Cooperativity of Glucocorticoid Response Elements Located Far Upstream of the Tyrosine Aminotransferase Gene

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Summary

Two glucocorticoid response elements (GREs) located 2.5 kb upstream of the transcription initiation site of the tyrosine aminotransferase gene were identified by gene transfer experiments and shown to bind to purified glucocorticoid receptor. Although the proximal GRE has no inherent capacity by itself to stimulate transcription, when present in conjunction with the distal GRE, this element synergistically enhances glucocorticoid induction of gene expression. Cooperativity of the two GREs is maintained when they are transposed upstream of a heterologous promoter. An oligonucleotide of 22 bp representing the distal GRE is sufficient to confer glucocorticoid inducibility. As evidenced by the mapping of DNAase I hypersensitive sites, local alterations in the structure of chromatin at the GREs take place as a consequence of hormonal treatment.

Introduction

We have undertaken an analysis of the expression of the tyrosine aminotransferase (TAT) gene because this system allows us to study the role of glucocorticoids and cAMP in cell-specific and developmental regulation of transcription (Granner and Beale, 1985). Tyrosine aminotransferase is synthesized exclusively in parenchymal cells of the liver (Hargrove and Granner, 1985). Whereas expression of the gene is undetectable before birth, transcription increases rapidly within the first hours after birth (Greengard, 1970). Premature activation of the TAT gene can be elicited by administration of glucocorticoid hormones and cAMP, pointing to the potential role of these two inducers in the developmental control of expression of this gene (Ruiz-Bravo and Ernest, 1982).

Genetic and biochemical analysis of albino lethal mutations and somatic cell genetic experiments have revealed two genetic loci that influence the expression of the TAT gene in vivo. A control region required for the expression and inducibility of TAT and several other liver-specific enzymes has been assigned to a region close to the albino locus on chromosome 7 of the mouse (Gluecksohn-Waelsch, 1979). In the absence of this locus, expression of the TAT gene, which resides on chromosome 8 of the mouse, is severely reduced and is no longer affected by glucocorticoids and cAMP (Schmid et al., 1985). An additional locus that affects expression of TAT was identified by somatic cell hybrids in which chromosome 11 of fibroblasts was introduced into hepatoma cells expressing TAT (Kiliary and Fournier, 1984). Expression of TAT is selectively extinguished suggesting that a repressor-like factor acting in 

To understand more completely the role of glucocorticoids and cAMP in the cell-specific and developmental regulation of TAT expression, a detailed analysis of the hormonal regulation of this gene has been performed. Increased expression of the gene following glucocorticoid and cAMP administration is a consequence of direct transcriptional activation (Hashimoto et al., 1984; E. Schmid et al., submitted). To identify those sequences that mediate transcriptional control by steroids, we introduced TAT fusion genes containing specific DNA fragments of the TAT upstream region into L cells and studied hormone-dependent expression of these recombinants. Using DNA transfection techniques (for review see Yamamoto, 1985; Ringold, 1985), sequences mediating steroid induction have been identified in the mouse mammary tumor virus (MMTV) genome (Yamamoto, 1985; Buetti and Kühnel, 1986), the human metallothionein IIa gene (Karim et al., 1984a), the chicken lysozyme gene (Renkawitz et al., 1984), the human growth hormone gene (Slater et al., 1985), and the Moloney murine sarcoma virus (M-MuSV) genome (Miksicek et al., 1986; DeFranco and Yamamoto, 1986). In each case, these elements have been found by in vitro DNA binding experiments to correspond to binding sites for the appropriate steroid receptors (Payvar et al., 1983; Scheideireit et al., 1983) and to be characterized by a well-conserved consensus sequence (Karim et al., 1984b). They are typically located within several hundred bp upstream of the transcription initiation sites or, alternately, within the coding region of the genes, and they have been found to possess enhancer-like properties (Chandler et al., 1983; Karin et al., 1984b). These studies suggest that by binding to specific sites in the promoter regions of target genes, steroid receptors are able to elicit an increased rate of transcription initiation; however, it remains unclear precisely how this effect is exerted.

A novel property of the glucocorticoid response elements (GREs) of the TAT gene is their ability to activate transcription in a cooperative manner. The glucocorticoid control region of the TAT gene is composed of two elements representing glucocorticoid receptor binding sites. One element, which in itself has no inducing capacity, will enhance glucocorticoid induction in the presence of the other.

