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Participation of Ets Transcription Factors in the Glucocorticoid Response of the Rat Tyrosine Aminotransferase Gene

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We have previously shown that two remote glucocorticoid-responsive units (GRUs) of the rat tyrosine aminotransferase (TAT) gene contain multiple binding sites for several transcription factor families, including the glucocorticoid receptor (GR). We report here the identification of two novel binding sites for members of the Ets family of transcription factors in one of these GRUs. One of these binding sites overlaps the major GR-binding site (GRBS), whereas the other is located in its vicinity. Inactivation of the latter binding site leads to a twofold reduction of the glucocorticoid response, whereas inactivation of the site overlapping the GRBS has no detectable effect. In vivo footprinting analysis reveals that the active site is occupied in a glucocorticoid-independent manner, in a TAT-expressing cell line, even though it is located at a position where there is a glucocorticoid-dependent alteration of the nucleosomal structure. This same site is not occupied in a cell line that does not express TAT but expresses Ets-related DNA-binding activities, suggesting the existence of an inhibitory effect of chromatin structure at a hierarchical level above the nucleosome. The inactive Ets-binding site that overlaps the GRBS is not occupied even in TAT-expressing cells. However, this same overlapping site can confer Ets-dependent stimulation of both basal and glucocorticoid-induced levels when it is isolated from the GRU and duplicated. Ets-1 expression in COS cells mimics the activity of the Ets-related activities present in hepatoma cells. These Ets-binding sites could participate in the integration of the glucocorticoid response of the TAT gene with signal transduction pathways triggered by other nonsteroidal extracellular stimuli.

The glucocorticoid receptor (GR) is a member of the steroid/thyroid hormone receptor superfamily, which, upon binding its cognate hormone, regulates transcription of target genes (for reviews, see references 3, 14, and 24). Sequences responsible for the glucocorticoid-mediated activation of transcription can be located far from the promoter and are composed of multiple binding sites for numerous *trans*-acting factors, including the GR (15, 16, 22, 28). This arrangement of sites forms the so-called glucocorticoid-responsive unit (GRU [52]). The various *trans*-acting factors that participate in the function of the GRU may contribute to the integration of multiple regulatory inputs (22, 28, 39).

The tyrosine aminotransferase (TAT [EC 2.6.1.5]) gene is expressed specifically in parenchymal cells of the liver, in which its transcription is stimulated by glucocorticoids (for a review, see reference 23). Full glucocorticoid induction of the rat TAT gene is achieved through cooperative interaction of two remote GRUs (21). These GRUs are located far upstream from the transcription initiation site; one maps around nucleotide −2,500, and the other maps around −5,500 (21, 29). The −2,500 GRU corresponds to a glucocorticoid-dependent DNase I-hypersensitive site (4, 21) that results from the displacement (or alteration) of two specifically phased nucleosomes (13), whereas the −5,500 GRU corresponds to a glucocorticoid-independent DNase I-hypersensitive site (21). In the −2,500 GRU, there are three GR-binding sites (GRBSs), termed GRE 1 to 3 (29), whereas in the −5,500 GRU there is a single GRBS (21). These GRBSs are associated with multiple contiguous and overlapping binding sites for other *trans*-acting factors; several of them are expressed preferentially in liver cells. Many of these transcription factors belong to two families: those of C/EBP and HNF3 (22) (the HNF3 activity was termed HNF5 in references 22 and 49, but these activities are identical [49a]). The liver-enriched factors, particularly HNF3, are believed to be responsible for the preferential activity of the GRUs in liver cells (22). Unexpectedly, one of the HNF3-binding sites overlaps with a GRBS, such that the interactions of these factors are mutually exclusive (49). When distinct *trans*-acting factors compete for overlapping binding sites in vitro, it is tempting to speculate that one negatively regulates the action of the other (for a review, see reference 48). However, in some cases, it has been shown that both factors can activate transcription (12, 33, 50). Indeed, HNF3 participates positively in the function of the −2,500 GRU (49a). In vivo footprinting analyses suggest that coactivation results from a dynamic exchange of HNF3 and the GR (49).

The Ets proto-oncogene family is one of the most recently identified class of eukaryotic sequence-specific DNA-binding proteins that contains already more than 10 different members (for reviews, see references 25, 36, and 58). Ets proteins bind specifically to purine-rich DNA sequences, which are characterized by an invariant GGA core sequence, and a number of them function as transcriptional regulators (58). Ets family members have been implicated in various developmental processes and in the response of cells to a variety of extracellular signals (58).

In the present report, we show that two binding sites for members of the Ets family are located in the −2,500 GRU of the TAT gene. Even though one of these sites overlaps a

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GRBS, members of the Ets family efficiently cooperate with the GR in transcriptional activation.

