

## *In vivo* protein–DNA interactions in a glucocorticoid response element require the presence of the hormone

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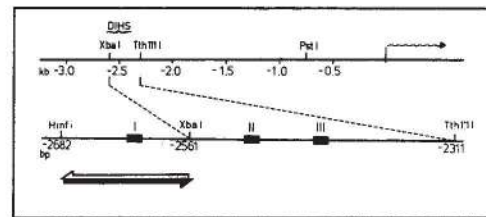
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Transcriptional activation of gene expression by glucocorticoid hormones is mediated by the interaction of hormone–receptor complexes with specific DNA sequences called glucocorticoid responsive elements (GREs) (refs 1–3, see ref. 4 for review). Deletion of this sequence abolishes glucocorticoid induction of transcription<sup>4–8</sup>. According to a current model, activation of the cytoplasmic receptor protein by hormone binding leads to its increased affinity for and translocation to the nucleus<sup>4</sup>. However, recent reports that the oestradiol and progesterone receptors are localized in the nucleus in the absence of steroid<sup>9–11</sup> led us to examine whether the free receptor interacts *in vivo* with its DNA binding site in the absence of hormone binding. We used the genomic footprinting technique<sup>12–15</sup> to show that changes in *in vivo* protein–DNA interactions within the GREs of the tyrosine aminotransferase gene (*TAT*) can be detected only after hormone treatment in hepatoma cells. Such changes are not detected in fibroblast cells, in which the *TAT* gene is not expressed. Many of the changes in dimethylsulphate reactivity observed in the living cell are also found *in vitro* using cloned DNA and a partially purified glucocorticoid receptor.

The GREs of the rat *TAT* gene are located unusually far upstream, at –2.5 kilobases (kb), from the transcriptional start site (Fig. 1). These elements were initially indicated by the appearance of a DNaseI-hypersensitive site following hormone treatment<sup>16</sup> and then identified by gene transfer experiments and DNaseI footprinting using partially purified glucocorticoid receptor complex *in vitro*<sup>17</sup>. Sequence analysis revealed several sequence motifs that fit the established GRE consensus sequence<sup>1,2,4</sup>. In this study we focus on those elements (II and III, Fig. 1) that have been shown to be essential for glucocorticoid induction by gene transfer experiments.

The instability during purification of the glucocorticoid receptor in the absence of ligand has hindered the analysis of its potential interaction with DNA sequences by *in vitro* methods, but the recent development of the genomic footprinting technique<sup>12,15</sup> has made possible the analysis of receptor–DNA interactions *in vivo* in the absence of steroid. Cells of the *TAT*-expressing hepatoma cell line FTO-2B were withdrawn from glucocorticoids by incubation for at least 16 hours in serum-free medium before induction with the steroid dexamethasone for various times. Trypsinized cells suspended in medium were reacted with dimethyl sulphate (DMS) to probe for the reactivity of the N7 position of guanines in the major groove of the DNA helix<sup>15,19,20</sup>. Patterns of guanine (G) residues were obtained using the probe fragment indicated in Fig. 1.

Figure 2a shows an example of such an analysis of uninduced and induced FTO cells. The band intensity reflects the reactivity of a given guanine or a group of unresolved guanines for the chemical modification. Protection from methylation at guanines is most readily explained by protein binding whereas enhanced modification of guanines has been interpreted as a local increase in reagent concentration in a hydrophobic pocket caused by close contacts of protein to DNA<sup>20</sup>. Methylation protection and enhancement after hormone treatment were analysed quantitatively for each guanine (Fig. 2c). The data were collected from four independent experiments, each DNA sample being analysed on at least two blots. A number of changes in band intensity