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Results

The GREs of the TAT Gene Are Located 2.5 kb Upstream of the Transcription Initiation Site

To identify sequences mediating glucocorticoid induction of the TAT gene, we have constructed a fusion gene containing the TAT 5′-flanking sequences (−2950 to +62) up-stream of the bacterial chloramphenicol acetyltransferase (CAT) gene (Figure 1A). Expression of this plasmid was studied after transient introduction into mouse L cells in the presence or absence of the synthetic glucocorticoid dexamethasone. To localize the essential regulatory elements within the TAT 5′-flanking sequences, appropriate 5′ and 3′ deletion mutants were constructed and their glucocorticoid-dependent expression was investigated. Expression of these plasmids was analyzed by following CAT enzymatic activity, and CAT mRNA levels were measured using the ribonuclease protection assay shown schematically in Figure 1B. A labeled antisense RNA probe that overlaps with the start site of transcription of the TAT gene was used for hybridization with total RNA from transfected cells. Figure 1B shows the protected fragments expected for correctly initiated transcripts and for transcripts originating upstream of the initiation site.

Analysis of the kinetics of TAT-CAT mRNA accumulation following hormone addition indicated that strong induction of RNA occurred 12 hr after the addition of dexamethasone (data not shown). We therefore analyzed expression of the 5′ and 3′ deletion mutants following 12 hr of dexamethasone treatment. Figure 1C shows an analysis of the expression of a set of 5′ deletion mutants using the ribonuclease protection assay. The parental plasmid and derivatives with deletion end points ending at −2561 and −2527 give rise to a strong increase in correctly initiated CAT-CAT mRNA following dexamethasone treatment. In addition, RNA originating upstream of the TAT transcription unit is also induced, though to a lesser extent. In contrast, the deletion mutants ending at −2504 and farther downstream showed no dexamethasone-induced expression, indicating that an element essential for glucocorticoid induction is located far upstream of the transcription initiation site. The extent of hormonal induction resulting from this element has been estimated to be about 15-fold based upon quantitative measurements of CAT enzymatic activity with and without dexamethasone treatment (data not shown). Transfection of a plasmid that contains 3 kb of 5′-flanking sequence, the complete TAT gene, and 4 kb of 3′-flanking sequence failed to give a higher ratio of induction as judged by Northern analysis of the RNA (data not shown). Therefore we conclude that all of the IA1 GREs are likely to be confined to the 5′-flanking region.

Two GREs Act Cooperatively

To delineate precisely the DNA sequences conferring glucocorticoid inducibility, internal deletion mutants were also constructed. A 5′ deletion end point of −2527 was chosen since this mutant still conveys full inducibility (see Figure 1C). The 3′ end point of this deletion series is located at nucleotide 2127. This end point was chosen in order to progressively delete sequence elements IV
(−2194 to −2178), III, and II (see Figure 7), which display strong sequence homology to the glucocorticoid receptor binding site (Payvar et al., 1983; Scheidereit et al., 1983). The set of internal deletion mutants was transfected into Ltk− cells, and expression in the presence and absence of dexamethasone was monitored by analysis of CAT activity and RNA start site mapping (Figure 2). Full inducibility is preserved up to deletion −2419/−2127, i.e., as long as sequences comprising homology II and III are intact. These results show that homology IV is dispensable for the observed regulatory response. Deletion of an additional 18 bp (−2439 to 2127) leads to an approximately 4-fold reduction in inducibility. This residual hormonal response is maintained up to deletion −2493/−2127 (Figure 2A). As can be seen from the sequence in Figure 7A, this internal deletion is located at the 3′ end of homology region II, but leaves intact the functionally important hexanucleotide TGTTCT. If the deletion extends farther into homology region II (−2510 to −2127), inducibility is lost completely (Figure 2A). From the analysis of the 5′ and internal deletion mutants we conclude that element II alone is capable of conferring inducibility; however, this is not the case for element III. From the quantitative analysis based on CAT enzymatic activity measurements (Figure 2B), it is evident that these two elements act in a cooperative fashion. Element III, which by itself is not able to convey glucocorticoid inducibility, strongly enhances glucocorticoid induction when present alongside element II.