MATERIALS AND METHODS

Plasmid constructions, cell culture, and transfections. Expression vectors were derived from pUTKat4 (21). The herpes simplex virus tk promoter was replaced by a promoter fragment (−49 to +94 [16a]) from the mouse RNA polymerase II gene (1). The −2,500 GRU fragment (−2,630 to −2,341 [21]), its mutated derivatives, or the dimerized GRE2 sites were inserted just upstream of the promoter fragment. Site-directed mutagenesis was performed on the −2,500 GRU fragment subcloned in pTZ18 (Pharmacia) using single-stranded DNA produced in a *dut*$^-$ *ung*$^-$ strain as described elsewhere (31); the following oligonucleotides were used: 5′-TAAATAACAA GAAGCCCAA-3′ for the generation of pHMa, 5′-TGCTG TACAAGATGTTCTA-3′ for the generation of pHMb, and both for the generation of pHMab (the mutated positions are underlined). The GRU that was mutated in the two Ets- and the two HNF3-binding sites was obtained by using simultaneously the two aforementioned oligonucleotides and the two oligonucleotides used to mutate the HNF3-binding sites (49a). The entire nucleotide sequence of each mutant fragment was verified. The dimerized GRE2 sites resulted from the annealing of the following oligonucleotides: CTAGATGTACAGG ATGTTCTACTAGTTGTACAGGATGTTCTAG and GAT CCTAGAACATCCTGTACAACTAGTAGAACATCCTGT ACATCTAGAGC for the generation of p2GRE2 and CTA GATGTACAAGATGTTCTACTAGTTGTACAAGATGTT CTAG and GATCCTAGAACATCTTGTACAACTAGTA GAACATCTTGTACATCTAGAGC for the generation of p2GM (the mutated bases are underlined). The fragments were cloned between the *Sac*I and *Bam*HI sites, with the *Bam*HI site lying just upstream of the −49 position of the promoter. The nucleotide sequences of the cloned fragments were verified.

Plasmid preparation, cell culture and $CaPO_4$ transfection of rat hepatoma cells (H4IIEC3) were performed as described elsewhere (21). For COS cells, the experimental conditions were similar except for the following modifications: the cells were grown in Dulbecco modified Eagle medium supplemented with 8% fetal calf serum in a 5% CO_2 incubator; they were seeded at 2×10^6 cells per 10-cm-diameter plate the day before DNA addition; and the amounts of DNA added onto each plate were 5 μg of the test plasmid, 10 μg of a sense or antisense Ets-1 expression vector (10), 1 μg of a rat GR expression vector (6RGR [43]; a gift of K. Yamamoto), and 4 μg of Bluescript (Stratagene).

Nuclear extract preparation, in vitro footprinting, and electrophoretic mobility shift assay (EMSA). Crude nuclear extracts used in DNase I footprinting were prepared from highly purified nuclei exactly as described elsewhere (22), except that buffer H also contained 40 mM $(NH_4)_2SO_4$. DNase I footprinting was performed as described elsewhere (22). Dimethyl sulfate (DMS) footprinting was performed under similar conditions, except that DMS (1%) was used instead of DNase I for 2 min at 4°C. The reaction was stopped with the addition of 3 volumes of 400 mM sodium acetate–140 mM β-mercaptoethanol–10 mM EDTA–100 μg of tRNA per ml, and this was followed by ethanol precipitation. The material was treated with piperidine before loading on a gel (38).

Crude nuclear extracts used in EMSAs were prepared by a rapid small-scale procedure. Cells (10^7) were collected from plates by a mild trypsin treatment, washed with phosphate-buffered saline–0.1 mM phenylmethylsulfonyl fluoride, and resuspended in 100 μl of buffer C (10% glycerol, 20 mM Tris-HCl [pH 7.2], 50 mM KCl, 2 mM dithiothreitol [DTT], 0.1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM Pefablock SC [Pentapharm], 10 μg of aprotinin per ml). Cell membranes were disrupted by a freeze-thawing treatment (55). The nuclei were pelleted by a 15-s centrifugation at $5{,}500 \times g$, were washed with 100 μl of buffer C, and were resuspended in buffer C containing 600 mM KCl (30). After a 10-min incubation at 4°C, the debris were pelleted by a 15-min centrifugation at $16{,}000 \times g$, and the supernatant was diluted threefold with buffer C without KCl.

EMSAs were performed using as a probe a 5′-^{32}P-labeled blunt-end double-stranded oligonucleotide generated by the annealing of two complementary oligonucleotides, the sequence of one being CTGCTGTACAGGATGTTCTAGC TACT. The probe (10,000 cpm) was incubated for 15 min at 4°C in 10 μl of 10% glycerol–10 mM Tris-HCl (pH 7.2)–100 mM KCl–1 mM DTT containing 4 μg of nuclear extract that was preincubated for 10 min with 0.5 μg of poly(dI-dC) and 10 μg of bovine serum albumin (BSA). Competition was performed with unlabeled blunt-end double-stranded oligonucleotides (8 ng), which were added simultaneously with the labeled probe. The sequences of one strand of the competitor oligonucleotides were as follows: GM, CTGCTGTACAA GATGTTCTAGCTACT (mutated position is underlined); and PEA3, TCGAGCAGGAAGTTCGA (60). The samples were loaded on a 4% polyacrylamide gel in 25 mM Tris–25 mM boric acid–0.5 mM EDTA (pH 8.3). The gel was run for 2 h at 200 V and 4°C.

In vivo footprinting and genomic sequencing. Before in vivo treatment, H4II and FR3T3 (54) cells were cultured for 60 min in serum-free medium and then treated for 60 min with either 10^{-7} M dexamethasone or 0.1% ethanol as a control. In vivo methylation with DMS and DNA preparation were performed as described elsewhere (47). Chemical sequencing reactions and piperidine cleavage were performed by the Maxam and Gilbert procedures (38).

