
Two remote glucocorticoid responsive units interact cooperatively to promote glucocorticoid induction of rat tyrosine aminotransferase gene expression

Thierry Grange, Jeanne Roux, Gildas Rigaud and Raymond Pictet

Institut Jacques Monod du CNRS, Unité INSERM 257, Université Paris 7, Tour 43, 2 place Jussieu, 75251 Paris CEDEX 05, France

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ABSTRACT

Tyrosine aminotransferase (TAT) gene transcription is specifically activated by glucocorticoid hormones in liver cells. This regulation involves a glucocorticoid responsive region located 2,500 bases upstream from the transcription start site of the rat gene. By transient transfection of TAT-CAT fusion genes into a rat hepatoma cell line expressing the TAT gene we found that this region promotes only 30% of the glucocorticoid stimulation. We have identified a new cis-acting region far upstream (-5,400) from the transcription start site that is essential to achieve the physiological level of glucocorticoid stimulation of endogenous TAT gene expression. This region corresponds to a tissue-specific DNase I hypersensitive site which is constitutive despite the fact it possesses a glucocorticoid receptor binding site. It is by itself almost inactive on a promoter but it cooperatively enhances the action of the proximal glucocorticoid responsive region. Its activity requires both the glucocorticoid receptor binding site and its flanking sequences.

INTRODUCTION

The study of steroid hormone effects has contributed to a large extent to our understanding of eucaryotic transcription regulation (for reviews see 1,2). Upon binding of the hormone, the receptor is able to activate transcription of its target genes through interaction with specific DNA sequences (1,2). A consensus binding site for the glucocorticoid receptor has emerged from the analysis of many genes positively regulated by glucocorticoid hormones. It is a 15 nucleotide-long imperfect palindrome comprising two 6 base-long arms, which canonical sequence is TGGTCT, separated by a 3 base-long spacer (1,2). This palindrome allows the binding of a receptor dimer (1,2). When placed in close proximity to a promoter, these binding sites are usually able to confer glucocorticoid inducibility (1,2). In other cases the level of induction conferred by these binding sites is low and is increased when such sites are associated with other trans-acting factor binding sites (3,4). This association defines a glucocorticoid responsive unit (GRU; ref 4) which is the functional entity when placed at a promoter distant location (5). *In vivo*, chromatin structural features may participate in the function of these GRUs. Indeed the interaction of the activated glucocorticoid receptor with DNA often leads to the appearance of a DNase I hypersensitive site (6,7) that has been shown in the case of mouse mammary tumor virus enhancer to correspond to a nucleosome displacement or alteration (8).

Subsequently to this displacement other trans-acting factors bind DNA sequences located in the vicinity of the glucocorticoid receptor binding site (9).

Tyrosine aminotransferase (TAT, E.C.2.6.1.5) gene is expressed specifically in liver where its transcription is increased by glucocorticoids and it has long been a model system to study glucocorticoid action (for a review see 10). A complex region located 2,500 bases upstream of the transcription start site is involved in glucocorticoid regulation of rat TAT gene expression (11). Using transient expression assays we have studied glucocorticoid regulation of TAT gene expression in a differentiated rat hepatoma cell line in which the endogenous TAT gene is faithfully regulated by this hormone (10,12). The present study reveals another level of complexity in the glucocorticoid response of the rat TAT gene: two (rather than one) remote units cooperatively interact to promote full corticoid induction.

MATERIAL AND METHODS

Plasmid constructions and DNA preparation

Plasmid pTC10 was derived from the promoterless CAT plasmid pSB1 (13) as follows: the Hind III site upstream from the CAT gene was converted to an Sst I site using a synthetic linker previously described (14), the TAT 5' flanking region was inserted in this new site as a 10 kb-long Sst I fragment originating from lambda TAT3 (14). This allowed reconstitution of the TAT promoter up to position +3. pTC3 originated from pTC10 by Hind III digestion followed by recircularization of the plasmid which led to deletion of the -10,095 to -3,337 region of the TAT gene 5' flanking sequence. The exact location of the deletion end points was deduced from the nucleotide sequence of the entire 10 kb fragment (15).

Plasmid pKT10 was constructed by insertion of a Sst I-BamH I fragment of pTC10 containing TAT gene 5' flanking region upstream from the thymidine kinase (tk) promoter of herpes simplex virus in the pUTKAT4 plasmid (16) digested by Sst I and BamH I. pKT3 originated from pKT10 by Hind III digestion followed by recircularization of the plasmid. pKT513 and pKT523 were obtained by insertion of the -6,100 to -5,180 region into the Hind III site of pKT3. For this insertion Hind III linkers were added on the Nco I and Bgl II sites surrounding the hypersensitive site n°5 (HS V, see below). pKT51 and pKT52 were obtained by addition of a BamH I linker on the aforementioned Nco I site and insertion of the resulting BamH I - Bgl II fragment into the BamH I site of pUTKAT4.

