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

Hans-Michael Jantzen,* Uwe Strähle, Bernd Gloss,
Francis Stewart, Wolfgang Schmid, Michael Boshart,
Richard Miksicek, and Günther Schütz
Institute of Cell and Tumor Biology
German Cancer Research Center
Im Neuenheimer Feld 280
D-6900 Heidelberg, Federal Republic of Germany

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 puri-
fied 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 glu-
cocorticoid induction of gene expression. Coopera-
tivity 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 sys-
tem allows us to study the role of glucocorticoids and
cAMP in cell-specific and developmental regulation of
transcription (Granner and Beale, 1985). Tyrosine amino-
transferase is synthesized exclusively in parenchymal
cells of the liver (Hargrove and Granner, 1985). Whereas
expression of the gene is undetectable before birth, tran-
scription increases rapidly within the first hours after birth
(Greengard, 1970). Premature activation of the TAT gene
can be elicited by administration of glucocorticoid hor-
mones 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 muta-
tions and somatic cell genetic experiments have revealed
two genetic loci that influence the expression of the TAT
gene in trans. A control region required for the expression
and inducibility of TAT and several other liver-specific en-
zymes 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 addi-
tional locus that affects expression of TAT was identified
by somatic cell hybrids in which chromosome 11 of fibro-
blasts was introduced into hepatoma cells expressing TAT
(Killary and Fournier, 1984). Expression of TAT is selec-
tively extinguished suggesting that a repressor-like factor
acting in trans guarantees that the TAT gene remains tran-
scriptionally inactive in non-liver cells. The precise mech-
anism by which these two trans-acting loci modulate the
expression of the TAT gene is unclear. In particular it is un-
known whether glucocorticoids and cAMP act together
with these trans-acting factors in establishing cell-specific
expression of TAT.

To understand more completely the role of glucocorti-
coids 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. In-
creased expression of the gene following glucocorticoid
and cAMP administration is a consequence of direct tran-
scriptional activation (Hashimoto et al., 1984; E. Schmid
et al., submitted). To identify those sequences that medi-
ate 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 II_A gene (Karin 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; Scheidereit et al., 1983) and to be characterized by
a well-conserved consensus sequence (Karin et al.,
1984b). They are typically located within several hundred
bp upstream of the transcription initiation sites or, alterna-
tively, within the coding region of the genes, and they have
been found to possess enhancer-like properties (Chan-
dler et al., 1983; Karin et al., 1984b). These studies sug-
gest 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 re-
mains unclear precisely how this effect is exerted.

A novel property of the glucocorticoid response ele-
ments (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 ele-
ments representing glucocorticoid receptor binding sites.
One element, which in itself has no inducing capacity, will
enhance glucocorticoid induction in the presence of the
other.

* Present address: Department of Biochemistry, University of Califor-
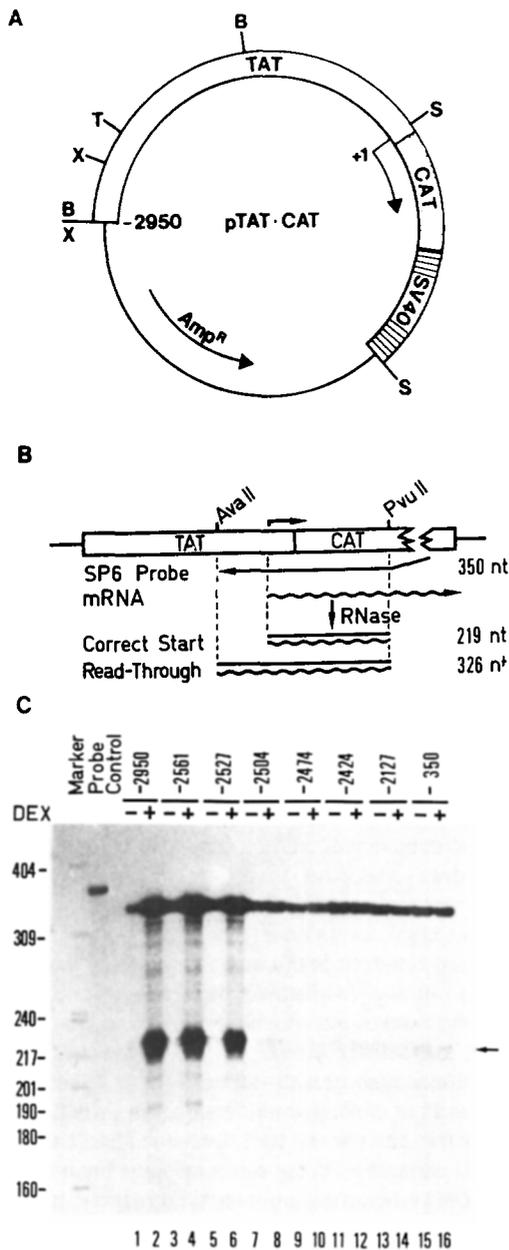nia, Berkeley, California 94720.

A

B

C

Figure 1. Structure of the Plasmid pTAT-CAT and Analysis of Expression of 5' Deletion Mutants

(A) In the plasmid pTAT-CAT expression of CAT is under the control of 5'-flanking DNA (−2950 to +62) of the TAT gene, with processing (splicing and polyadenylation) signals derived from SV40. The thin line represents prokaryotic vector sequences (pUC18). Restriction sites indicated are as follows: X, Xbal; B, BamHI; T, Tth111 I; S, SstI. The start site of transcription is indicated as +1.