**Fig. 1** The 5'-flanking region of the rat *TAT* gene. Upper line, 3 kb of the 5'-flanking region of the rat *TAT* gene: Numbering, distance from the cap-site; wavy arrow, the start of transcription; DMS, region of dexamethasone-induced DNaseI hypersensitivity<sup>16</sup>. The lower line, enlargement of the region around the dexamethasone-inducible DNaseI-hypersensitive site: black boxes, sequence homologies to the GRE consensus sequence labelled with roman numerals; arrows, single-stranded DNA probes used to generate genomic sequence synthesized from an M13 vector containing the *Hinfl*–*XbaI* fragment.

appear to be correlated with hormone treatment. DMS protections are evident at the sites of glucocorticoid interaction as identified by DNaseI footprinting<sup>17</sup>. Additional changes in reactivity map to neighbouring sequences (see Fig. 4). In a non-expressing rat fibroblast cell-line (XC cells) no such changes could be discerned after hormone treatment (Fig. 2a), nor were they observed when FTO-2B cells were treated with the glucocorticoid antagonist RU486 (Fig. 2b).

To ascertain whether the changes in DMS reactivity observed *in vivo* at the *TAT* gene GREs are caused by the glucocorticoid receptor, we performed *in vitro* methylation experiments, using cloned *TAT* DNA fragments containing the GREs and a partially purified glucocorticoid receptor (Fig. 3). The most prominent G protections map to the conserved TGTTCT motif and are identical with those shown to be contacted by the receptor in the GRE of mouse mammary tumour virus (MMTV)<sup>21</sup>.

Figure 4 summarizes the data obtained *in vivo* and *in vitro*. Comparison of *in vivo* G reactivity patterns from uninduced and induced FTO-2B cells clearly shows that protection of guanines in the binding site is seen after dexamethasone treatment of cells (Fig. 2). They are accompanied by a number of hormone-induced changes in reactivity in neighbouring sequences. The most dramatically enhanced G reactivities are seen at four adjacent guanines (–2,459 to –2,456) between the two receptor binding sites. Most of the changes in DMS reactivity following hormone treatment are also found *in vitro* using a partially purified glucocorticoid receptor. The changes are not found in XC cells, where the *TAT* gene is not transcribed. These findings strongly indicate that changes in reactivity at guanines within the GRE are indeed caused by interaction of the glucocorticoid receptor with its binding site and that its affinity in the absence of hormone binding is too low to lead to a tight interaction with a GRE at receptor concentrations found in the living cell.

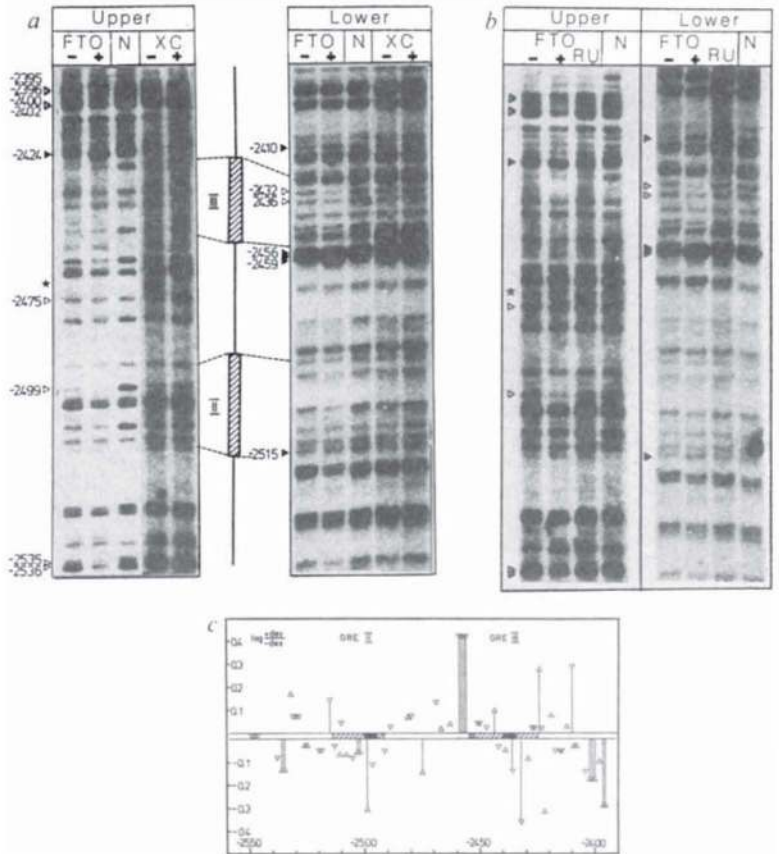
Receptor binding is accompanied by changes in chromatin structure that render a region of 200 base pairs (bp) surrounding the GREs hypersensitive to DNaseI digestion<sup>16</sup>. Whether this altered structure is a prerequisite for receptor binding or rather a consequence of binding is not known. For the MMTV GREs a similar DNaseI hypersensitivity has been shown to precede or parallel hormonal stimulation of MMTV promoter function<sup>18</sup>.