The GREs Elicit a Stronger Response When Positioned Close to the TAT Promoter

The results presented so far clearly demonstrate that the GREs of the TAT gene confer inducibility from a position far upstream. This observation strongly supports the concept that GREs act as hormonally regulated enhancers (Chandler et al., 1983; Yamamoto, 1984). To test whether the TAT GREs conform to this notion we have measured their activity in their native context, when located closer to the TAT promoter (Figure 3), and when transposed upstream of the HSV thymidine kinase (TK) promoter (Figure 4). We were particularly interested in evaluating whether the cooperative behavior of the two GREs is maintained when positioned closer to the TAT promoter or when linked to a heterologous promoter. The experiments depicted in Figure 3 compare the effect of the two GREs, alone or in combination, at a site close to (−351) and remote from (−2127) the TAT promoter. Two interesting observations can be made. First, inducibility increases approximately 4-fold when the fragment containing elements II and III is moved closer to the TAT promoter. This is also true for element II alone. The increase is observed in either orientation of the fragments conferring inducibility. Element III, which is inactive by itself in its natural position, remains inactive when placed closer to the TAT promoter. Second, since element II alone displays only 30% of the induction in comparison with the effect of both elements, it is clear that the cooperativity of elements II and III is maintained when they are moved 2 kb closer to the TAT promoter.

Cooperativity of the GREs Is Maintained When Positioned Upstream of a Heterologous Promoter

Does the cooperative behavior of the GREs depend on specific interactions with the TAT promoter and bound proteins or does this element also activate a heterologous promoter in a cooperative manner? To answer this question, the effect of GREs II and III, alone or in combination, was tested in front of the heterologous TK promoter. Fragments containing GRE II, GRE III, or both GREs were inserted in both orientations 5′ and 3′ to the TK-CAT fusion gene (Miksicek et al., 1986). The TK promoter was chosen since it has been demonstrated to respond to a variety of hormonal and nonhormonal transcriptional control sequences (Chandler et al., 1983; Hynes et al., 1983; Karin et al., 1984b; Pelham and Bienz, 1982; Searle et al., 1985). Measurements of CAT activity from two independent experiments (Figure 4) show that the TAT GREs display a similar behavior upstream of the heterologous TK pro-
Figure 3. The GREs Elicit a Stronger Response When Positioned Closer to the TAT Promoter

Ltk- cells were transfected with the pTAT-CAT recombinants schematically outlined. A and B denote the natural and inverse orientation of the GRE-containing fragments, respectively. Cells were cultured with or without 10^{-9} M dexamethasone (DEX) for 24 hr before harvesting. Extracts were prepared and assayed for CAT enzymatic activity as described in Experimental Procedures. The values were derived from duplicate transfections.

Figure 4. Cooperative Effect of the GREs on a Heterologous Promoter

Ltk- cells were transfected with the GRE-TK-CAT recombinants schematically shown. In A and B the GRE-containing fragments are linked at -105 with the TK sequence; in C and D they are inserted at the Smal site downstream of the TK-CAT cassette. A and C, as well as B and D, indicate the natural and inverse orientation of the GRE-containing fragments, respectively. The 22-mer oligonucleotide represents the sequence of GRE II protected against DNAase I digestion by the glucocorticoid receptor. Cells were processed and CAT activity was determined as described in Figure 28. The results shown are from two independent transfection experiments. The ratios of induction were derived from duplicate transfections.

A 22 bp Oligonucleotide Is Sufficient to Render the TK Promoter Responsive to Glucocorticoids

To define the minimal sequence required for glucocorticoid induction, a synthetic 22 bp oligonucleotide representing the region of GRE II protected against DNAase I digestion by glucocorticoid receptor (see Figure 5) was placed immediately upstream of the complete HSV TK promoter. As seen in Figure 4, this oligonucleotide leads to an approximately 10-fold increase in expression of CAT activity when cells are treated with dexamethasone. In the inverse orientation the oligonucleotide is less effective than the larger fragment encompassing footprint II, indicating a requirement for additional sequences or for a particular alignment of the GRE with respect to other promoter elements. This experiment demonstrates that, at least in the context of the TK promoter, the sequence protected against DNAase I digestion by the glucocorticoid receptor is sufficient to give glucocorticoid inducibility.
Cooperativity of Glucocorticoid Response Elements