The Exonuclease III method was used to generate the 5' and 3' deletion mutants of the -6,100 to -5,180 fragment (17). This fragment was subcloned as a Hind III fragment into the Hind III site of pTZ18 (Pharmacia). After digestion with BamH I and Kpn I, the DNA was successively treated with Exonuclease III, S1 nuclease, Klenow fragment and T4 DNA ligase. The extent of the deletion was determined by DNA sequencing (18). The deleted fragments were excised and inserted into pKT3 as Sst I - Hind III fragments. The plasmids obtained are referred as pKT123, pKT153 and pKT146 for the 5' deletions and pKT233 and pKT253 for the 3' deletions.

A 290 bp region (-2,630 to -2,341) that covers the whole glucocorticoid responsive region of hypersensitive site n°3 (HS III, see below and Grange *et al*, in preparation) was subcloned into the BamH I site of pTZ18 as a Bgl II - Sau3A I fragment obtained after addition of a Bgl II linker on the Ava II site located at -2,630. In pKT123 this 290 bp fragment was substituted for the 2 kb fragment containing HS III giving rise to pKT531. This substitution was achieved by replacing the Hind III - Sal I fragment by a Hind III - Sst I fragment, after filling-in of the Sal I and Sst I sites. pKT31 was obtained by deletion of the -5,857 to -5,180 region (a Hind III to Sst I fragment) in pKT531.

Site-directed mutagenesis was performed as described (19) with the following modifications. The single stranded form of a plasmid was used that corresponds to the fragment extending from nucleotides -5,857 to -5,180 subcloned into pTZ18 (Pharmacia). The nucleotide sequences of the mutagenic oligonucleotides used were AATAAAAGATCACGAAGATCACCC for the generation of pMD5*3 and AAGAACACGATGTTCCCTGGAG for the generation of pMD5* (the mutated bases are underlined). After *in vitro* elongation, the reaction mixture was used to transform *E. coli* C600 mut L, the plasmids were allowed to replicate for 5 hours, reextracted by the alkaline lysis procedure (20) and then transferred into *E. coli* JM101. The entire nucleotide sequence of the TAT gene fragment of both mutants was verified. The fragments obtained were then transferred into their respective test plasmid.

For transfection experiments plasmids were prepared by the alkaline lysis procedure (20). The DNA was purified on two successive CsCl/ethidium bromide gradients. After extensive dialysis, phenol extractions and ethanol precipitation, the DNA was quantified by UV absorption and its purity verified by gel electrophoresis.

Transfection of cells.

Rat hepatoma cells (H4IIEC3; 12) were cultured in Coon's modified Ham's F12 medium supplemented with 5% fetal bovine serum, penicillin and streptomycin in a 10% CO₂, 36.5°C incubator (21). For transfection cells were seeded at 5.10⁶ cells per 10-cm plate and incubated overnight in 10 ml of growth medium. Three hours before addition of DNA the medium was renewed. The DNA was applied as a calcium phosphate precipitate (22,23) prepared as follows: 30 µg of test plasmid (various constructions upstream from the CAT gene) and 10 µg of control plasmid (pCH110 which allows expression of the β galactosidase gene from SV40 derived sequences; ref 24) were mixed in 1 ml of 250mM CaCl₂. This solution was added dropwise to 1 ml of 2XHBS (Hepes 50mM, NaCl 280mM, Na₂HPO₄ 1.5mM, pH 6.95 extemporaneously) under constant agitation. After incubation for 15 minutes at room temperature, 1 ml of suspension was applied dropwise to two 10-cm plates which were incubated for 24 hours in a 5% CO₂, 36.5°C incubator. The medium was replaced and incubation pursued for 18 hours in a 10% CO₂, 36.5°C incubator. Dexamethasone was then added in one of the two plates at a final concentration of 10⁻⁶M. The cells were collected 24 hours later by trypsinization for the analysis of the protein content.

For 7 of the tested constructions we have measured the ratio of induction by dexamethasone as described in the legend of figure 1 using more than one plasmid preparation. No significant differences were observed between different preparations of the same plasmid.

Cell-free extracts and enzymatic assays.

After trypsinization the cells originating from one plate were washed twice with PBS and resuspended in 100 μ l of 250mM Tris HCl pH 7.6, 5mM DTT. The cells were lysed by three cycles of freezing and thawing. The supernatant of a 15 minute-centrifugation in a microcentrifuge was directly assayed for enzymatic activity.

Protein concentration was determined using the Bio-Rad Protein Assay. CAT, TAT and β galactosidase activity were assayed as described (13, 25, 26).

Nuclei preparation and DNase I digestion.