For genomic sequencing, we used the ligation-mediated PCR (LMPCR) procedure (19, 40, 49) as follows. One microgram of genomic DNA was incubated with 0.3 pmol of primer 1 in 25 μl of first-strand buffer (10 mM Tris-HCl [pH 8.8], 40 mM NaCl, 5 mM $MgSO_4$, 20 μg of DNase-free BSA per ml) for 5 min at 95°C and then for 30 min at 50°C. Five microliters of first-strand buffer containing 1.2 mM dXTP and 0.5 U of Vent, exo$^-$, DNA polymerase (Biolabs) were then added, and the mixture was incubated in a water bath set at 50°C. The temperature of the bath was raised to 76°C in 10 min, and the incubation was continued for 10 min at 76°C. The extension mixture was transferred to ice, and then 20 μl of ligase dilution buffer (110 mM Tris-HCl [pH 7.5], 17.5 mM $MgCl_2$, 50 mM DTT, 125 μg of BSA per ml) and 25 μl of ligase mix (10 mM $MgCl_2$, 20 mM DTT, 3 mM ATP, 50 μg of BSA per ml, 4 μM annealed linker, 120 U of T4 DNA ligase per ml; Boehringer) were added. The mixture was incubated for 16 h at 17°C and was then ethanol precipitated. The exponential amplification was performed as described elsewhere (49), with the following modifications: the amplification buffer was 65 mM Tris-HCl (pH 8.8)–10 mM β-mercaptoethanol–40 mM NaCl–2.5 mM $MgCl_2$–100 μg of BSA per ml, the samples were amplified for 25 cycles (40 s at 94°C, 2 min at 69°C, and 3 min at 76°C), and visualization of the LMPCR products was carried out with 0.3 pmol of ^{32}P-labeled primer 3 for five cycles (40 s at 94°C, 3 min at 72°C, and 5 min at 76°C). We used the primer set previously described (49).

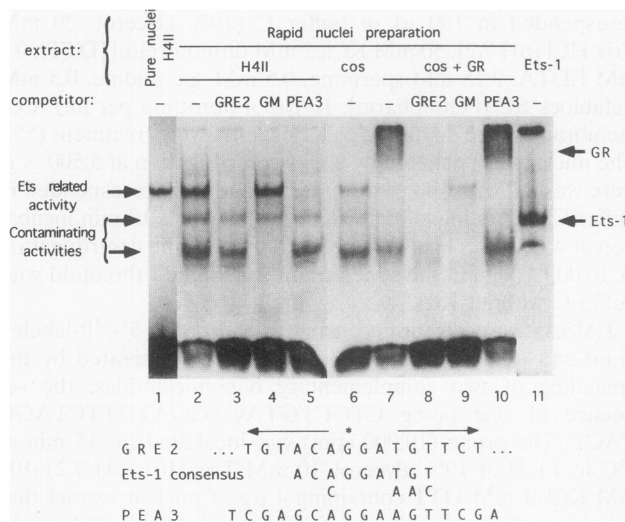

FIG. 1. In vitro characterization of the different GRE2 binding activities. EMSAs were performed using as a probe a synthetic oligonucleotide corresponding to the GRE2 site. The probe was incubated with various combinations of nuclear extracts or baculovirus-produced Ets-1 (9) and competitor oligonucleotides as indicated at the top of the figure. The positions of the various retarded bands are indicated by arrows alongside their presumptive identities. Note that Ets-1 (lane 11) gives rise to a complex that migrates at a position similar to that of one of the contaminating activities present in the nuclear extract obtained from rapidly prepared nuclei. However, this contaminating activity is distinct from Ets-1, since it is not affected by anti-Ets-1 antibodies (data not shown). The nucleotide sequences of the GRE2 site, the Ets-1-binding site consensus (41, 64), and the PEA3 motif (60) are represented below. In the GRE2 site, the star indicates the G that is replaced by an A in the GM site.

RESULTS

The −2,500 GRU contains two binding sites for Ets-related proteins. One of the GRBSs (GRE3) of the −2,500 GRU of the TAT gene is also a binding site for another *trans*-acting factor that appears to contribute to the transcriptional activation triggered by the GR (22, 49). To assess the generality of this type of arrangement, we looked for factor interaction with the other major GRBS of this GRU, the GRE2 site (29). Factor interaction with this site was analyzed by an EMSA using nuclear extracts from a differentiated rat hepatoma cell line (H4IIEC3 [44]). Nuclei were rapidly prepared by freezing and thawing, by a protocol (see Material and Methods) that provides a good yield of GR (data not shown). This allowed comparative analysis between complexes due to the GR and to other DNA-binding proteins. Figure 1 shows that this preparation gives rise to the presence of three bands, whereas a single one is observed when the extract is prepared from highly purified nuclei (sedimented through a 2 M sucrose cushion; compare lanes 1 and 2). The GR is responsible for a complex of slower mobility that is visible when a nuclear extract from COS cells transfected with a GR expression vector is used (compare lane 2 with lanes 6 and 7 of Fig. 1). The nature of the specific complex that is obtained even with highly purified nuclei was analyzed in more detail. Analysis of the nucleotide sequence of the GRE2 site revealed that it contains a motif matching perfectly the consensus binding site for the Ets-1 protein (Fig. 1). The invariant GGA core sequence is the 3-bp spacer that separates the inverted repeat characteristic of the GRBS. Replacement of the first G of this core (indicated by a star in Fig. 1) by an A is known to inhibit Ets-1 interaction with the PEA3 motif found in polyomavirus enhancer (61). We used as competitors either a mutated GRE2 site that carries this G-to-A mutation (the GM site) or the PEA3 motif that does not contain a sequence homologous to a GRBS. Figure 1 shows that the GM site is a good competitor for the GR complex (compare lanes 7 and 9) but not for the faster specific complex (compare lanes 2 and 4), whereas the PEA3 motif abolishes formation of this complex (compare lanes 2 and 5) but does not affect the one involving the GR (compare lanes 7 and 10). Ets-1 is able to interact stably with the GRE2 site, since it is responsible for a retarded complex (lane 11). DMS interference analyses performed with the GRE2 site revealed that both Ets-1 and the specific activity present in hepatoma cell nuclei contact in the major groove the two Gs of the central core (data not shown). Altogether, these data indicate that this activity has a specificity of interaction with DNA similar to that of Ets-1. However, the proteins are distinct. The mobilities of the corresponding complexes are different (compare lanes 1 and 11), and antibodies that react with both Ets-1 and Ets-2 do not interfere with the formation of hepatoma cell complex(es) (data not shown).