Induction Leads to Changes in Chromatin Structure at the GREs

To acquire additional evidence that the GREs defined in this study function as such in vivo, precise mapping of the glucocorticoid responsive DNAase I hypersensitive site previously observed upstream of the TAT gene (Becker et al., 1984) was performed (Figure 6). This analysis was performed on nuclei isolated from FTO-2B cells, a rat hepatoma line that expresses TAT and is responsive to glucocorticoid induction (Killary and Fournier, 1984). Use of either DNAase I or methidiumpropyl-EDTA-iron(II) (MPE.Fe(III)) shows that this hypersensitive site can be resolved into a triplet of intense cleavages confined to the region between −2600 and −2300. The two GREs defined above fall within these boundaries. These hypersensitive sites are not seen in the absence of dexamethasone. Furthermore, no additional dexamethasone-dependent changes in DNAase I sensitivity can be discerned within a 40 kb region encompassing the TAT gene (data not shown). These glucocorticoid-dependent alterations in chromatin structure are consistent with the premise that elements II and III represent the sites of interaction of the glucocorticoid receptor with the TAT gene in vivo as well as in vitro.

Discussion

Sequence of the GREs of the TAT Gene

Analysis of glucocorticoid-dependent expression, together with in vitro and in vivo (Becker et al., 1986) foot-printing experiments, has allowed the identification of the sequences of the TAT gene important for glucocorticoid control. Figure 7 presents the sequence of the TAT gene in the vicinity of the functional glucocorticoid regulation elements (7A) and a comparison of this sequence to GREs of other steroid controlled genes (7B). Of the three regions identified by in vitro binding studies with the purified glucocorticoid receptor, only sequences of footprints II and III are required for full glucocorticoid inducibility, while footprint I is dispensable. It is not clear why site I does not contribute to inducibility, although it is capable of binding to the receptor in vitro. A possible explanation for the inactivity of this sequence is that this homology is shortened in comparison to footprints II and III and represents only one-half of the palindromic sequence characteristic of functional GREs (see below). Footprint I therefore has properties quite similar to nonfunctional footprint sequences in the MMTV genome (Scheiderleit et al., 1983; Buetti and Kühnel, 1986). The importance of sequence elements II and III is strongly underscored by the changes in the DNAase I hypersensitivity pattern that occur upon glucocorticoid induction (Figure 6). No other hormone-inducible changes in chromatin structure, as evidenced by DNAase I hypersensitive site mapping, occur within 20 kb of 5'-flanking DNA, within 9 kb of 3'-flanking DNA, or within the transcribed region (F. Stewart, unpublished results). Within the limits of resolution, the DNAase I cleavages at −2.5 kb correspond to the regions that represent binding sites for the glucocorticoid receptor in vitro and that are important for conferring inducibility in gene
Figure 6. Glucocorticoid Induction of the TAT Gene Leads to Chromatin Changes at the GREs

Nuclei were isolated from FTO-26 cells cultured in the absence (lanes 1–11) or presence (lanes 12–22) of dexamethasone (Dex) (1 μM). The nuclei were digested with increasing amounts of DNAase I for 10 min on ice (lanes 1 and 12, 0.075 U/μg; lanes 2 and 13, 0.15 U/μg; lanes 3 and 14, 0.3 U/μg; lanes 4 and 15, 0.6 U/μg; lanes 5 and 16, 0.9 U/μg; lanes 6 and 17, 1.2 U/μg) or with 50 μM MPE.Fe(II) at 25°C for increasing times (lanes 7 and 18, 0 min; lanes 8 and 19, 10 min; lanes 9 and 20, 20 min; lanes 10 and 21, 30 min; lanes 11 and 22, 40 min). After EcoRl digestion, DNA (20 μg per lane) was electrophoresed in a 1.7% agarose gel, blotted, and indirectly end-labeled to the EcoRl site at -3050 of the TAT gene. M denotes end-labeled marker DNAs whose sizes in bp are indicated to the left. Lanes 23 and 24 display internal markers derived from EcoRI-digested genomic DNA. The cleavage sites of these enzymes with respect to the start site of TAT transcription are indicated to the right. Lane 23 contains 14 μg of EcoRI-cut genomic DNA plus 2 μg of EcoRI/XbaI (-2561); 2 μg of EcoRI/Hinfl (-2582) and 3 μg of EcoRI/Aval (-2633) double-digested genomic DNAs. Lane 24 contains 14 μg of EcoRI-cut genomic DNA plus 3 μg of EcoRI/Hinfl (-2561) and 3 μg of EcoRI/Aval (-2633) double-digested genomic DNAs. The probe used was a T7 polymerase transcript from subclone pBX406 (-2950 to -2561 of the TAT gene). The positions of GRE II and GRE III and the positions of the other hypersensitive sites are indicated with arrows.