(B) Transcripts derived from pTAT-CAT were mapped using a uniformly ^{32}P-labeled 350 nucleotide antisense RNA probe generated by in vitro transcription from an SP6 plasmid (see Experimental Procedures). Transcripts originating from the start site protect 219 nucleotides of the probe, whereas transcripts initiated upstream of the AvaII site protect 326 nucleotides.

(C) Expression of 5' deletion mutants of pTAT-CAT. Total RNA (20 µg) isolated from LtK⁻ cells transfected with the 5' deletion mutants of pTAT-CAT was analyzed as described in Experimental Procedures. Cells were cultured with (+) or without (−) 10⁻⁶ M dexamethasone (DEX) for 12 hr before harvesting. The arrow indicates transcripts originating from the start site at +1. ^{32}P-labeled HpaII fragments of pBR322 were used as marker. The control corresponds to hybridization of the probe with 20 µg of RNA from untransfected L cells.

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) upstream 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 TAT-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 TAT-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 TAT 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
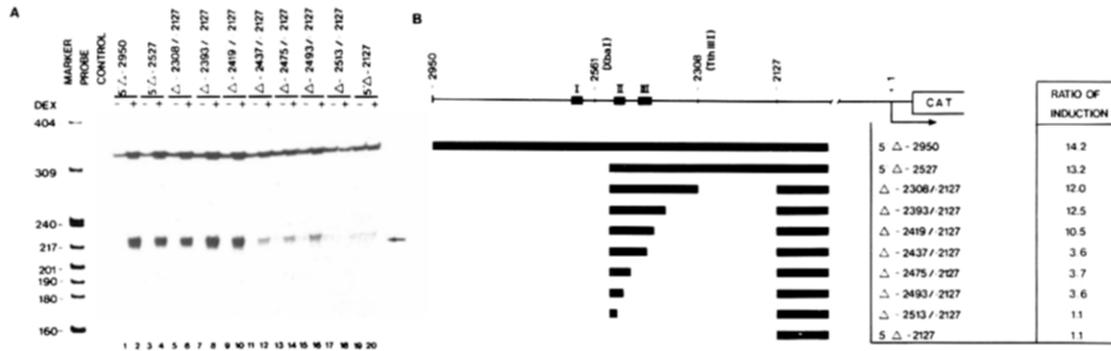
Figure 2. Expression of Internal Deletion Mutants of pTAT-CAT

(A) Ribonuclease protection assay. Analysis of total RNA isolated from Ltk⁻ cells transfected with 5' and internal deletion mutants was performed as described in Experimental Procedures and Figure 1. The numbers designate the position of TAT nucleotides flanking the deletions. Cells were cultured with (+) or without (−) 10^{-6} M dexamethasone (DEX) for 12 hr.

(B) CAT enzyme assay. Cells transfected with the indicated plasmids were induced with 10^{-6} M dexamethasone for 24 hr as described in Experimental Procedures. Values indicate the ratio of induction of CAT activity as determined in the extracts derived from duplicate transfections. Boxes I, II, and III indicate binding sites of the glucocorticoid receptor.

(−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, 1985). 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 up-

stream 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 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-
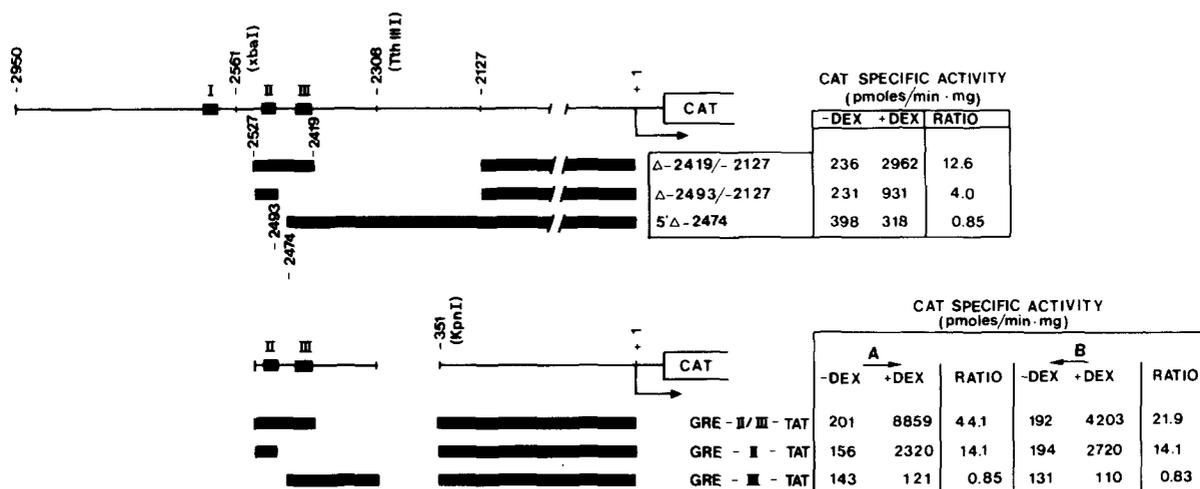
Figure 3. The GREs Elicit a Stronger Response When Positioned Closer to the TAT Promoter