H4IIEC3 cells were grown on plates as described above until they reach 80 % confluence. After 15 hours of serum depletion, the cells were treated or not with 10^{-6} M dexamethasone for 2 hours and then scraped in PBS (whithout $MgCl_2$ and $CaCl_2$) containing or not 10^{-6} M dexamethasone. Nuclei preparations were performed as described (27), except that 2 strokes were given with the dounce homogeneizer using pestle A. DNase I digestion was performed as described (27) with the following modifications: 1 ml of nuclei suspension (corresponding to 15 u OD_{260nm}/ml measured in 1% SDS) was incubated with various amounts of DNase I (Worthington-Cooper) for 10 minutes at $0^\circ C$ (7). The reaction was stopped by adding 1 ml of stop solution (1.2 mg/ml Proteinase K, 25mM EDTA, 2% SDS) preheated at $55^\circ C$. After 15 hours of incubation at $37^\circ C$, the DNA was purified by three phenol/chloroform (3/1) and one chloroform extraction, RNase A treated, reextracted with phenol/chloroform and twice with chloroform, and finally ethanol precipitated.

RESULTS

10 kb of TAT gene 5' flanking region confers to a reporter gene a glucocorticoid response identical to that of the endogenous TAT gene.

We have studied the regulatory regions involved in the glucocorticoid stimulation of tyrosine aminotransferase (TAT) gene transcription in a differentiated rat hepatoma cell line (H4IIEC3; ref 12) which expresses the TAT gene at a similar level as normal hepatocytes. The functional test was transient expression assays using, as reporter gene, the bacterial chloramphenicol acetyltransferase (CAT; ref 28) gene placed under the control of TAT gene flanking sequences. To correct for variations of transfection efficiency we cotransfected the bacterial β galactosidase gene under the control of viral (SV40) sequences (24) as an internal control.

We first tested if a 10 kb fragment of 5' flanking sequence (-10,095 to +3) is able to drive a glucocorticoid response identical to that of the endogenous TAT gene in our experimental conditions. CAT and TAT activities were measured at various dexamethasone (a glucocorticoid analog) concentrations. The superimposable dose response curves obtained (fig 1) indicate that

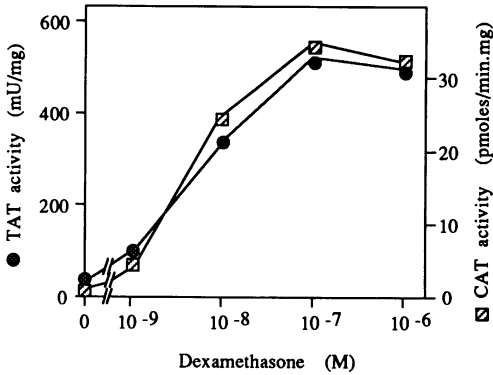


Figure 1- 10 kb of the TAT gene 5' flanking region confers to a reporter CAT gene a glucocorticoid inducibility similar to that of the endogenous TAT gene. Rat hepatoma cells (H4IIEC3) were transfected with plasmid pTC10 (see fig 2). TAT and CAT activities were measured in cell-free extracts 24 hours after dexamethasone addition at various concentrations. The curves presented here are issued from one representative experiment.

the magnitude of stimulation is identical for the endogenous and the transfected gene products at identical glucocorticoid receptor occupancy. This demonstrates that all regulatory sequences necessary for the glucocorticoid regulation of the TAT gene are present and functional in the 10 kb-long fragment tested.

Since a glucocorticoid responsive region has been described around position -2,500 (ref 11)

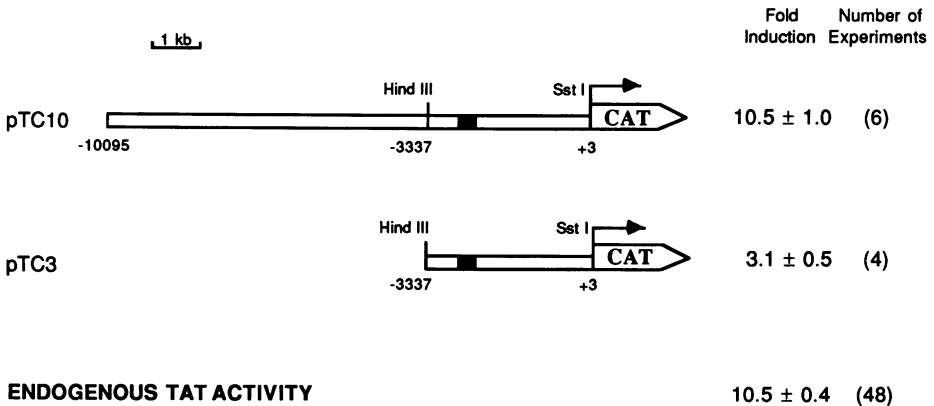


Figure 2- Two remote regions are involved in the glucocorticoid response.

The TAT gene 5' flanking regions placed upstream from the CAT gene are represented as empty boxes drawn to scale. The numbers under these boxes indicate the position of the boundaries of the TAT gene fragments relative to the TAT gene transcription initiation site referred as position +1. The black box indicates the location of the previously described glucocorticoid responsive region (11). The restriction sites used for plasmid constructions are indicated. Their precise location has been deduced from the complete nucleotide sequence of the 10 kb fragment (15). After normalization of the CAT activity with respect to the β galactosidase activity for each transfection plate, the ratio of induction of CAT activity by dexamethasone was determined for each transfected plasmid in each series of independent experiments. The mean value of these independent ratios of induction is indicated as fold induction ± the S.E.M. The number of independent experiments is indicated between brackets.