The interaction of Ets-1 with the −2,500 GRU was analyzed by in vitro footprinting assays. Figure 2A shows that Ets-1 protects the GRE2 site (site b) and an additional upstream site (site a) from DNase I digestion. Both sites are homologous to the Ets-1 consensus, and both are characterized by the presence of a DNase I-hyperreactive band at a similar position in the middle of the footprint. The location of the corresponding phosphodiester bond with respect to the Ets-binding site core sequence is identical for both sites (Fig. 2C). DNase I hyperreactivity at an identical location has also been described for another Ets-1-binding site (41) as well as for several E74A (a distantly related Ets protein of *Drosophila melanogaster*) binding sites (57). No such hyperreactive bands are visible in the middle of the footprints on the other strand (data not shown) (41, 57). The affinities of Ets-1 for its two binding sites are similar, as assessed by the similar low occupancy levels observed when the amount of Ets-1 is decreased (compare lanes 3 and 4 in Fig. 2A).

When a hepatoma cell nuclear extract is used for in vitro footprinting assays, the Ets-1-hyperreactive bands are also obtained at both sites (Fig. 2B; compare lanes 4 and 6). This reinforces the notion that the corresponding activity in hepatoma cells is related to the Ets family. The footprints, however, are less apparent because of a partial occupancy of the sites that may be linked to the presence of other factors interacting with adjacent sequences.

A point mutation that inactivates the interaction of Ets-1 and Ets-related activities (Fig. 1A) (59) was introduced by site-directed mutagenesis into the two Ets motifs of the −2,500 GRU. To determine the effect of the mutations on the overall binding pattern, wild-type and mutant fragments were analyzed by in vitro footprinting assays using either a hepatoma cell nuclear extract or recombinant Ets-1 (Fig. 2B). The mutation of both motifs does not alter the control DNase I pattern (compare lanes 2 and 3) but leads to the disappearance of the footprints and hyperreactive bands characteristic of the Ets proteins (compare lanes 4 and 5 and lanes 6 and 7). The interaction of other proteins appears to be unaffected by the mutations, since the rest of the footprinting pattern obtained with hepatoma cell nuclear extract is not altered.

Among the Ets-related activities expressed in liver cells, one, GABP, was identified following purification of the major DNA-binding activity (32). GABP is a dimer with two unrelated subunits, α and β. The α subunit carries the Ets homol-