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^{-6} 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.
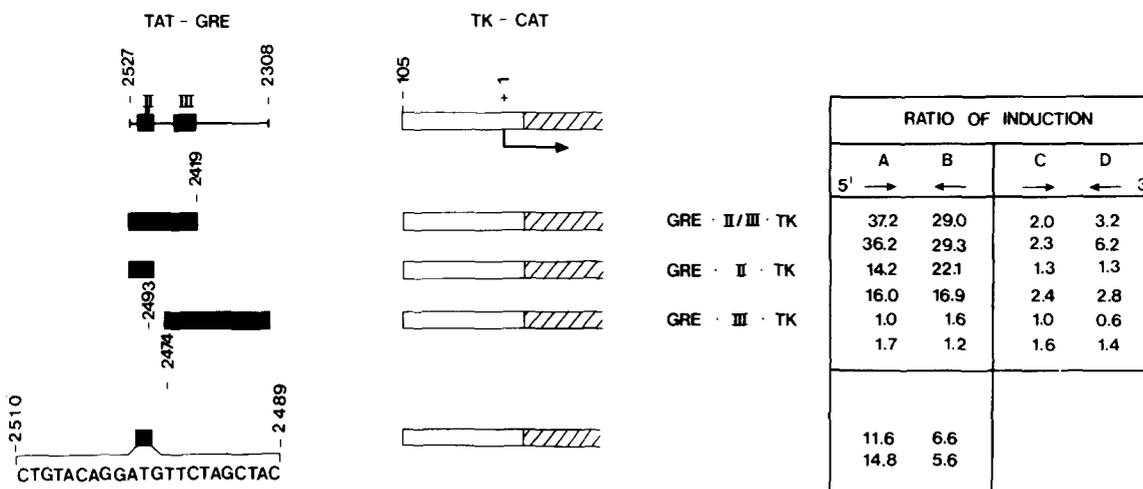
CAT SPECIFIC ACTIVITY (pmoles/min · mg)

	-DEX	+DEX	RATIO
Δ-2419/-2127	236	2962	12.6
Δ-2493/-2127	231	931	4.0
5'Δ-2474	398	318	0.85

CAT SPECIFIC ACTIVITY (pmoles/min·mg)

	A -DEX	A +DEX	A RATIO	B -DEX	B +DEX	B RATIO
GRE - II/III - TAT	201	8859	44.1	192	4203	21.9
GRE - II - TAT	156	2320	14.1	194	2720	14.1
GRE - III - TAT	143	121	0.85	131	110	0.83

CTGTACAGGATGTTCTAGCTAC

	RATIO OF INDUCTION			
	A 5' →	B ←	C →	D ← 3'
GRE · II/III · TK	37.2	29.0	2.0	3.2
	36.2	29.3	2.3	6.2
GRE · II · TK	14.2	22.1	1.3	1.3
	16.0	16.9	2.4	2.8
GRE · III · TK	1.0	1.6	1.0	0.6
	1.7	1.2	1.6	1.4
	11.6	6.6		
	14.8	5.6		

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 SmaI 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 2B. The results shown are from two independent transfection experiments. The ratios of induction were derived from duplicate transfections.

moter as upstream of the TAT promoter. The inducing effect of the active elements, however, is drastically diminished when they are inserted downstream of the TK-CAT gene. Of particular interest is the cooperativity of the elements. Element III alone is without effect, whereas GRE II has about one-third of the glucocorticoid regulatory activity of both elements together. The two GREs of the TAT gene therefore can confer their intrinsic characteristic of cooperativity upon a heterologous promoter.

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 repre-

senting 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.
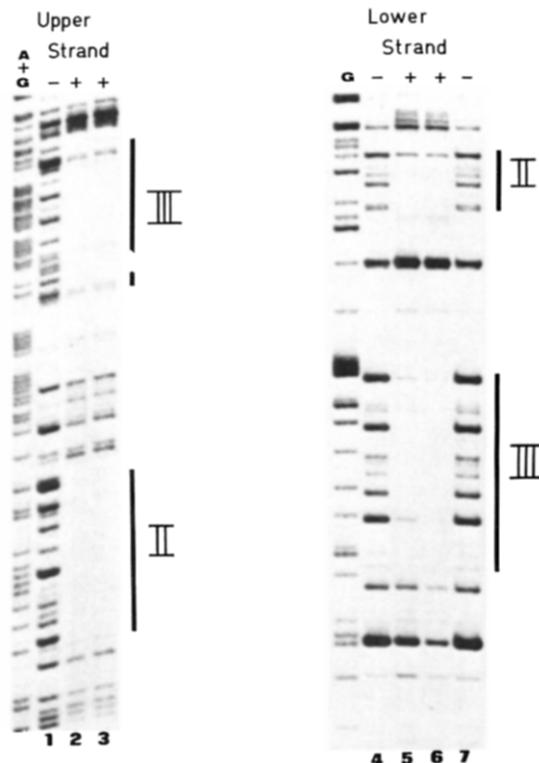
Figure 5. DNAase I Footprinting of Glucocorticoid Receptor Binding Sites in the GREs of the TAT Gene

The XbaI–Tth111 I fragment (−2561 to −2308) was asymmetrically labeled at the XbaI site for analysis of the upper strand and at the Tth111 I site for analysis of the lower strand. Labeled fragments were incubated with partially purified glucocorticoid receptor, subsequently digested with DNAase I, and analyzed on 6% polyacrylamide gels (Miksicek et al., 1986). The left panel shows an analysis of the upper strand in which the left-most lane represents an A+G sequencing reaction, followed by a control reaction containing BSA and lacking glucocorticoid receptor (lane 1). Lanes 2 and 3 represent reactions containing glucocorticoid receptor with 1 fmol and 2 fmol of asymmetrically labeled fragment, respectively. The right panel shows an analysis of the lower strand in which the left-most lane represents a G sequencing reaction, followed by a control reaction containing BSA and lacking glucocorticoid receptor (lanes 4 and 7). Lanes 5 and 6 represent reactions containing glucocorticoid receptor with 2 fmol and 1 fmol of asymmetrically labeled fragment, respectively.