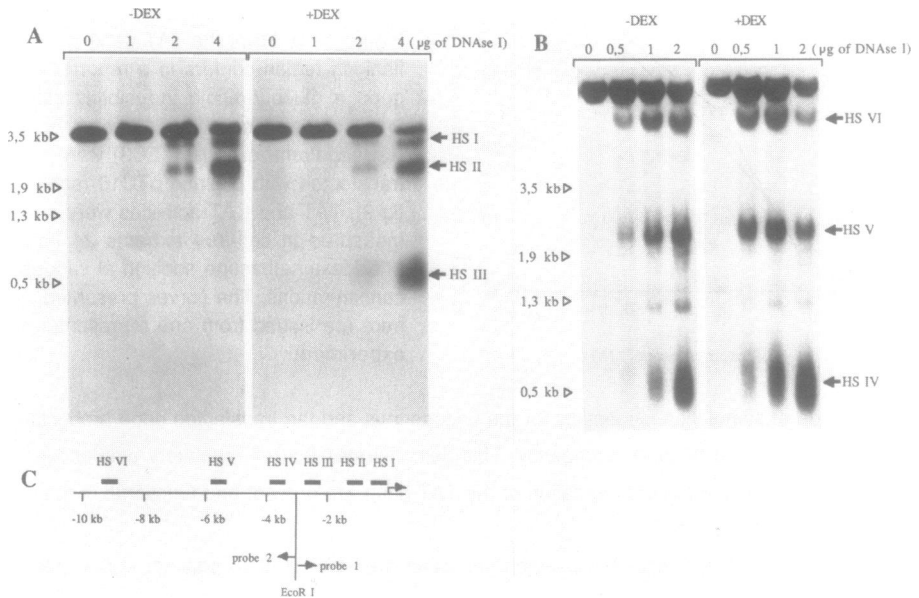


Figure 3- Multiple DNase I hypersensitive sites are distributed along 10 Kb of the TAT gene 5' flanking sequences

A and B: Genomic DNA (50 µg) prepared from nuclei of hepatoma cells (H4IIEC3) stimulated or not by dexamethasone and treated with DNase I were digested with EcoRI (position: -3062), separated on agarose gel (1.6 %: fig 3A; 1%: fig 3B) and transferred onto nylon membranes that were further processed as described (30). Hybridization was performed with "oligolabeled" (31) fragments ranging either from nucleotides -2,917 to -2,558 (probe 1, fig 3A) or from nucleotides -3,337 to -3,062 (probe 2, fig 3B). The amount of DNase I added to 1 ml of nuclei suspension is indicated. In A and B the nuclei preparation and the DNase I batch were different, thus the amounts of DNase I required for the appearance of the hypersensitive sites are not comparable. The arrows on the right show the hypersensitive sites numbered HS I to VI. The location of relevant bands of a size marker is indicated on the left.

C: Hypersensitive sites within the 5' flanking sequences of the rat TAT gene. Positions are determined relative to the start site of transcription. Hypersensitive sites are represented by bars over the sequence. The probes used for indirect end labelling are indicated by arrows. The location of the EcoRI site from which the hypersensitive sites have been mapped is indicated.

we also tested the activity of a 3 kb fragment of the 5' flanking sequence (-3,337 to +3) which includes this region. This fragment alone is not sufficient to allow full dexamethasone stimulation in a cell line expressing TAT (fig 2). Thus at least two separate regions are involved in the TAT gene glucocorticoid response. Progressive 5' end deletions were tested and this analysis showed that full glucocorticoid inducibility is also obtained with the -8,925 to +3 region while a low glucocorticoid response is obtained with the -3,918 to +3 region and no glucocorticoid response with the -1,300 to +3 region (data not shown). This indicates that a