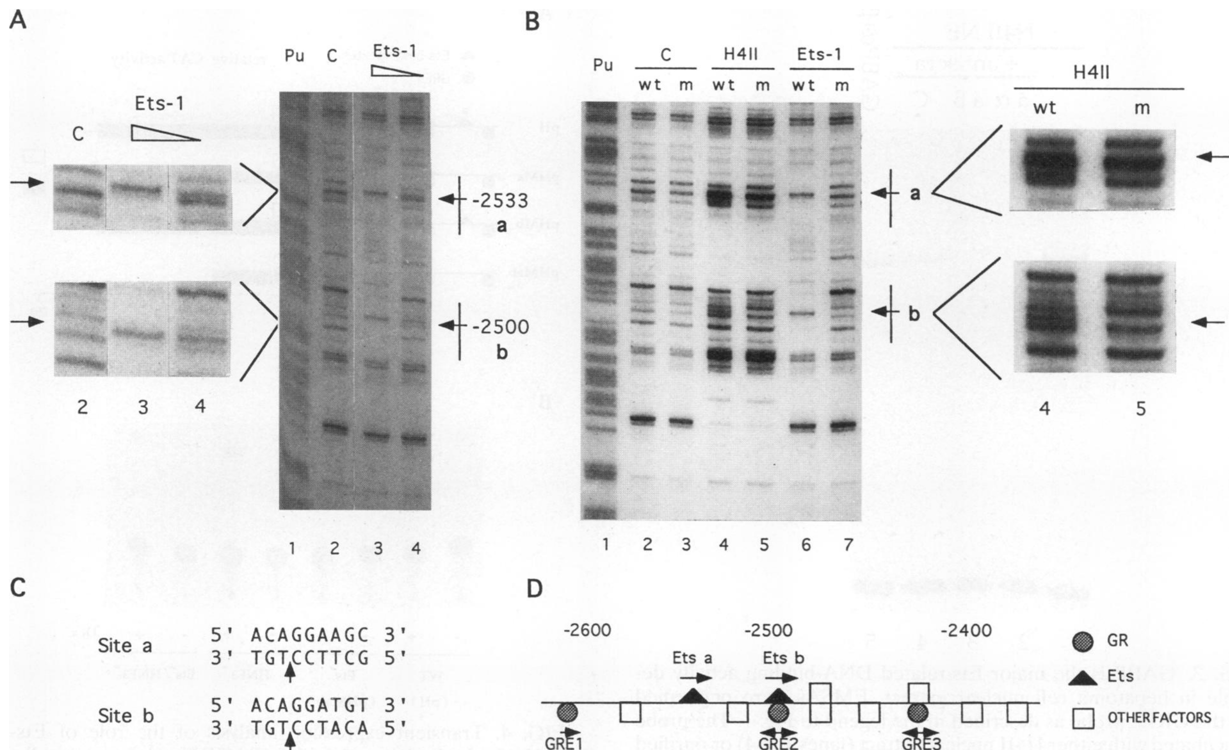

FIG. 2. In vitro analysis of the binding sites for Ets-related proteins in the −2,500 GRU. (A) DNase I footprinting analysis of the −2,500 GRU (lower strand). Lanes: 1, chemical cleavage at purines (Pu); 2, the control (C), i.e., naked DNA treated with DNase I; 3 and 4, DNA cleaved by DNase I after incubation with various amounts of baculovirus-produced Ets-1 (6 and 1 arbitrary units, respectively). The DNase I-hyperreactive bands specific of Ets-1 interaction are represented by horizontal arrows, and the corresponding footprints are indicated by vertical bars. The numbering indicates the position of the bands with respect to the start site of transcription (42); a and b indicate the identities of the sites (see panels C and D). The regions corresponding to the hyperreactive bands are enlarged on the left of the figure. (B) Comparative DNase I footprinting analysis of the wild-type (wt) and mutated (m) −2,500 GRU (lower strand). Lanes: 2 and 3, control; 4 and 5, DNA treated with DNase I after incubation with hepatoma cell (H4II) nuclear extract; 6 and 7, DNA cleaved after incubation with baculovirus-produced Ets-1 protein (the amount used corresponds to 6 arbitrary units as in panel A, lane 3). The regions corresponding to the Ets-hyperreactive bands obtained with H4II nuclear extracts are enlarged on the right. (C) Nucleotide sequences of the two Ets-binding sites. The arrows indicate the phosphodiester bonds whose sensitivity to DNase I cleavage is increased upon Ets-related protein interaction. (D) Recapitulation of the arrangement of the nuclear factor binding sites in the −2,500 GRU. The arrows above the Ets sites (represented by black triangles) indicate the orientation of the binding sites and the bidirectional arrows indicate the palindromic GRBSs (represented by stippled circles). The binding sites for other factors are represented by empty squares, irrespective of the identities of the factors for clarity (see reference 22 for a detailed analysis).

ogy and the DNA-binding domain (32). To test if the Ets activity detected in hepatoma cell nuclear extract is related to GABP, we used antisera specific for each GABP subunit (a gift of S. McKnight). We also directly compared by an EMSA the hepatoma cell activity with purified GABP of bacterial origin (a gift of S. McKnight). Figure 3 shows that the complex due to the major hepatoma cell DNA-binding activity (lane 1) is disrupted by antibodies directed against either the α or the β subunit (lanes 2 and 3) but not by control antibodies (lane 4). Furthermore, the mobility of the hepatoma cell activity is similar to that of purified GABP (compare lanes 1 and 5). Thus, GABP subunits α and β (GABP α + β) are responsible for the DNA-binding activity detected in the hepatoma cell nuclear extract.

In conclusion, there are two binding sites for Ets-related proteins within the −2,500 TAT gene GRU, and one of them overlaps the GRE2 site (see recapitulation in Fig. 2D).

Ets-related proteins modulate the glucocorticoid response mediated by the −2,500 GRU. The role of the Ets-related activities in the regulation of rat TAT gene transcription was analyzed by transient expression assays. We used as a reporter the −2,500 GRU linked upstream to a minimal promoter (region −49 to +94 of the human gene coding for the large subunit of RNA polymerase II [1]) driving the CAT gene. This promoter fragment was used instead of the thymidine kinase promoter of herpes simplex virus (21) because it displays a higher ratio of induction by glucocorticoids (16a). The activities obtained with the wild-type GRU and GRU mutated in either one or in both Ets-binding sites were compared. The differences in the activities observed should reveal the contribution of the Ets-related proteins, since the point mutations interfere specifically with the binding of these proteins.

In the H4II hepatoma cell line, the mutations affect the magnitude of the hormonal induction but not the basal level (Fig. 4A). The ratio of induction is decreased twofold by mutation of the upstream Ets motif (motif a [plasmid pHMa]); in contrast, it is not affected by mutation of the downstream motif (motif b [plasmid pHMb]). Mutation of both motifs produces an effect similar to that of mutation of motif a alone (plasmid pHMab).