The GREs of the TAT Gene Function as a Transcriptional Enhancer

Although enhancer properties have previously been attributed to GREs (Chandler et al., 1983; Karin et al., 1984...
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-2520 -> c
-2470 -> i
-2420

A

TTTGAACATGACTTGACATGCATTCATG

CONSENSUS

GCTACANNNNTGTTCT

Figure 7. Sequence of the TAT GREs and Comparison with GREs of Other Glucocorticoid Controlled Genes

(A) The sequence of the TAT gene between -2620 and -2371 is shown. Overlining and underlining are used to indicate the sequences protected from DNAase I digestion after binding of the glucocorticoid receptor. Footprinted regions are labeled by Roman numerals. The palindromic nature of the GREs is indicated by arrows, with a star used to denote the center of symmetry. The thick arrows indicate nucleotides homologous to the consensus sequence. The arrows above the sequence indicate the incidence of 5' deletions (arrows pointing to the right) and 3' deletions (arrows pointing to the left).

(D) Sequences of GREs that have been identified by gene transfer experiments are compared by alignment with the most conserved hexanucleotide. A consensus sequence is derived from nucleotides conserved with a frequency of 60% or more, and homology of each GRE with this consensus is indicated by the thick horizontal arrows. The previously determined response elements of the lysozyme gene (Renkawitz et al., 1984) have not been included since this analysis has only been conducted at a qualitative level. The sequence of the GREs of the tryptophan oxygenase gene, TO (U. Danesch et al., submitted), the human metallothionein gene, HMT Ia (Karlin et al., 1984b), the murine sarcoma virus, MSV (Miksic et al., 1986), the human growth hormone gene, hGH (Slater et al., 1985), and the mouse mammary tumor virus, MMTV (Chandler et al., 1983; Buetti and Kühnle, 1986) are shown.

1984a), the finding that the TAT GREs are located 2.5 kb upstream of the transcription initiation site is in itself remarkable and immediately presents several potential implications. When the GREs are moved closer to their upstream of the transcription initiation site (G. Scherer, personal communication). This suggests that the TAT GREs, for unknown reasons, are restricted from maximal activity and/or that sequences between the GREs and the cognate promoter or to a heterologous promoter inducibility of widely dispersed transcriptional control elements, which can be independently shuffled via recombination, may have been crucial for the establishment of complex gene control circuits in a manner analogous to "exon shuffling" in the generation of structural genes (Gilbert, 1978). However, the spatial separation of those elements 2.5 kb away from the site of interaction of RNA polymerase poses the question as to how they affect the rate of transcription. Several mechanisms have been proposed to explain how such transcription factors may act at a distance (Serfling et al., 1985). Although it remains formally possible, it is unlikely that the altered chromatin configuration induced by glucocorticoid receptor binding to the GRE is propagated over kilobases of 5'-flanking sequence toward the promoter. Similarly, it is improbable that the receptor molecules themselves become physically displaced from their primary binding sites at the GREs until they reach the site of transcription initiation. A third possibility raised by analogy to the bacteriophage lambda system (Ptashne, 1986) is that specific contacts are established between proteins binding to the upstream GREs and additional factors interacting with the promoter. Since the minimal DNA sequence required for glucocorticoid induction has now been established, it is possible to determine whether such direct protein-protein interactions with the GREs are involved in the mechanism of action. Positional flexibility of widely dispersed transcriptional control elements, which can be independently shuffled via recombination, may have been crucial for the establishment of complex gene control circuits in a manner analogous to "exon shuffling" in the generation of structural genes (Gilbert, 1978). However, the spatial separation of those elements 2.5 kb away from the site of interaction of RNA polymerase poses the question as to how they affect the rate of transcription. Several mechanisms have been proposed to explain how such transcription factors may act at a distance (Serfling et al., 1985). 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interactions actually occur within the TAT upstream region.