It should be emphasized that these effects on guanine reactivities have been observed within a region which is unusually remote (at –2.5 kb) from the cap-site that is selectively rendered hypersensitive to DNase I digestion after dexamethasone treatment. Using the same DNA samples no hormone-dependent DMS effects were seen within several hundred nucleotides surrounding the *TAT* gene cap-site or within a region of DNaseI hypersensitivity at –1,000 bp (data not shown). Within the first

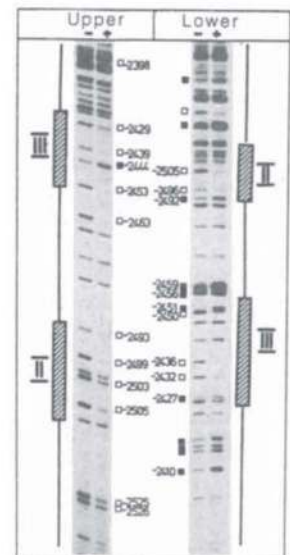


**Fig. 2** Glucocorticoid-induced changes in DMS reactivity *in vivo*. *a*, G ladders for both strands of a region of genomic DNA between -2,380 and -2,540 bp (upstream) of the transcriptional start site of the *TAT* gene from DMS-treated FTO-2B or XC cells (+, induced with dexamethasone; -, uninduced); N, *in vitro* methylated protein-free DNA. Protection ( $\Delta$ ) and enhancement ( $\blacktriangle$ ) seen when comparing dexamethasone-induced with uninduced FTO-2B cells; numbers, distance from the cap-site. Only those reactivity changes that appeared highly significant in the quantitative analysis (*c*) are indicated. \*, Band originating from hybridization to *lacZ* sequences on a contaminating fragment; hatched bars, positions of the two major DNaseI footprints obtained *in vitro* with partially purified glucocorticoid receptor<sup>17</sup>. *b*, As in *a*, but only FTO-2B cells were used and incubation with the glucocorticoid antagonist RU486 (RU) was carried out in parallel with dexamethasone inductions. *c*, Quantitative analysis of hormone-dependent changes in G methylation; summary of results. Nucleotide numbers (from -2,560 to -2,390) upstream of the *TAT* cap-site are given on the abscissa. Hatched bars, sites of receptor binding identified by DNaseI footprinting *in vitro*<sup>17</sup>; black bar, conserved hexanucleotide TGTCT. For each G residue the logarithm between the median of induced divided by the median of uninduced samples ( $\log +\text{dex}/-\text{dex}$ ) is plotted.  $\Delta$  and  $\nabla$  symbolize this value for each guanine on the upper and lower strand, respectively. Positive values indicate dexamethasone-dependent enhancements, negative values represent inducible protections. The significance of the deviations obtained was checked by comparing induced and uninduced values using a Kruskal-Wallis test<sup>24</sup>. Those values that fell below a threshold of 5% probability of error (1st order error) are indicated with vertical lines.