The Glucocorticoid Receptor Binds to Sequences Conferring Steroid Responsiveness

To ascertain whether the sequences important for glucocorticoid induction are capable of binding to the glucocorticoid receptor, in vitro footprinting experiments using purified glucocorticoid receptor were performed. As can be seen in Figure 5 two regions upstream of the TAT gene are protected against DNAase I digestion. The protected regions contain the hexanucleotide TGTTCT, which is indicative of glucocorticoid receptor binding sites (Yamamoto, 1985). In addition to these binding sites, a third protected region containing homology I (see Figure 7A) was also identified (data not shown). As shown above, however, this region does not appear to contribute to the glucocorticoid regulatory potential of the TAT promoter in the constructions tested. Attempts to footprint homology region IV have been unsuccessful (data not shown).

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(II)) 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) footprinting 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 (Scheidereit 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
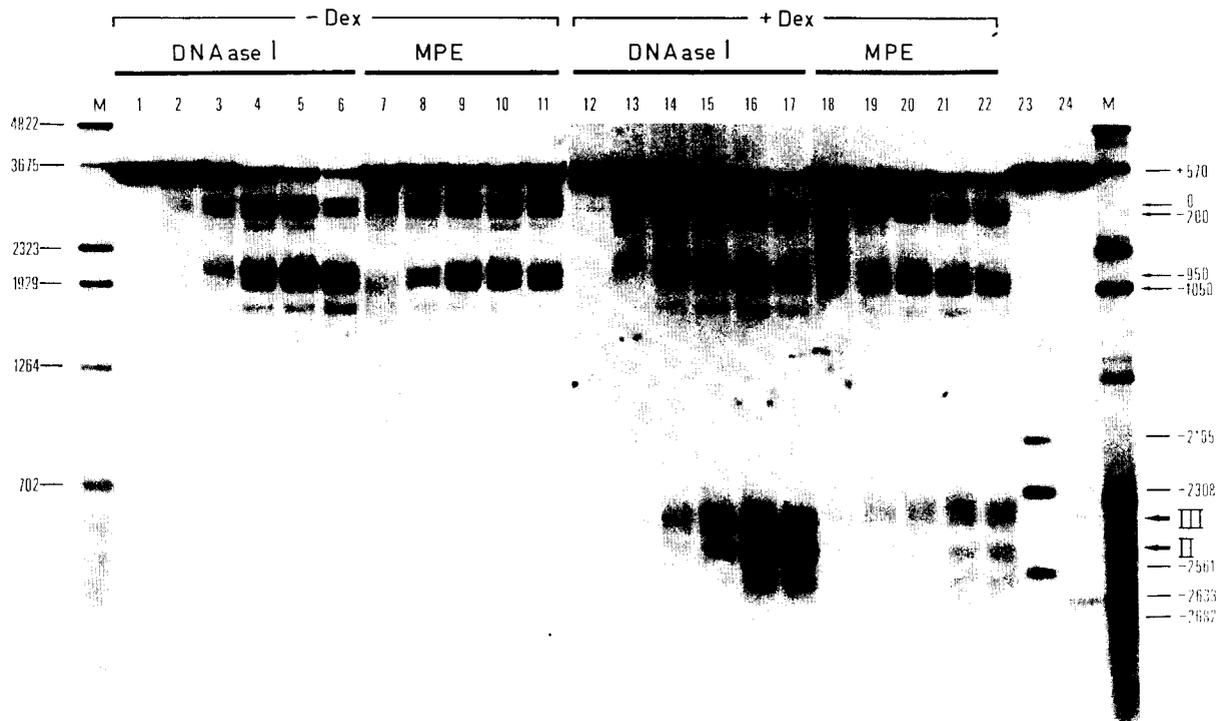
Figure 6. Glucocorticoid Induction of the TAT Gene Leads to Chromatin Changes at the GREs

Nuclei were isolated from FTO-2B 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 U/μg; lanes 2 and 13, 0.075 U/μg; lanes 3 and 14, 0.15 U/μg; lanes 4 and 15, 0.3 U/μg; lanes 5 and 16, 0.45 U/μg; lanes 6 and 17, 0.60 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 EcoRI digestion, DNA (20 μg per lane) was electrophoresed in a 1.7% agarose gel, blotted, and indirectly end-labeled to the EcoRI 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/Tth111 I (–2308), and 2 μg of EcoRI/MboII (–2105) double-digested genomic DNA. Lane 24 contains 14 μg of EcoRI-cut genomic DNA plus 3 μg of EcoRI/HinfI (–2682) and 3 μg of EcoRI/AvaII (–2633) double-digested genomic DNAs. The probe used was a T7 polymerase transcript from subclone pBX400 (–2950 to –2561 of the TAT gene). The positions of GRE II and GRE III and the position of the other hypersensitive sites are indicated with arrows.

transfer experiments. Analysis of protein–DNA interactions in vivo with the genomic sequencing technique (Becker et al., 1986) has shown that changes in dimethylsulfate reactivity, indicative of glucocorticoid receptor binding to the TAT GREs, occur upon dexamethasone administration to hepatoma cells. It remains unclear whether these structural alterations precede the receptor–GRE interaction or whether they represent a consequence of receptor binding. Similar changes in DNAase I hypersensitivity around the MMTV GREs have been shown to be concomitant with the stimulation of MMTV expression by glucocorticoids (Zaret and Yamamoto, 1984).