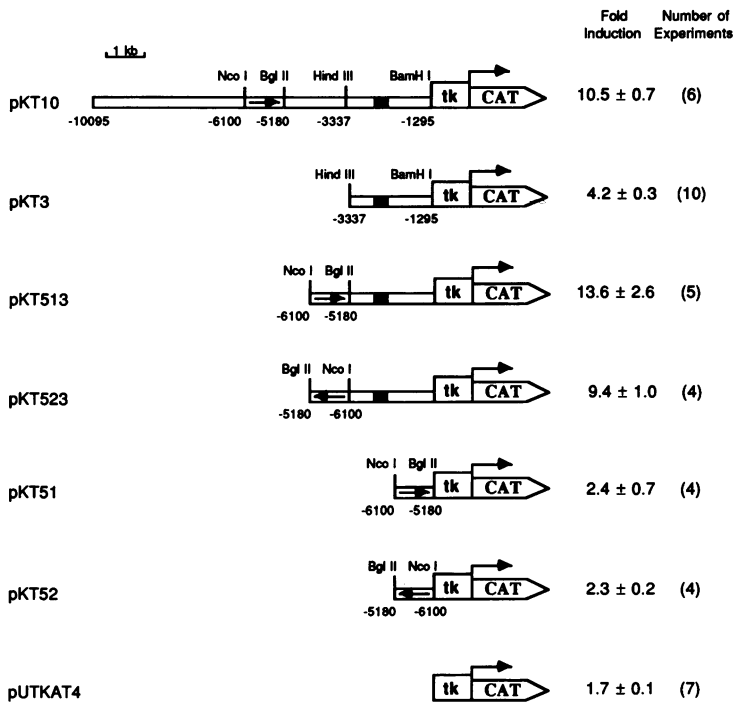


Figure 4- HS V cooperates with HS III to achieve full glucocorticoid inducibility. The data are presented as in fig 2. The tk box indicates the tk promoter of herpes simplex virus (from nucleotides -207 to +56; ref 16). The arrow inside the box indicates the orientation of the HS V containing fragment relatively to the direction of TAT gene transcription. The black box indicates the location of HS III.

region necessary for full glucocorticoid inducibility is located between positions -8,925 and -3,918.

Multiple DNase I hypersensitive sites are spread over the 10 kb of TAT gene 5' flanking sequences.

Since regulatory sequences have very often been mapped at or near DNase I hypersensitive sites (for a review see 29) we have localized these sites over the 10 kb of the 5' flanking sequence as a means to simplify the search for regulatory regions.

Our analysis reveals the existence in TAT expressing hepatoma cells of six major DNase I hypersensitive sites located around positions -100 (HS I), -1,000 (HS II), -2,500 (HS III), -3,600 (HS IV), -5,400 (HS V), -9,000 (HS VI) (fig 3). HS I, II and III have also been detected in rat liver (7) as well as HS IV, V and VI (G.R. unpublished results). None of these sites are detectable in fibroblasts (G.R. unpublished results). HS III which has been previously described (11) is the only site whose appearance is glucocorticoid dependent. Thus the

additional region involved in the glucocorticoid response does not correspond to a region where glucocorticoid hormones induce a change in chromatin structure detectable with DNase I.

A glucocorticoid independent DNase I hypersensitive site (HS V) is involved in the glucocorticoid response.

The preliminary deletion analysis reported above suggested that HS V which is located between -8,925 and -3,918 could be involved in the glucocorticoid response. This prompted us to test the activity of fragments containing HS III and HS V in the absence of any other DNase I hypersensitive sites. First we substituted the -1,294 to +3 region by the well-characterized promoter of the thymidine kinase (tk) gene of herpes simplex virus. The remaining 5' flanking region exerted on the tk promoter the same activity as on the TAT promoter: full glucocorticoid inducibility with sequences up to -10,095 and low glucocorticoid response with sequences up to -3,337 (pKT10 and pKT3 respectively, fig 4). Then we inserted into pKT3 upstream from the fragment containing HS III, a fragment containing HS V. This construct (pKT513) confers to the tk promoter a glucocorticoid response as strong as the longer fragment (pKT10, fig 4). If the orientation of HS V relative to HS III and the tk promoter is reversed (pKT523), HS V still possesses the ability to enhance the glucocorticoid response of HS III (fig 4).

HS III and HS V individually or in combination have no effect on the activity of the tk promoter in the absence of dexamethasone (not shown). However in contrast to HS III, HS V alone has little or no effect on the tk promoter in the presence of dexamethasone (pKT51 and pKT52, fig 4; see also pKT5, fig 7). Thus both HS III and HS V activities are glucocorticoid dependent but while HS III directly enhances the transcriptional activity of a promoter, HS V acts by enhancing the activity of HS III.

A glucocorticoid receptor binding site is involved in the activity of HS V.

The nucleotide sequence of the HS V region reveals the existence of a putative binding site for a glucocorticoid receptor dimer (1,2,32) located between nucleotides -5,450 and -5,436 (fig 5). It is a 15 nucleotide-long imperfect palindrome with two base-changes in one arm (AGTTCA instead of TGTTC). In order to narrow down the localization of the regulatory regions involved in the activity of HS V and to determine the importance of the putative glucocorticoid receptor binding site (GRBS), unidirectional deletions were performed from either one of the two ends on the 920 bp fragment containing HS V. Since HS V is not active by itself on the tk promoter, the deleted fragments were tested in the presence of HS III. In order to keep constant the distance separating HS V from HS III, the deletions performed from the downstream end were tested in the reverse orientation. Figure 6 shows the results of this analysis. The activity of the deleted fragments ranged from no activity, i.e. the level of induction by dexamethasone of HS III alone (pKT3), to full enhancing activity i.e. the level of induction by dexamethasone of HS V + HS III where HS V is in the same orientation as the tested deleted fragment (either pKT513 or pKT523). When the deletions were performed from the upstream end (relative to the natural orientation of HS V) the activity was not significantly affected by the deletion of the -6,100 to


