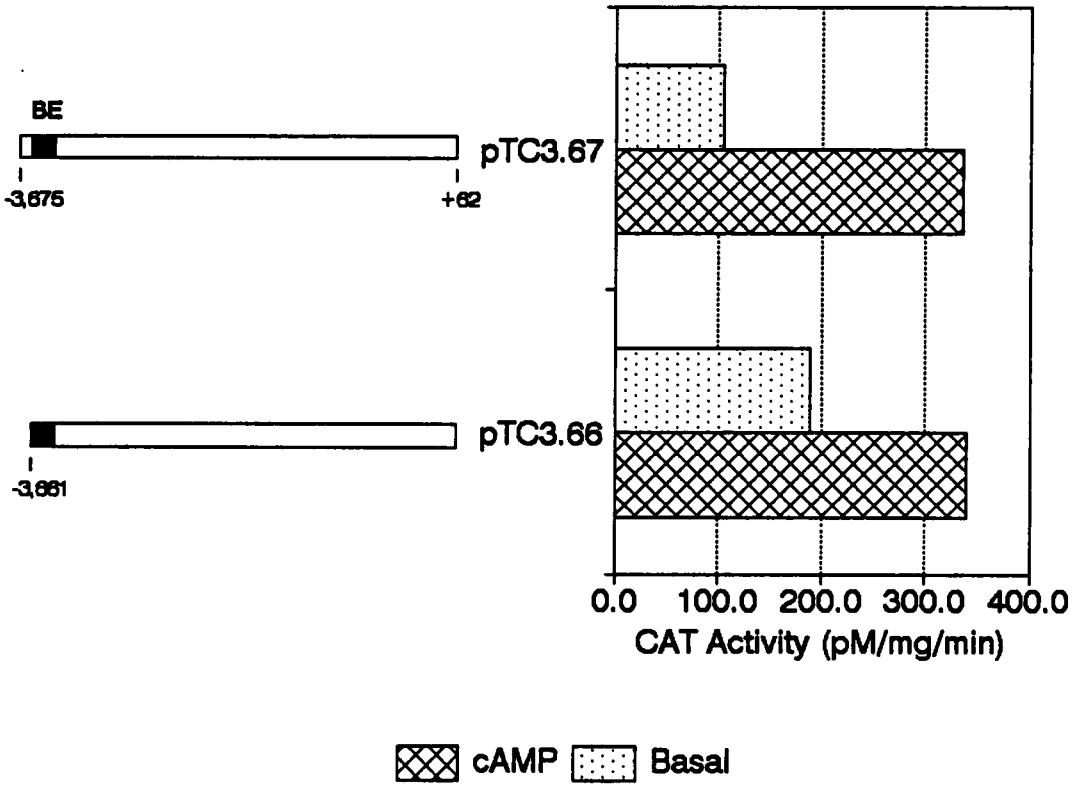


FIGURE 18 - The IRE Does Not Potentiate the cAMP Response. Shown are absolute CAT activities accumulated over the 12 hour treatment period. These same data are expressed in **Figure 12** (page X) as relative hormone effects. n=6, 2 experiments. IRE = insulin-responsive element; BE = basal enhancer.

The distortion produced by fold change descriptions could conceivably minimize the observed insulin effect on basal as well, "cloaking" regions required for insulin inhibition. For example, pBE1.30's basal expression is two fold greater than pTC3.92's (See **Figure 13**, page 56). The negative insulin effect seen with pBE1.30 is reported as a statistically insignificant fold change in **Figure 9** (page 51). However, the actual number of initiation events prevented by insulin with this plasmid may exceed those "subtracted" by insulin from pTC3.92's basal expression. (All plasmids which contained the IRE (-3,675 to -3,661) mediated a statistically significant relative insulin inhibition.)

The TAT IRE and Other IRE's

The TAT IRE has not yet been fully delimited, nor tested in the context of a heterologous promoter, thus its acceptance into the family of IRE's may be questioned. However, the recreation of endogenous function in a heterologous DNA sequence environment may be an unreasonable expectation for some systems, particularly those that require multiple promoter regions for full function.

The list of IRE's from other genes is slowly growing (85); only a few are able to recreate their function in heterologous contexts. The IRE from the gene encoding phosphoenolpyruvate carboxykinase (PEPCK) can

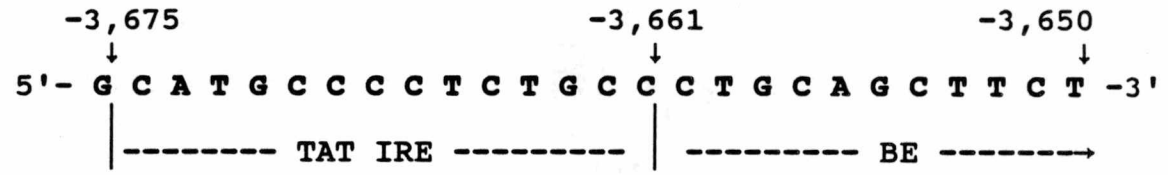
apparently function in HSV TK constructs only if placed within the viral promoter (86).