**Methods.** FTO-2B and XC cells were grown to about 80% confluency in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf-serum and 10 mM HEPES pH 7.4, withdrawn in serum-free DMEM for at least 16 h and incubated for 4 h with serum-free DMEM with or without  $10^{-6}$  M dexamethasone or  $10^{-6}$  M RU486. Induction times were 20 min-12 h, no significant time-dependent changes in the DMS reactivity pattern were observed (data not shown). DMS treatment of whole cells and *in vitro* methylation of protein-free DNA were as published<sup>15,19</sup>. After isolation of cellular DNA, 25  $\mu$ g of each sample was restricted with *HinfI* (Boehringer), reacted with piperidine and separated on a sequencing gel<sup>12</sup>. Details of DNA blotting onto Gene Screen membrane (NEN) UV crosslinking, hybridization and washing of filters were as in ref. 12. Probe synthesis from a single-stranded M13 template was performed as described<sup>12</sup> with modifications: 100 mM NaCl was included in primer annealing and elongation reactions, 250  $\mu$ Ci of [ $\alpha^{32}$ P]dATP (NEN), 5,000 Ci mmole<sup>-1</sup> was used per probe synthesis. The newly-synthesized probe DNA was separated from the template strand in a 6% denaturing gel and recovered from the polyacrylamide by isotachopheresis<sup>22</sup>. Exposure times were 7-14 days with 'lightning plus' intensifying screens (DuPont). Quantitative analysis was done as Giniger *et al.*<sup>23</sup>; four independent DMS treatments of FTO-2B cells were performed under either dexamethasone-induced or uninduced conditions. The resulting DNAs were analysed on at least two independent blots. Genomic sequencing ladders were scanned with an Elscript 400 densitometer (Fisher Scientific) at 16  $\mu$ m resolution. Peak integrals were calculated and median values determined for each guanine band.



**Fig. 3** Changes in DMS reactivity following glucocorticoid receptor binding *in vitro*. Left panel, results obtained for the upper strand and right panel, for the lower strand with (+) and without (-) triamcinolone-saturated glucocorticoid receptor. Protected guanines are indicated by open squares, enhanced methylation at a guanine as a filled square. Guanines that are influenced in their reactivity when comparing the '- and '+' lanes are labelled with their numbers upstream of the *TAT* cap-site. Hatched bars, regions protected against DNaseI-digestion by bound glucocorticoid receptor *in vitro*<sup>17</sup>. **Methods.** Purification of the glucocorticoid receptor<sup>25</sup> included as a final step DEAE-Sepharose 6 CL column chromatography in which the receptor eluted as a single peak at 150 mM NaCl. A *XbaI*-*Tth111I* fragment containing both functional GREs was isolated from a subclone and end-labelled using T4 kinase polynucleotide and [ $\gamma^{32}$ P]ATP either at the *XbaI* (upper strand) or at the *Tth111I* (lower strand) sites. Labelled fragment (0.5-1.0 fmol) was incubated with (+) or without (-) 90 fmoles of triamcinolone-saturated receptor in a volume of 25  $\mu$ l containing 100 mM NaCl, 0.6 mM EDTA, 7% glycerol (w/v), 22 mM Tris-Cl, 1 mM MgCl<sub>2</sub>, 0.5  $\mu$ g bovine serum albumin 6 mM  $\beta$ -mercaptoethanol, 0.1 mM dithiothreitol, 1  $\mu$ M triamcinolone, 15% polyethyleneglycol 6000 (Serva), pH 8.2. After 45 min at room temperature, 2  $\mu$ l of 10% DMS (Fluka) was added and reaction stopped after 2 min incubation at room temperature by adding 75  $\mu$ l buffer containing 400 mM sodium acetate, 140 mM  $\beta$ -mercaptoethanol and 270  $\mu$ g ml<sup>-1</sup> transfer RNA. DNA was extracted with phenol/chloroform (1:1), precipitated and subjected to piperidine cleavage according to Maxam and Gilbert<sup>19</sup>, and finally dissolved in formamide containing loading buffer and analysed on a 6% sequencing gel.