In addition to the Ets family, members of the HNF3 family activate the glucocorticoid response mediated by this GRU through two binding sites (49a). Figure 4B shows that the combined action of these factors is necessary for the activity of

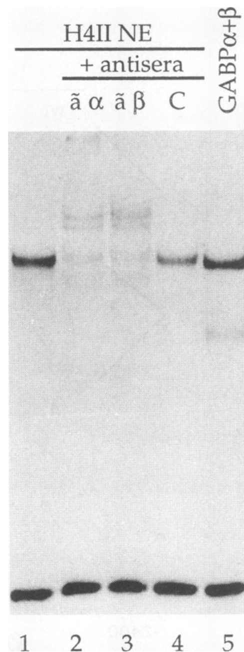

FIG. 3. GABP is the major Ets-related DNA-binding activity detectable in hepatoma cell nuclear extract. EMSAs were performed using the GRE2 probe as described in the legend to Fig. 1. The probe was incubated with either H4II nuclear extract (lanes 1 to 4) or purified GABP α + β produced in *Escherichia coli* (lane 5). Specific antisera directed against the α or the β subunit of GABP (ã α and ã β [lanes 2 and 3, respectively]) or a control antiserum (C [lane 4]) was added to the extract where indicated.

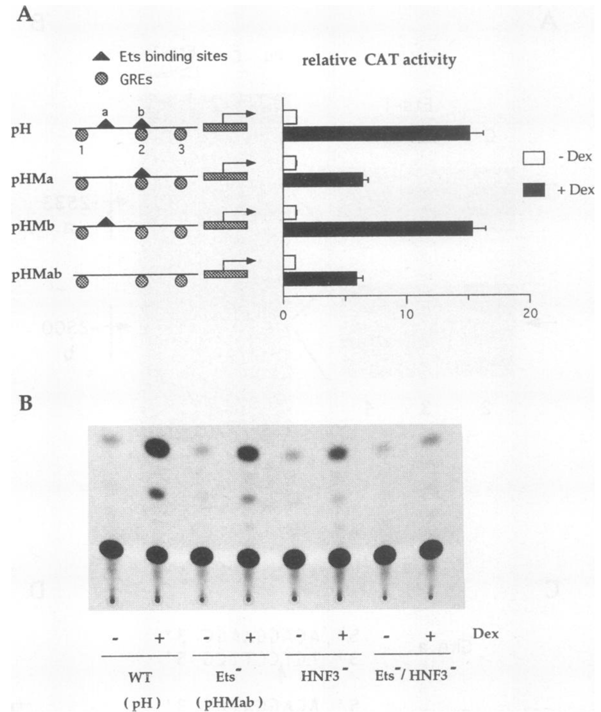

FIG. 4. Transient expression analysis of the role of Ets-related activities in the function of the −2,500 GRU in hepatoma cells (H4II). (A) Contribution of the Ets-binding sites. The reporter plasmids tested are represented on the left; the GR- and Ets-binding sites are represented by circles and triangles, respectively, as in Fig. 2D; the absence of a triangle indicates the mutation of the corresponding Ets-binding site; the stippled box represents the minimal promoter. The chloramphenicol acetyltransferase (CAT) activities obtained in each experimental condition are expressed relatively to the CAT activity obtained in the same set of experiments with pHMab in the absence of hormone. The values are the means of at least three independent experiments. The bars indicate the standard errors of the means. When indicated, cells were treated by dexamethasone (+ Dex) (10^{-6} M) 24 h prior to collection. (B) Combined action of Ets- and HNF3-binding sites. Cells were transfected with reporter CAT constructs containing either the wild-type GRU (WT, corresponding to the pH plasmid) or a mutant GRU with inactivated Ets sites (Ets^-) and/or HNF3 sites ($HNF3^-$). A cotransfected β-galactosidase expression vector was used to correct for variation of transfection efficiency, and the amount of extract used for measurement of CAT activity was normalized accordingly. The CAT assay of a representative experiment is shown.

this GRU. When either the Ets sites or the HNF3 sites are mutated, the glucocorticoid response decreases. When the four mutations are combined, there is almost no response left. The contribution of the Ets-binding sites is not significantly modified by the mutation of the HNF3-binding sites. However, the physiological importance of the twofold contribution becomes clear when the HNF3 sites are mutated because the Ets sites account for most of the remaining activity.

To test whether another member of the Ets family could similarly stimulate the glucocorticoid response, cotransfection assays with an Ets-1 expression vector were performed. The experiments were carried out in COS cells, a cell line that can be transfected with high efficiency and that contains a low level of Ets-related DNA-binding activity (compare lanes 2 and 6 in Fig. 1). To obtain glucocorticoid induction in COS cells, a GR expression vector was also cotransfected. Since the expression of Ets-1 leads to a two- to threefold increase of the activity of the minimal promoter (data not shown), the data were expressed relative to the activity obtained in the absence of hormone with the −2,500 GRU mutated in both Ets-binding sites. Figure 5 shows that the endogenous Ets-related protein has almost no effect on the activity of the −2,500 GRU. Indeed, mutations of either one or both Ets-binding sites do not significantly modify the basal and hormone-induced levels. The expression of Ets-1 does not lead to an increase in the activity of the −2,500 GRU in the absence of hormone, whereas the hormone-induced activity is slightly increased. The specificity of this effect can be assessed by the behavior of the various GRU mutants. Upon Ets-1 overexpression in COS cells, each mutant behaves as in H4II cells; mutation of the upstream motif produces a 2.5-fold decrease in the hormone-induced level, whereas mutation of the downstream motif has no effect.

In conclusion, both the endogenous Ets-related activities in hepatoma cells and the Ets-1 protein overexpressed in COS cells stimulate the glucocorticoid response mediated by the −2,500 GRU. Even though Ets-1 appears to have a similar affinity for both binding sites, only one of them participates in this stimulation in these analyses. Despite its strategic location, the site that overlaps the GRBS is dispensable.