Two Separate but Interacting Domains Constitute the Functional GRE of the TAT Gene

Analysis of mutants deleting elements I and III, alone or together, shows that both elements are required for maximal induction of transcription and that they have a strong synergistic effect on promoter activity. Whereas element II alone is capable of eliciting about one-third of the maximal response, element III by itself is inactive. The cooperative behavior is preserved when the GREs are positioned upstream of the heterologous TK promoter, suggesting that no additional elements specific to the TAT gene are required for the hormonal response. This result appears to be different from that obtained by mutational analyses of the MMTV hormonal response elements, which have to be different from that obtained by mutational analyses of other (Yamamoto, 1985; Buetti and Kühnel, 1986). It is not suggested that the GREs function independently of each other (Hochschild and Ptashne, 1986). Cooperative binding of the glucocorticoid receptor to nonadjacent sites may be the basis for the observed synergistic effect. Replacement of a single GREs and their bound receptor molecules is achieved.

We have not yet varied the relative orientation and spacing of the elements and therefore do not know whether cooperativity is established by direct contact of the receptor proteins, as is the case for the cooperativity of phase lambda repressor bound to operator sites (Hochschild and Ptashne, 1986). Cooperative binding of the glucocorticoid receptor to nonadjacent sites may be the basis for the observed synergistic effect. Replacement of a single binding site with two sites to which the hormone receptor complex binds cooperatively may strongly increase the efficiency of interaction of the receptor with its target sequences.

Experimental Procedures

Plasmid Constructions

pTAT-CAT

pTAT-CAT was constructed by inserting the 5'-flanking sequence of the rat TAT gene (~2950 to +62) in front of the bacterial CAT gene. The SalI-BamHI fragment from a genomic TAT clone carrying this sequence (Scherer et al., 1982; I. Scheffler, unpublished results) was inserted into the XhoI site of pBL-CAT-3 (kindly provided by B. Luckow). In pBL-CAT-3 the CAT coding region and SV40 splice and polyadenylation signals from pSV2-CAT (Gorman et al., 1982) are inserted into the polylinker of pUC18. In addition, BglII and XhoI sites have been engineered into the untranslated leader of CAT.

Construction of 5’ Deletion Mutants

The plasmid pTATBS16 containing the TAT upstream region between ~2950 and the BamHI site at ~1300 in pUC19 was digested with SalI. The linearized pTATBS16 was treated with exonuclease Bal31 (0.15 U/μg plasmid DNA; BFLC) in Bal31 digestion buffer (0.5 mM EDTA, 4 mM Tris-HCl [pH 7.5], 1 mM MgCl2, 1 mM CaCl2, and 20 mM NaCl) at 30°C. At various time points, aliquots were removed and the reaction was stopped by phenol-chloroform extraction. After generation of blunt ends by T4 polymerase (Maniatis et al., 1982) the TAT inserts were excised by BamHI digestion and isolated after separation on a low melting agarose gel. The shortened fragments were recombinated with the plasmid pTAT-CAT after excision of sequences between ~2950 and ~1300.

Construction of Internal Deletions

The 5’ deletion mutant of pTAT-CAT ending at ~2527 was linearized with SphI and XhoI sites have been engineered into the untranslated leader of CAT.

Construction of GRE-TAT and GRE-TK Recombinants

Fragments of the TAT 5'-flanking region containing GREs, as indicated in Figures 3 and 4, were cloned in both orientations into the polylinker region upstream of the TK deletion mutant ending at ~351. The GRE-TK recombinants were obtained by cloning the same fragments into pBL-CAT-2 (a gift of B. Luckow). The vector harboring the HSV-TK promoter into pTAT-CAT. Insertion sites were upstream and downstream of the TK-CAT cassette. The oligonucleotide CCGCGGAGCTGCTTCTGCACACAT was inserted into the BamHI site of pBL-CAT-2. Details of the strategies used for construction of the GRE-TK recombinants are available on request.