When the sequences of regions II and III are compared with those of other sequences mediating glucocorticoid responsiveness, it becomes evident that the consensus sequence derived by Karin et al. (1984b) by comparing two footprints from MMTV and one from the human metallothionein II_A gene is characteristic of all the functionally characterized elements listed (Figure 7B). This sequence comparison defines an imperfect palindrome of 15 nucleotides as the active GRE. Indeed, both a 22-mer (Figure 4) and a 15-mer sequence with degenerate symmetry patterned after the TAT GRE II, as well as a rotationally

symmetrical oligonucleotide in which the hexanucleotide TGTTCT is precisely repeated, confer glucocorticoid induction when positioned upstream of the TK promoter (U. Strähle, unpublished results). Changes in dimethyl reactivity in vivo following hormone administration, which are characteristic of receptor–DNA interaction, argue that the receptor interacts with GRE III (Becker et al., 1986). It cannot be excluded that a protein other than the receptor binds at the sequence of GRE III interacting with the receptor bound to GRE II. It is unclear why element III alone is not functional, but this may be related to the fact that the palindromic sequence is not well conserved. An obvious implication of the partial symmetry shown by functional GREs is that a productive interaction of the glucocorticoid receptor with its regulatory element may require the binding of a dimer of identical or nonidentical subunits; however, there is no direct experimental evidence to support this contention.

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.,

A

-2620 TCTTCT̲C̲A̲G̲T̲G̲T̲T̲C̲T̲C̲T̲A̲T̲C̲A̲C̲ASGGASAGCTGTCAGCCCCTGGAATGTG

AGAAGA̲G̲T̲C̲A̲C̲A̲A̲G̲A̲G̲A̲T̲A̲G̲T̲GTCCCTCTCGACAGTCGGSSACCTTACAC

I

'CC'