Only the active Ets site is occupied in vivo, and occupancy is independent of glucocorticoid activation. The different activities of the two Ets-binding sites could be due either to differences in their occupancy levels in vivo or to differences in the activities of the factors interacting with each site. To distinguish between these two possibilities, we performed in vivo footprinting experiments using DMS treatment of intact

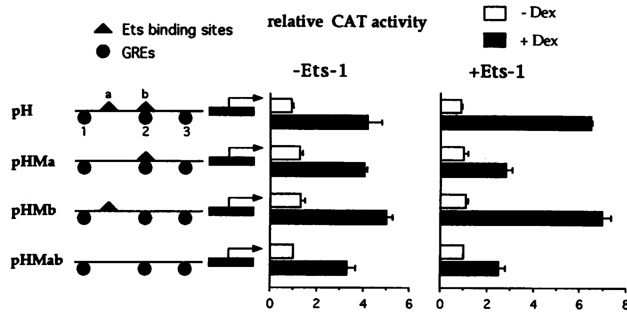

FIG. 5. Transient expression analysis of the role of Ets-1 in the function of the −2,500 GRU in COS cells. The data are presented as described in the legend to Fig. 4. −Ets-1 and +Ets-1, cotransfections with an antisense or a sense Ets-1 expression vector (9, 10), respectively. The CAT activities in each class of experimental conditions (either −Ets-1 or +Ets-1) are expressed relatively to that obtained with pHMab in the absence of hormone. Dex, dexamethasone.

H4II cells. To interpret unambiguously the in vivo data, the in vitro footprinting patterns of some members of each family of factors interacting with the GRU were analyzed. Figure 6A shows the DMS footprinting pattern obtained in vitro on the upper strand of the −2,500 GRU with either Ets-1- or the DNA-binding domain of the GR. On this strand, Ets-1 protects essentially both Gs of the central core of the Ets motif, in agreement with DMS interference analyses (41) (data not shown), whereas on the other strand, the reactivity of none of the bases is modified by Ets-1 interaction (data not shown). The DMS protection pattern of the GR on the overlapping Ets/GR-binding site is distinct from that of Ets-1 (Fig. 6A), and neither C/EBP nor HNF3 modifies the reactivity of the bases corresponding to the Ets-binding sites (data not shown).

In vivo, in hepatoma cells, essentially two Gs are protected from DMS methylation on only one of the strands of the region analyzed, irrespective of the presence of glucocorticoids in the medium (Fig. 6B and data not shown). These Gs correspond to those that are protected in vitro by Ets-1 interaction with the active upstream site. No occupancy of the inactive downstream Ets-binding site can be detected, irrespective of hormone addition. Furthermore, in contrast to a previously published report on another cell line (5), no glucocorticoid-dependent change of reactivity toward DMS is observed on either strand of the −2,500 GRU (Fig. 6B and data not shown). Since the GR leaves a clear in vitro footprint (Fig. 6A) (20), these results confirm that, upon hormonal stimulation, the GR does not interact permanently with these target sites in this cell line (49).

In a rat fibroblast cell line that does not express the TAT gene (FR3T3 [54]), no in vivo protection was observed within the region (Fig. 6B). Therefore, the Ets-related activities present in this cell line (data not shown) do not interact with either of the Ets-binding sites.

Ets-related proteins can activate glucocorticoid induction via the overlapping Ets/GR-binding site if it is isolated from the context of the GRU. It is likely that the mutation that abolishes the binding of Ets to the downstream binding site has no effect because Ets-related factors do not interact with that site in H4II cells. This absence of interaction could be due to the Ets-binding site on its own or to the interaction of factors with flanking sequences. To analyze the behavior of the overlapping binding site, in the absence of the rest of the −2,500 GRU, we tested the activity of a synthetic dimerized site. This site was dimerized because this is essential to obtain