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-6100 CATGGACCATCTCCTGCTGGGCATGGGGCCCGCCCCTAAGAGTAGTTTGTTCCTCCACGCGAGACTCCCTTGAGAAAAGT
      513 ←
-6020 AAAATTTTATTTGCCAGTGTTTTTAAGTGGTGATAACTTCTGGATTAGGGATGGGGTACGTGTCCAACCTCTCCITTTCAAC
-5940 TCTAGGACCCATCTGGTGCAGCCCTGTGACAGCCCTGCACGTGCTGCCACAGTCTCTGTGAGTTCCTGTGTGGATCAGTC
-5860 CTGTTGACCAGAGGGCTTGTTCCTCGGGCTCTTCCATCGCCTCTGGCTCTCTGCCTCCTCTTCTGCAGGGTTCACAGA
      123 ←
-5780 GACACCCATTTAAGCCGGGTATGCCAAGTTTCTCACGCTCTGCATGCTGTCTGGCTACGGCTTTCTATTTGTCCCATC
-5700 TGCCGAAGAAGGAAGCCTCTCTGATGATGACTGAGCAGGGCACTGATCTACTTCCCTGACTGAAGCACACACTGGATTTTC
                                     ← 253
-5620 TTTACGAAAGCTCAGGGTACTAAGCGAGATTTGGTAGACGAACCTGGCCTGTGTTCAGAACCGCTCAGAATGACTCCTCTG
                                     153 ←
-5540 GCTTTCATCGGTCAACCCAGTCATGGCAAACCTATTTCTGATAAAATGCTCAGGATGAGGACTGGATTGCAGATGCGGC
-5460 CACAAATAAAAGAACACGAAGTTCCACCTGGAGACAAACGGCTGAGGTAAGATCATGGTTGCCACAGTTATGCAAAAACA
      *****          *****
                                     ← 233
-5380 CAAAACAATAAGGTGGTCTATTTGCTAAACTCCGTGAGTGGGGCACCCAGAAGGCACCTCAGTCTGCAAAACAGATGAA
      146 ←
-5300 GATTTTATTTAGCTTTTGAAGAGCAGAGTGCTGAAGTAGAGAGAAAAGCCACAACCTTTAAAGGGTGAGCAGCCCTTTGA
-5220 AAACCCACTATCCCTTTATACCATGAACAAAACAGCAGATC
                                     ← 523

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Figure 5 - A putative GRBS is present in HS V.

The nucleotide numbering is relative to the TAT gene transcription start site (15,34). The stars indicate the location of the palindromic glucocorticoid receptor binding site. The arrows indicate the location of the deletion end point of each fragment whose activity has been measured and reported in fig 6, the associated numbers correspond to the various pKT... plasmids.

-5,857 region (pKT123) but was decreased by the deletion of the -5,857 to -5,554 region (pKT153) and was drastically affected by the deletion of the -5,554 to -5,362 region (pKT146). When the deletions were performed from the downstream end, deletion of the -5,180 to -5,411 region totally abolishes the activity of HS V (pKT233) and thus was not affected by further deletion (pKT253). These results show that sequences upstream from the putative GRBS participate slightly into the activity of HS V (pKT153), that this GRBS is not sufficient (pKT233), and that the region located downstream from it, between nucleotides -5,411 and -5,362, is absolutely necessary for the activity of HS V (pKT146 and pKT233).

In order to test the contribution of the putative GRBS to the activity of HS V, we introduced in each half of the palindrome a point mutation which totally inactivate a perfect palindrome (33). To measure the effect of this mutation, we used a plasmid containing shorter fragments of the TAT gene 5' flanking region. The 2 kb-long fragment containing HS III (-3,337 to -1,295) was reduced to a 300 base-long fragment (-2,630 to -2,341) without detectable change of activity either when tested alone (compare pKT3 fig 6 with pKT31 fig 7A) or in combination with HS V (compare pKT123 and pKT513 fig 6 with pKT531 fig 7A). Mutation of the GRBS drastically affected the ability of HS V to enhance the HS III-driven glucocorticoid response (compare pMD5³ to pKT531, fig 7A). Thus the GRBS is necessary to the activity of HS V.