-2570 GTTCTATGTCTASAAAACTATCCCATAAATAACASSAAGCCCAASGTTTA

CAAGATACAGATCTTTTGATAGSGTATTTATTGTCCTTCGGGTTCCAAAT

0%   0%   25%
-2520 CCAATCTCTG̲C̲T̲G̲T̲A̲C̲A̲G̲G̲A̲T̲G̲T̲T̲C̲T̲A̲G̲CTACTTTATTTGCAATAGAAAA

GGTTAGAGAC̲G̲A̲C̲A̲T̲G̲T̲C̲C̲T̲A̲C̲A̲A̲G̲A̲T̲CGATGAAATAAACGTTATCTTTT

II   25%

-2470 TCTGAAAGTTTCCCCATGTCCAACAAGACTAGAACAAACAAGTCCTGCGT

AGACTTTCAAAGGGG̲T̲A̲C̲A̲G̲G̲T̲T̲G̲T̲T̲C̲T̲G̲A̲T̲C̲T̲T̲G̲T̲T̲T̲G̲T̲T̲C̲A̲G̲G̲A̲C̲GCA

III

100%
-2420 AG̲T̲CGCCTGTCGGTTTCTGGGTGTGGTGGTATAGCCCTGTAATCCCAGCA

TCAGCGGACAGCCAAAGACCCACACCACCATATCGGGACATTAGGGTCGT

B

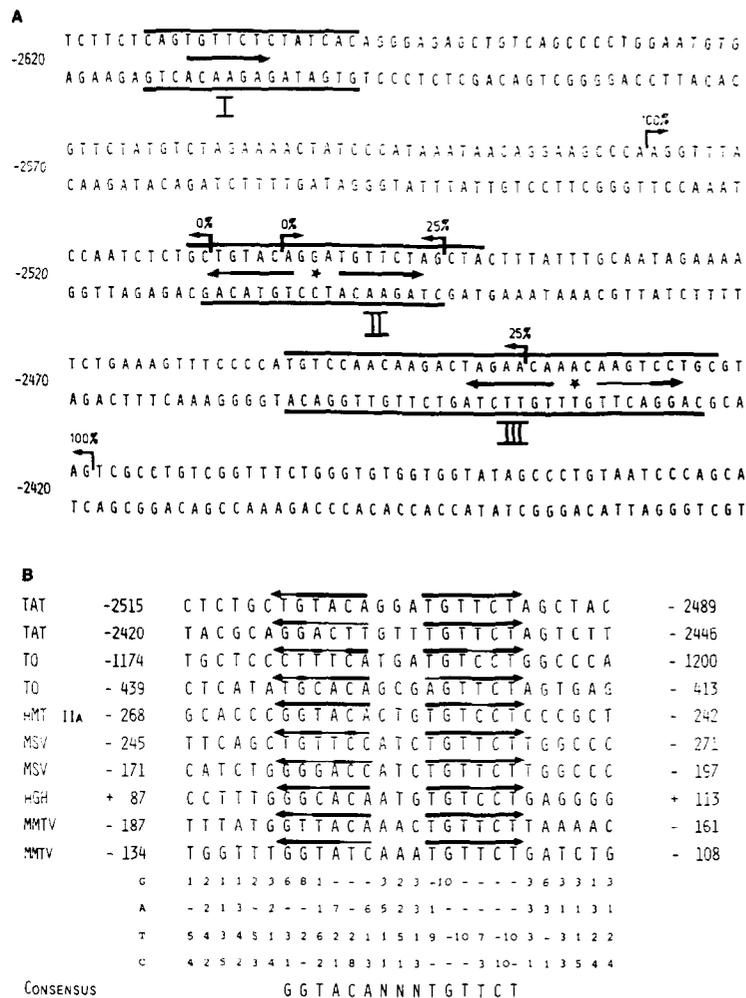|			
TAT	-2515	CTCTGC̲T̲G̲T̲A̲C̲A̲G̲G̲A̲T̲G̲T̲T̲C̲T̲AGCTAC	- 2489
TAT	-2420	TACGCA̲G̲G̲A̲C̲T̲T̲G̲T̲T̲T̲G̲T̲T̲C̲T̲AGTCTT	- 2446
TO	-1174	TGCTCC̲C̲T̲T̲T̲T̲C̲A̲T̲G̲A̲T̲G̲T̲C̲C̲T̲GGCCCA	- 1200
TO	- 439	CTCATA̲T̲G̲C̲A̲C̲A̲G̲C̲G̲A̲G̲T̲T̲C̲T̲AGTGAG	- 413
HMT IIA	- 268	GCACCC̲G̲G̲T̲A̲C̲A̲C̲T̲G̲T̲G̲T̲C̲C̲T̲CCCGCT	- 242
MSV	- 245	TTCAGC̲T̲G̲T̲T̲C̲C̲A̲T̲C̲T̲G̲T̲T̲C̲T̲TGGCCC	- 271
MSV	- 171	CATCTG̲T̲G̲G̲A̲C̲C̲A̲T̲C̲T̲G̲T̲T̲C̲T̲TGGCCC	- 197
HGH	+ 87	CCTTTG̲G̲G̲C̲A̲C̲A̲A̲T̲G̲T̲G̲T̲C̲C̲T̲GAGGGG	+ 113
MMTV	- 187	TTTATG̲G̲T̲T̲A̲C̲A̲A̲A̲C̲T̲G̲T̲T̲C̲T̲TAAAAC	- 161
MMTV	- 134	TGGTTT̲G̲G̲T̲A̲T̲C̲A̲A̲A̲T̲G̲T̲T̲C̲T̲GATCTG	- 108
G		1 2 1 1 2 3 6 8 1 - - - 3 2 3 -10 - - - - 3 6 3 3 1 3	
A		- 2 1 3 - 2 - - 1 7 - 6 5 2 3 1 - - - - - 3 3 1 1 3 1	
T		5 4 3 4 5 1 3 2 6 2 2 1 1 5 1 9 -10 7 -10 3 - 3 1 2 2	
C		4 2 5 2 3 4 1 - 2 1 8 3 1 1 3 - - - 3 10- 1 1 3 5 4 4	
CONSENSUS		G G T A C A N N N T G T T C T	

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 inducibility of 5′ deletions (arrows pointing to the right) and 3′ deletions (arrows pointing to the left).

(B) 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 IIA (Karin et al., 1984b), the murine sarcoma virus, MSV (Miksicek 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 Kühnel, 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 cognate promoter or to a heterologous promoter inducibility strongly increases. Preliminary evidence from the human TAT gene indicates that its GRE is also located far 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 promoter are functionally important.

In this context it is of great interest to us that the DNAase I hypersensitive site at −1000 (Figure 6), which is present both in the rat and the mouse genes, is characterized by sequence-specific in vivo cleavage sites for topoisomerases I and II and lies in the immediate vicinity of a 70 bp region found to be complexed with proteins in vivo (F. Stewart, P. Becker, and G. Schütz, unpublished results). The location of the GREs of the TAT gene 2.5 kb upstream of the initiation site and their ability to exert transcriptional control at a distance also raises important questions with regards to their evolution and their possible mechanism of action. Positional flexibility of widely dispersed tran-

scriptional 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 these 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 propogated 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 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 II 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 suggested that the GREs function independently of each other (Yamamoto, 1985; Buetti and Kühnel, 1986). It is not clear how this cooperative interaction between the two 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 phage 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 Sall–BstN1 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 pTATSB1.6 containing the TAT upstream region between −2950 and the BamHI site at −1300 in pUC19 was digested with SalI. The linearized pTATSB1.6 was treated with exonuclease Bal31 (0.15 U/μg plasmid DNA; BRL) in Bal31 digestion buffer (0.2 mM EDTA, 4 mM Tris–HCl [pH 8], 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 recombined 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 Tth111 I (−2308). After incubation with exonuclease III for different times, the DNA was treated with S1 nuclease and T4 polymerase to generate blunt ends and restricted with ApaI (−52). The resulting frag-

ments were separated on an agarose gel, and appropriately sized fragments were isolated. The plasmid pTAT-CAT 5'Δ −2127 was cleaved in the polylinker region with AccI, the ends were filled in with T4 polymerase. The plasmid was subsequently cleaved with ApaI. The AccI–ApaI fragment containing 5'-flanking sequences from −2127 to −52 was isolated and ligated with the fragments carrying deletion upstream of the Tth111 I site.

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 5' 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), a vector harboring the HSV-TK promoter up to −105 fused to the CAT coding region at +57. Insertion sites were upstream and downstream of the TK-CAT cassette. The oligonucleotide CTGTACAGGATGTTCTAGCTAC 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).