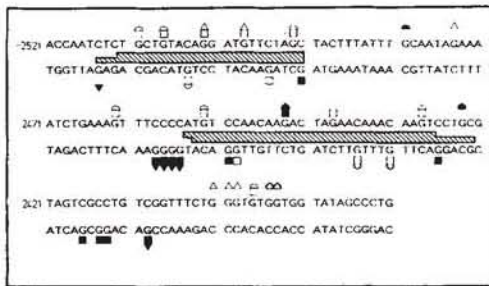


200 bp upstream of the transcriptional start site several protections and enhancements can be identified that are strictly correlated with the active state of the gene in FTO-2B cells but are not seen in XC cells. However, none of them are influenced by hormone treatment (P.B., unpublished data).

Comparison of the methylation patterns of the two analysed GREs *in vivo* reveals qualitative and quantitative differences.

Whereas the protection of the central guanine (-2,499) in element II is clearly evident, it is less prominent in element III (-2,436). A surprising finding is a very strong protection of a TGT (-2,432) upstream of the conserved hexanucleotide TGTCT of GRE III. Why the two binding sites behave so differently is not known. Recent results suggest that this difference in DMS reactivities might reflect differences in the





**Fig. 4** Summary of results obtained by DMS reactivity experiments *in vivo* and *in vitro*. Changes in DMS reactivity at guanines in the DNA sequence between nucleotides -2,521 and -2,382, upstream of the TAT cap-site, are shown. Hatched bars, sites of glucocorticoid receptor interaction as identified by *in vitro* footprinting experiments<sup>17</sup>. Filled symbols, G residues showing enhanced methylation; open symbols, guanines showing protections from methylation. Squares, *in vitro* data; triangles, dexamethasone-dependent changes *in vivo*; half-circles, changes in reactivity found *in vivo* comparing the pattern obtained from uninduced cells with that from protein-free DNA. For the *in vivo* data only those guanines are indicated that by quantitative analysis have been shown to be significantly influenced in their reactivity (Fig. 2c).

inducing capacity of the two GREs (M. Jantzen and U.S., unpublished data).

In addition to effects in the receptor binding site, a number of changes in reactivity are observed in neighbouring sequences after dexamethasone treatment. Most prominent among these are the enhancements of guanines at -2,515, -2,459 to -2,456, and at -2,424 and protections of guanines at -2,475, -2,402 to -2,400, -2,396, and -2,395, *in vivo* and to some extent *in vitro*. Whether these changes are due to alterations in DNA structure as a consequence of glucocorticoid receptor binding or interaction with other factors not yet defined remains unclear.

These changes in DMS reactivity are clearly correlated with hormone induction and other changes are evident when the pattern of DMS reactivity of uninduced FTO-2B cell chromatin is compared with that of protein-free FTO-2B DNA (quantitative analysis not shown). Analysis of the reactivity of single guanines in naked genomic DNA at various sites and in plasmid DNA revealed that guanines flanked by thymidines or followed by a thymidine are often unusually sensitive to methylation. This enhanced reactivity is not observed *in vivo*, which might be explained by the difference in reaction conditions inside living cells compared with protein-free DNA *in vitro*. Because of these differences in methylation conditions naked genomic DNA cannot serve as a suitable standard for comparisons of DMS reactivity obtained *in vivo*. The methylation pattern in uninduced FTO-cells is identical to that obtained from induced or uninduced XC-cells. In contrast to observations at the cap-site, there is no evidence for an interaction of protein factors with the receptor binding site in the absence of hormone that discriminates TAT in FTO-cells from the gene in nonexpressing fibroblast cells.

We have applied genomic sequencing methodology to obtain information on the *in vivo* interaction of the glucocorticoid receptor with its recognition sequence in the TAT gene. Results support the concept that glucocorticoid increases the affinity of the receptor for its target sequence. These experiments do not exclude the possibility that the unliganded receptor can interact with a GRE, albeit with lower affinity and selectivity.

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