The inability of HS V to activate the tk promoter in a glucocorticoid dependent way can be due

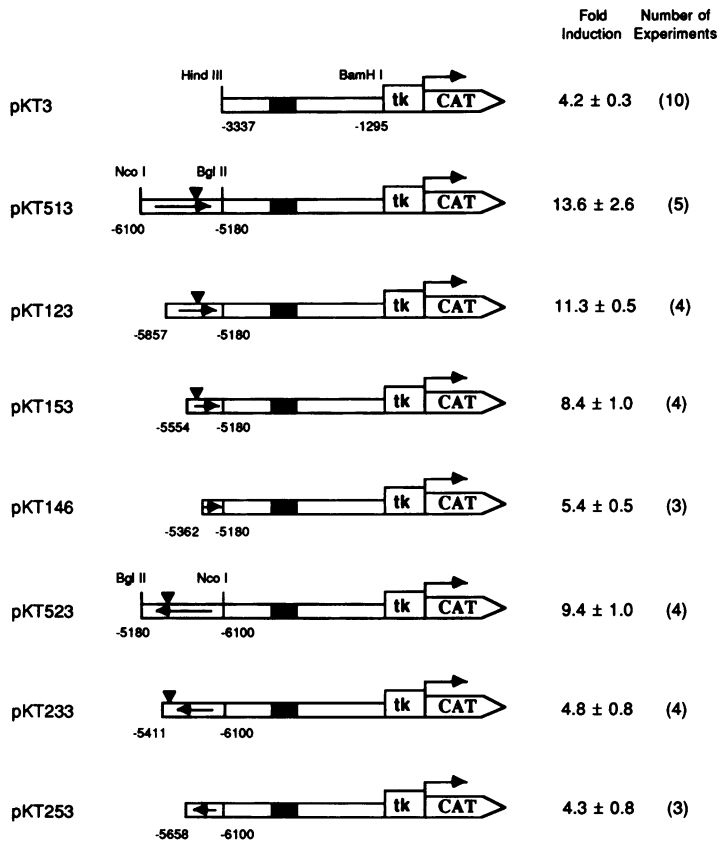


Figure 6- The putative GRBS is not sufficient to account for HS V activity. The data are presented as in fig 4. The location of the putative GRBS is indicated by a filled triangle over the HS V box. Details of plasmid constructions are presented in Material and Methods.

either to an inadequate structure of its GRBS or to the sequences flanking this GRBS. To distinguish between these two possibilities we modified in HS V the GRBS such as to transform it into a perfect palindrome of the motif TGTTCT. This modification of two bases transformed HS V into a glucocorticoid responsive region that is even more active than HS III (compare pMD5⁺ with pKT5, fig 7B and with pKT31, fig 7A).

DISCUSSION

The glucocorticoid receptor is present in most cell types; however in a given cell type glucocorticoid hormones regulate the transcription of a tissue-specific set of genes (for reviews see 35,36). The modality of this tissue-specificity remains to be explained. One necessary condition to study this specificity is to use cells in which the endogenous gene is

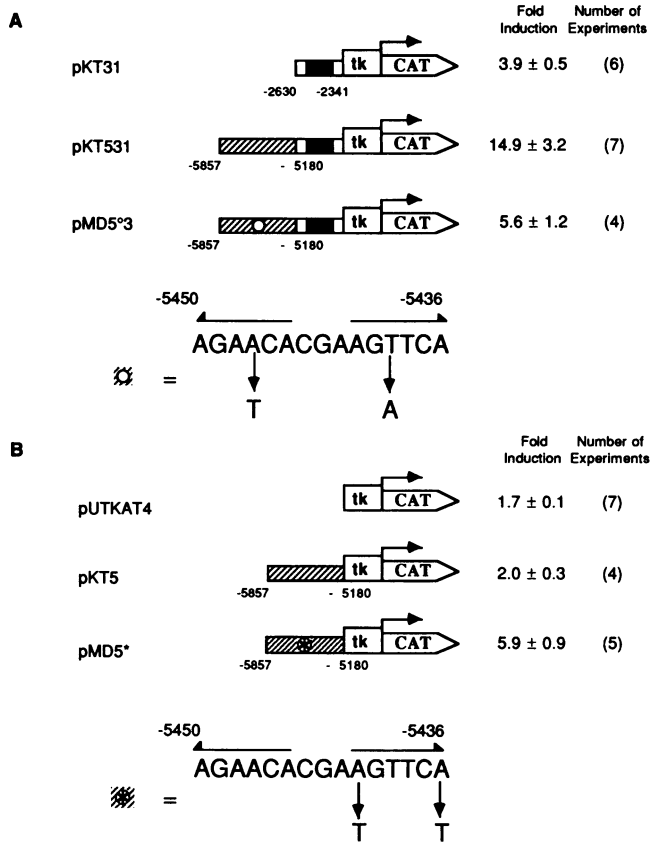


Figure 7- Mutation in the GRBS of HS V modify its activity.

A- Inactivation of HS V by mutation of its GRBS.

B- Conversion of HS V into a glucocorticoid responsive unit.

The data are presented as in fig 4 except that the fragment containing HS V is represented by a hatched box. The two bases modified in each mutant are represented in the lower part of the figure; the sequence above is the original sequence of the GRBS in HS V, the arrows under this sequence indicate the location and the nature of the changes introduced in HS V by site directed mutagenesis.

expressed and normally regulated. We have studied glucocorticoid regulation of the liver-specific tyrosine aminotransferase (TAT) gene in a differentiated rat hepatoma cell line in which the endogenous TAT gene is expressed and regulated by glucocorticoid hormones. Analysis by transient expression assays reveals that a hybrid gene with 10 kb of TAT gene 5' flanking sequences is regulated by glucocorticoids like the endogenous TAT gene: the same level of stimulation at identical hormone concentration. This physiological level of glucocorticoid induction is conferred to a promoter by two regulatory regions which interact cooperatively.