DNA Sequencing

The TAT sequence up to ~2950 was determined by chemical sequencing of both strands (Maxam and Gilbert, 1980). The end points of deletion mutants were verified by the enzymatic sequencing method (Chen and Seeburg, 1985).

Enzyme Assays

CAT assays were performed as described (Gorman et al., 1982), with the following modifications. Protein (10–100 μg) from 10,000 x g supernatant fractions of sonic extracts was incubated at 37°C for 10–60 min in 200 μl of an assay mixture containing 0.25 M Tris-HCl (pH 7.8), 0.4 mM acetyl coenzyme A, and 0.25 μCi [3H]chloramphenicol (57.6 Ci/mmol). The linearity of the reaction under these conditions was verified. The acetylated and nonacylated forms of [3H]chloramphenicol were separated by thin layer chromatography and quantitated by liquid scintillation counting. Enzyme activities were calculated as pmol chloramphenicol acetylated per min per mg of extract protein.

Ribonuclease Protection Assay

Total cellular RNA was isolated from transfected cultures using the method described by Sergeant et al. (1984). Quantitation of TAT-CAT-specific mRNA was performed with a ribonuclease protection assay using a uniformly 32P-labeled SP6 antisense RNA probe. The template used for generating the probe was derived by cloning the KpnI-PvuII fragment of pTAT-CAT in the antisense orientation into plasmid pSP64 (Melton et al., 1984); this plasmid was linearized with Avall at ~108. For the ribonuclease protection assay, 10 fmol (~3.5 x 10^5 cpm) of probe was hybridized for 12 hr at 45°C with 20 μg aliquots of total cellular RNA in 20 μl of hybridization buffer (80% formamide, 0.4 NaCl, 40 mM PIPES [pH 6.4], and 1 mM EDTA). Following hybridization, the samples were digested with ribonuclease and processed as described previously (Zinn et al., 1983; Melton et al., 1984). Autoradiographic exposure times were 3–12 hr using intensifying screens.

DNase I Footprinting

The 253 bp XbaI-TthI1 fragment (positions ~2681 to ~2308) was asymmetrically 32P-labeled at the XbaI site (upper strand) or at the TthI1 site (lower strand). Asymmetrically labeled DNA fragment (1–2
50–100 fmol of partially purified glucocorticoid receptor. Incubations were performed in a volume of 25 μl containing 15% PEG-6000, 10 mM Tris (pH 7.5), 2.5 mM dithiothreitol, 1 mM MgCl₂, 0.1 mM EDTA, 0.1 mM mg/ml BSA, 5 mM DTT, and 1 μM trichloronil for 45 min at 30°C. After incubation, a partial DNAase I digest was done by adding 3 μl of a DNAase I mix containing 45 mM MgCl₂, 300 ng/μl calf thymus DNA, and 5 μg/ml DNAase I (Worthington). DNA was extracted with phenol-chloroform, recovered by ethanol precipitation, and analyzed on a 6% polyacrylamide gel. Glucocorticoid receptor was partially purified from livers of adrenalectomized male rats following the procedure of Singh and Moudgil (1985).

Mapping of DNAase I and MPE: Fe(I) Hypersensitive Sites in Nuclei

FTD-2B cells were cultured, nuclei were prepared, and DNAase I digestions were performed as described previously for H4.IIE-C3 cells (Becker et al., 1984), except that serum-free treatment was for 20 hr, dexamethasone induction was for 2 hr, and nuclei isolation buffer was altered by omitting EDTA, spermine, and spermidine and including 5 mM MgCl₂. MPE: Fe(I) digestions were performed according to the method of Cartwright and Elgin (1984), except that the nucleotide concentration was 10 fmol (mnl) and the MPE: Fe(I) concentration was 50 μM.

Electrophoresis of agarose gels was performed in Tris acetate buffer (Maniatis et al., 1982) at 2 V/cm for 22 hr. After a 10 min treatment in 0.5 M NaOH to nitrocellulose (Gene Screen, New England Nuclear) by soak blotting, the filter was then floated for 5 min on 25 mM sodium phosphate (pH 6.5) and 1 mM EDTA (DNA side up), followed by rinsing in the same buffer for 30 sec. After air drying on 3MM paper, hybridizations and washings were performed at 70°C.

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