Perplexingly, a universal concensus IRE has not emerged from these discoveries. This is unique in transcriptional regulation: all genes regulated by a common hormone usually share common (concensus) sequences. Although a few IRE's appear to group within a single tenuous family (85), most others, including this new TAT IRE, have no strong similarity to those sequences.

The PEPCK IRE (86) is the prototype sequence for the family mentioned above, by virtue of its earlier discovery (See **Figure 19**). It shares similarities with sequences within the IRE's of genes encoding α-amylase (92), prolactin (101), human growth hormone (102) and insulin-like growth factor binding protein 1 (103). Other insulin-regulated genes, including gene 33 (104), adipsin (105), glucokinase (106) and malic enzyme (107) have similarities to the PEPCK IRE within their sequences, but those regions have not yet been demonstrated to be functionally required for insulin sensitivity.

In addition to, and overlapping its PEPCK-like sequences, the large α-amylase IRE (92) contains a region similar to sequences within one of the two glyceraldehyde phosphate dehydrogenase (GAPDH) IRE's (IRE A) (88). The TAT IRE is similar to those shared sequences, which bear no

TAT IRE (coding strand)



PEPCK IRE (coding strand)

α-AMYLASE IRE (coding strand)

GAPDH IRE A (non-coding strand)

TAT IRE (non-coding strand)

FIGURE 19 - The TAT IRE Is Similar to the GAPDH IRE. Shown are the IRE's for TAT, PEPCK, α-amylase and TAT. Similarities between PEPCK and α-amylase IRE's are boxed; similarities between α-amylase and GAPDH IRE's are double underlined; similarities between GAPDH and TAT IRE's are boxed. TAT and GAPDH IRE's share 9/11 nucleotides over the indicated region. The T at -3,650 in the TAT IRE coding strand is the first base of the CRE in BI of the BE.

relation to the PEPCK IRE (See **Figure 19**). Together, GAPDH and TAT may provide the prototype for a new concensus IRE:

5' - G G C n G A G n G G C - 3'

The TAT IRE compares well with other IRE's from other perspectives. The PEPCK IRE is closely approximated to PEPCK's GRE's (86), which are critical for PEPCK's glucocorticoid sensitivity. The PEPCK IRE may modulate glucocorticoid-induced transcription by steric interference with glucocorticoid receptor binding (87). The similarity with TAT's IRE is clear, except that the CRE, not GRE, may be the target *cis* element. The PEPCK IRE also mediates inhibition of basal transcription, even though PEPCK's GRE's play no apparent role in basal expression. It is unclear whether the PEPCK IRE, which mediates only ~50% of the total insulin effect, also mediates insulin modulation of cAMP-induced transcription.

The presence of multiple IRE's in the PEPCK promoter (87) is mirrored in the GAPDH gene. There two distinct IRE's each mediate 50% of the positive insulin effect on basal levels of expression (88). A protein has been isolated which binds one of GAPDH's IRE's (IRE A) with increased affinity after insulin treatment (89). The DNA binding domain of that protein shows some similarity to the comparable domain of SRY, the testis determining factor (90).

In other examples, IRE's lie near or overlap other *cis*-acting regions. The IRE in the c-fos promoter appears to be identical with the serum response element there (91). In the PEPCK gene described above, regulation by insulin and phorbol esters is mediated by the same or closely approximated sequences (86). In an arrangement similar to TAT's, the IRE in the promoter of the amylase gene lies close to *cis* regions essential for basal expression (92). Although the mode by which insulin's effectors interact with those *cis* regions is not known for any system, the opportunities for steric interference or non-covalent interactions between *trans* factors seem clear.

Conclusion

This research has revealed that sequences between -3,675 and -3,661 are required for insulin's effect on basal and cAMP-modulated TAT transcription. In addition, a region in the proximal promoter between -208 and -52 is required for full function of the BE at -3,600.

TAT may be the most complex of all insulin-regulated genes. There, insulin responsiveness is functionally and structurally divided: Research conducted exclusively in this laboratory has revealed that different and widely separated regions are required to mediate different insulin effects. One of those responsive regions, identified in the work described here (the distal IRE), may modulate basal and

cAMP-induced levels by the same mechanism. The distal IRE's proximity to a region implicated in tissue-specific expression raises currently unanswerable questions about its role in that form of regulation as well. In addition, results of this study suggest that insulin acts through multiple positive and negative mechanisms in this gene.

The yearning that all researchers feel for an underlying simplicity in the living systems they study remains unfulfilled in those who choose to examine insulin and its effects. The inability to extrapolate to other genes newly acquired findings on insulin's mechanisms portends slow progress in this area of transcriptional regulation. Yet the exceptions to our expectations can often be revealing. Once fully illuminated, insulin's mysteries might urge us to restructure our understanding of how cells retrieve the information in their genes.

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