These regions are located respectively 2,500 and 5,400 bases upstream from the transcription start site. Both are able to act over long distances and in either orientations (for HS III, see also ref 11) and thus behave like glucocorticoid dependent enhancers. However they have distinctive properties.

The proximal region (HS III) corresponds to a tissue-specific glucocorticoid inducible DNase I hypersensitive site. Some of its properties have been previously analyzed in fibroblasts where the endogenous TAT gene is neither expressed nor induced in the presence of dexamethasone (11). This region is able to activate a promoter in a glucocorticoid dependent way. However we show here that in hepatoma cells this activation represents only one third of the stimulation level of the endogenous gene whether the promoter used is the natural TAT promoter or the heterologous tk promoter.

The distal region (HS V) corresponds to a tissue-specific glucocorticoid independent DNase I hypersensitive site. HS V is not active by itself on a promoter but it enhances the level of glucocorticoid stimulation exerted by the proximal region (HS III) to the level of the endogenous gene.

HS III is constituted of several trans-acting factor binding sites, some of which have been shown to interact (5, 11). Three glucocorticoid receptor binding sites (GRBS) have been characterized by *in vitro* footprinting performed with the purified glucocorticoid receptor (11). DNA regions encompassing two of these GRBS interact cooperatively (11). Full activity of the region requires sequences flanking the GRBS that are binding sites for other trans-acting factors (5, Grange *et al*, in preparation).

The distal region (HS V) contains an imperfect palindrome of the motif TGTCT, a sequence homologous to a GRBS (1,2). We introduced in this putative GRBS a mutation known to inactivate the function of a glucocorticoid responsive element presumably by decreasing the affinity of the interacting steroid receptor (32,33). Since the distal region is inactivated by this mutation we conclude that glucocorticoid receptor binding to that region is essential for the function of HS V. We believe that the mutation does not directly interfere with the binding of other trans-acting factors since there is no *in vitro* DNase I protection of the GRBS by hepatoma cells nuclear proteins prepared in the absence of glucocorticoid treatment (Grange *et al*, in preparation). The GRBS in HS V is however not sufficient to account for the activity of the distal region. Indeed deletion of the sequences located downstream from the GRBS inactivates HS V. These flanking sequences also bind multiple trans-acting factors (Grange *et al*, in preparation). The association of a GRBS with other trans-acting factors binding sites is designated glucocorticoid responsive unit (GRU; ref 4). HS III and HS V would thus correspond to GRUs. However each of these GRUs independently has little (HS III) or no (HS V) enhancer activity and it is their combination that generates the fully active enhancer. This organization is similar to the one of the SV40 enhancer which is constituted of two domains with little activity

on their own (37). Each domain is itself constituted of multiple trans-acting factor binding sites (38 and ref therein). In contrast to TAT gene GRUs, the two domains of the SV40 enhancer do not interact cooperatively when they are separated by a few hundred bases (37).

The interaction of several remote GRUs may be a general feature of glucocorticoid regulation. Indeed two remote glucocorticoid responsive regions have been described in several genes. They are located closer to the transcription start site than in the case of the rat TAT gene, for example -1,200 and -450 for the rat tryptophan oxygenase and phosphoenolpyruvate carboxykinase genes (39,40). Apart from their location the salient distinctive property of TAT gene GRUs is that they interact cooperatively, i.e. when associated their activity is higher than the sum of their individual activity. In contrast the effect of the two glucocorticoid responsive regions of the other genes is only additive (39,40).

The DNase I hypersensitive sites that characterize both GRUs are typical of alteration of chromatin structure (see 29). These hypersensitive sites are detectable in different conditions: their appearance does (HS III) or does not (HS V) depend on the presence of glucocorticoid hormones. Thus differences in nucleosome structure or position (relative to the GRBS) existing prior to the addition of hormone could be responsible for the different activities of the two GRUs. However this is unlikely since we could show here that the lack of activity of HS V towards the tk promoter is due to the GRBS structure rather than the overall sequence arrangement of HS V. Indeed the transformation of this GRBS into a perfect palindrome of the TGTTCT motif turns HS V into a GRU efficiently active on the tk promoter. Two possibilities could account for the effect of this mutation: 1) the changes introduced increase the affinity of the glucocorticoid receptor for its GRBS and thus increase its degree of occupancy *in vivo*, thereby accounting for the improved activity of the GRU; 2) conformational changes of the glucocorticoid receptor are induced by the target DNA and these changes are different depending on the precise sequence of the target. The different conformations would have different abilities to interact with other factors. These presently undemonstrated conformational changes have been hypothesized to explain the properties of a negative glucocorticoid responsive element from the bovine prolactin gene (41).

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