Cell Culture and DNA Transfections
LtK⁻ cells were plated at a density of 2 × 10⁶ cells per 10-cm dish in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and supplemented with 2 mM glutamine and 0.1 mg/ml penicillin and streptomycin. DEAE dextran–mediated DNA transfections were performed the next day as described (Banerji et al., 1983) with 2 pmol plasmid DNA per plate in 1 ml of 0.5 mg/ml DEAE dextran (MW 0.5 or 2 × 10⁶, Sigma). The DNA–DEAE dextran mixture was removed after 30 min, and the cells were treated for 5 hr with medium containing 0.1 mM chloroquine diphosphate. Cells were allowed a 12 hr recovery period in normal medium followed by an incubation of 16–24 hr in serum-free DMEM. When indicated the cells were treated with 1 μM dexamethasone in serum-free DMEM for 12 hr (RNA analysis) or 24 hr (CAT enzyme analysis) prior to harvesting.

Enzyme Assays
CAT assays were performed as described (Gorman et al., 1982), with the following modifications. Protein (10–100 μg) from 10,000 × 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 [¹⁴C]chloramphenicol (57.6 mCi/mmol). The linearity of the reaction under these conditions was verified. The acetylated and nonacetylated forms of [¹⁴C]chloramphenicol were separated by thin layer chromatography and excised 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 pSP64 (Melton et al., 1984); this plasmid was linearized with AvaII at −108. For the ribonuclease protection assay, 10 fmol (∼3.5 × 10⁵ 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.

DNAase I Footprinting
The 253 bp XbaI–Tth111 I fragment (positions −2561 to −2308) was asymmetrically ³²P-labeled at the XbaI site (upper strand) or at the Tth111 I site (lower strand). Asymmetrically labeled DNA fragment (1–2

fmol) was incubated with 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% glycerol, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mg/ml BSA, 5 mM DTT, and 1 μM triamcinolone for 45 min at 25°C. After incubation, a partial DNAase I digest was done by adding 3 μl of a DNAase I mix containing 45 mM $MgCl_2$, 300 ng/μl calf thymus DNA, and 5 μg/μl 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(II)
Hypersensitive Sites in Nuclei

FTO-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 $MgCl_2$. MPE · Fe(II) digestions were performed according to the method of Cartwright and Elgin (1984), except that the nuclei concentration was 10^8/ml (final) and the MPE · Fe(II) 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 0.25 M HCl, the gels were rinsed (3 times, 20 min) and transferred overnight 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, the filter was baked for 20 min at 80°C with vacuum and then UV crosslinked (25 sec, 20 cm from 6 × 15 W bactericidal lamps). Hybridizations were performed according to Church and Gilbert (1984), except that both hybridizations and washings were performed at 70°C.

References

Banerji, J., Olson, L., and Schaffner, W. (1983). A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. Cell 33, 729–740.

Becker, P. B., Renkawitz, R., and Schütz, G. (1984). Tissue specific DNaseI hypersensitive sites in the 5′-flanking sequences of the tryptophan oxygenase and the tyrosine aminotransferase genes. EMBO J. 3, 2015–2020.

Becker, P. B., Gloss, B., Schmid, W., Strähle, U., and Schütz, G. (1986). In vivo protein–DNA interactions within a glucocorticoid response element requires presence of the hormone. Nature 324, 686–688.

Buetti, E., and Kühnel, B. (1986). Distinct sequence elements involved in the glucocorticoid regulation of the mouse mammary tumor virus promoter identified by linker scanning mutagenesis. J. Mol. Biol. 190, 379–391.

Cartwright, I. L., and Elgin, S. C. R. (1984). Chemical footprinting of 5S RNA chromatin in embryos of Drosophila melanogaster. EMBO J. 3, 3101–3108.

Chandler, V. L., Maier, B. A., and Yamamoto, K. R. (1983). DNA sequences bound specifically by glucocorticoid receptor in vitro render a heterologous promoter hormone responsive in vivo. Cell 33, 489–499.

Chen, E. Y., and Seeburg, P. H. (1985). Laboratory methods: supercoil sequencing: a fast and simple method for sequencing plasmid DNA. DNA 4, 165–170.

Church, G. M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA 81, 1991–1995.

DeFranco, D., and Yamamoto, K. R. (1986). Two different factors act separately or together to specify functionally distinct activities at a single transcriptional enhancer. Mol. Cell. Biol. 6, 993–1001.

Gilbert, W. (1978) Why genes in pieces. Nature 271, 501.

Glucksohn-Waelsch, S. (1979). Genetic control of morphogenetic and biochemical differentiation: lethal albino deletions in the mouse. Cell 16, 225–237.

Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 2, 1044–1051.

Granner, D. K., and Beale, E. G. (1985). Regulation of the synthesis of tyrosine aminotransferase and phosphoenolpyruvate carboxykinase by glucocorticoid hormones. In Biochemical Actions of Hormones, Vol. XII, G. Litwack, ed. (New York: Academic Press), pp. 89–138.

Greengard, O. (1970). The developmental formation of enzymes in rat liver. In Mechanisms of Hormone Action, Vol. I, G. Litwack, ed. (New York: Academic Press), pp. 53–85.

Hargrove, J. L., and Granner, D. K. (1985). Biosynthesis and intracellular processing of tyrosine aminotransferase. In Transaminases, P. Christen and D. E. Metzler, eds. (New York: John Wiley & Sons, Inc.), pp. 511–532.

Hashimoto, S., Schmid, W., and Schültz, G. (1984). Transcriptional activation of the rat liver tyrosine aminotransferase gene by cAMP. Proc. Natl. Acad. Sci. USA 81, 6637–6641.

Hochschild, A., and Ptashne, M. (1986). Cooperative binding of λ repressors to sites separated by integral turns of the DNA helix. Cell 44, 681–687.

Hynes, N., van Ooyen, A. J. J., Kennedy, N., Herrlich, P., Ponta, H., and Groner, B. (1983). Subfragments of the large terminal repeat cause glucocorticoid responsive expression of mouse mammary tumor virus and of an adjacent gene. Proc. Natl. Acad. Sci. USA 80, 3637–3641.

Karin, M., Haslinger, A., Holtgreve, H., Cathala, G., Slater, E., and Baxter, J. D. (1984a). Activation of a heterologous promoter in response to dexamethasone and cadmium by metallothionein gene 5′-flanking DNA. Cell 36, 371–379.

Karin, M., Haslinger, A., Holtgreve, A., Richards, R. I., Krauter, P., Westphal, H. M., and Beato, M. (1984b). Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-IIA gene. Nature 308, 513–519.

Killary, A. M., and Fournier, R. E. K. (1984). A genetic analysis of extinction: trans-dominant loci regulate expression of liver-specific traits in hepatoma hybrid cells. Cell 38, 523–534.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York, Cold Spring Harbor Laboratory).

Maxam, A. M., and Gilbert, W. (1980). Sequencing end-labelled DNA with base-specific chemical cleavages. Meth. Enzymol. 65, 499–560.

Melton, D. A., Krieg, P. A., Rebagliatti, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucl. Acids Res. 12, 7035–7056.

Miksicek, R., Heber, A., Schmid, W., Danesch, U., Posseckert, G., Beato, M., and Schütz, G. (1986). Glucocorticoid responsiveness of the transcriptional enhancer of Moloney murine sarcoma virus. Cell 46, 283–290.

Payvar, F., DeFranco, D., Firestone, G. L., Edgar, B., Wrange, Ö., Okret, S., Gustafsson, J.-Å., and Yamamoto, K. R. (1983). Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. Cell 356, 381–392.

Pelham, H. R. B., and Bienz, M. (1982). A synthetic heat-shock pro-

moter element confers heat-inducibility on the Herpes simplex virus thymidine kinase gene. EMBO J. *1*, 1473–1477.

Ptashne, M. (1986). Gene regulation by proteins acting nearby and at a distance. Nature *322*, 697–701.

Renkawitz, R., Schütz, G., von der Ahe, D., and Beato, M. (1984). Sequences in the promoter region of the chicken lysozyme gene required for steroid regulation and receptor binding. Cell *37*, 503–510.

Ringold, G. (1985). Steroid hormone regulation of gene expression. Ann. Rev. Pharmacol. Toxicol. *25*, 529–566.

Ruiz-Bravo, N., and Ernest, M. J. (1982). Induction of tyrosineaminotransferase mRNA by glucocorticoids and cAMP in fetal rat liver. Proc. Natl. Acad. Sci. USA *79*, 365–368.

Scheidereit, C., Geisse, S., Westphal, H. M., and Beato, M. (1983). The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of mouse mammary tumor virus. Nature *304*, 749–752.

Scherer, G., Schmid, W., Strange, C. M., Röwekamp, W., and Schütz, G. (1982). Isolation of cDNA clones coding for rat tyrosine aminotransferase. Proc. Natl. Acad. Sci. USA *79*, 7205–7208.

Schmid, W., Müller, G., Schütz, G., and Glücksohn-Waelsch, S. (1985). Deletions near the albino locus on chromosome 7 of the mouse affect the level of tyrosine aminotransferase mRNA. Proc. Natl. Acad. Sci. USA *82*, 2866–2869.

Searle, P. F., Stuart, G. W., and Palmiter, R. D. (1985). Building a metal responsive promoter with synthetic regulatory elements. Mol. Cell. Biol. *5*, 1480–1489.

Serfling, E., Jasin, M., and Schaffner, W. (1985) Enhancers and eukaryotic gene transcription. Trends Genet. *1*, 224–230.

Sergeant, A., Bohmann, D., Zentgraf, H., Weiher, H., and Keller, W. (1984). A transcription enhancer acts in vitro over distances of hundreds of base pairs on both circular and linear templates but not on chromatin-reconstituted DNA. J. Mol. Biol. *180*, 577–600.

Singh, V. B., and Moudgil, V. K. (1985). Phosphorylation of rat liver glucocorticoid receptor. J. Biol. Chem. *260*, 3684–3690.

Slater, E. P., Rabenau, O., Karin, M., Baxter, J. D., and Beato, M. (1985). Glucocorticoid receptor binding and activation of a heterologous promoter by dexamethasone by the first intron of the human growth hormone gene. Mol. Cell. Biol. *5*, 2984–2992.

Yamamoto, K. R. (1985). Steroid receptor regulated transcription of specific genes and gene networks. Ann. Rev. Genet. *19*, 209–252.

Zaret, K. S., and Yamamoto, K. R. (1984). Reversible and persistent changes in chromatin structure accompany activation of a glucocorticoid-dependent enhancer element. Cell *38*, 29–38.

Zinn, K., D. DiMaio, D., and Maniatis, T. (1983). Identification of two distinct regulatory regions adjacent to the human β-interferon gene. Cell *34*, 865–879.