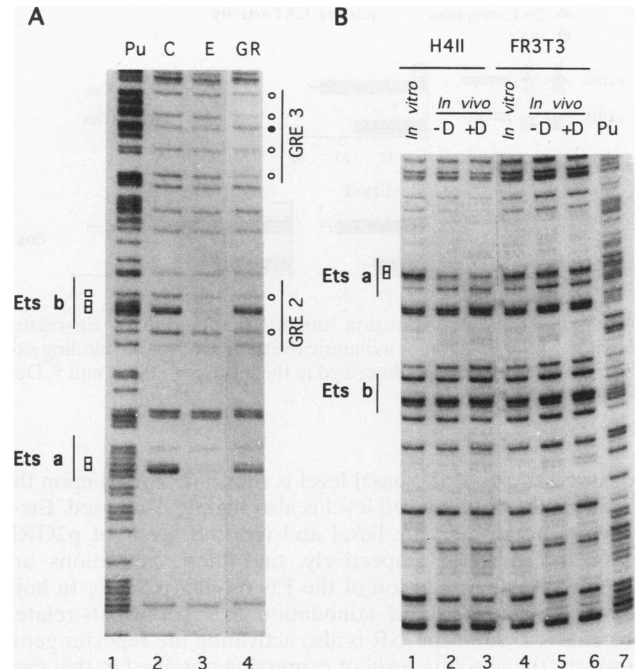

FIG. 6. Comparative in vitro and in vivo DMS footprinting analyses of the interaction of factors with the Ets-binding sites. (A) In vitro DMS footprinting analysis of the −2,500 GRU (upper strand). Lanes: 1, chemical cleavage at purines (Pu); 2, the control (C), i.e., naked DNA treated with DMS and piperidine, 3 and 4, DNA treated with DMS after incubation with, respectively, either baculovirus-produced Ets-1 (E [25 arbitrary units; see Fig. 2A]) or the purified bacterially produced GR DNA-binding domain (GR [500 ng; see reference 35; a gift of K. R. Yamamoto]). The protected guanosines are indicated by empty symbols; the hyperreactive one is indicated by a filled circle. Squares on the left side correspond to the changes of reactivity induced by Ets-1; circles on the right side correspond to those induced by the GR. (B) In vivo DMS footprinting analysis of the −2,500 GRU (upper strand) using LMPCR (40) for genomic sequencing. Lanes 1, 4, and 7 correspond to genomic DNA that was treated in vitro with either DMS (in vitro [lanes 1 and 4]) or formic acid (purine-specific reaction [Pu; lane 7]). Lanes 2, 3, 5, and 6 correspond to the genomic DNAs of cells treated with DMS in vivo, following an incubation with or without dexamethasone (+D and −D, respectively). The material originates either from hepatoma cells (H4II [lanes 1 to 3 and 7]) or fibroblasts (FR3T3 [lanes 4 to 6]). The protected guanosines are indicated by open squares on the left side. The ladders in panels A and B are inverted because the procedures used for visualization of the in vitro and in vivo footprints are different (direct end labeling and LMPCR).

significant activity (22, 56). The spacing between the two sites was selected such that the distance from center to center was 21 bp, because this is optimal for synergistic GR binding (51). We compared the activity of the dimerized wild-type site (plasmid p2GRE) with that of a dimerized mutant site (plasmid p2GM) that does not allow Ets binding (the above-mentioned mutation). These sites were inserted in place of the −2,500 GRU in the previously described reporter plasmid and were tested either in H4II cells or in COS cells for the analysis of coexpression of Ets-1.

In the two cell lines, Ets-related proteins activate transcription both in the absence and in the presence of hormone through the duplicated GRE2 motif (Fig. 7). In H4II cells, inactivation of the Ets motif leads to a threefold reduction of the basal level of transcription and to a less pronounced decrease of the induced level. In COS cells, in the absence of

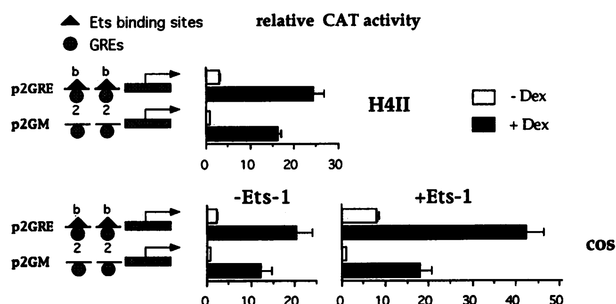

FIG. 7. Transient expression analysis of the role of Ets-related activities in the function of a dimeric overlapping Ets/GR-binding site. The data are presented as described in the legends to Fig. 4 and 5. Dex, dexamethasone.

coexpressed Ets-1, the basal level is reduced twofold upon the mutation and the induced level is also slightly decreased. Ets-1 expression activates the basal and induced levels of p2GRE four- and twofold, respectively, and these activations are abolished by the mutation of the Ets motifs (p2GM). In both cell lines, the factor of stimulation exerted by Ets-related proteins is lower if the GR is also activating the reporter gene; however, the absolute level of expression obtained in that case is higher than the sum of the level obtained following activation by each factor independently.

In conclusion, the duplicated GRE2 motif isolated from the context of the rest of the −2,500 GRU can confer Ets-dependent activation of both the basal and glucocorticoid-induced levels of expression of a linked reporter gene. Thus, the Ets motif overlapping the GRE2 site behaves differently, depending on the overall arrangement of the regulatory sequences.

DISCUSSION

Ets-related proteins and the TAT gene glucocorticoid response. The regulatory element analyzed here is a complex GRU containing numerous binding sites for several transcription factor families (22). Transient expression assays in cultured hepatoma cells indicate that, in addition to the GR, several of these factor families participate positively in the glucocorticoid response mediated by this GRU. Here, we show that members of the Ets family of transcription factors interact in vitro with two sites in the −2,500 GRU of rat TAT gene. Under the conditions used, only one of the two Ets-binding sites of the intact GRU participates in the activity. This site is located in the vicinity of the major GRBS (the GRE2 site [29]) at a position allowing classical cooperativity with the GR (52, 53, 56). The activity of the other site, which overlaps this GRBS, can be detected only when isolated from its natural flanking sequences and duplicated. Under these experimental conditions, it confers Ets-dependent activation of basal and hormone-induced levels. Although it was weak, the synergism found suggests that the GR and Ets-1 do not activate a fully independent population of reporter plasmid molecules. Since the two factors interact in a mutually exclusive way with the overlapping site (data not shown; see also the overlap of the DMS footprints of the two factors in Fig. 6A), transcriptional activation in that case may proceed through a dynamic interplay of the factors, similar to that observed for the overlapping HNF3-GR-binding site (49).

Functional analysis of this GRU shows that members of both the Ets and HNF3 families contribute to the transcriptional activation triggered by the GR (the present study and reference 49a). The net contribution of individual binding site for these factors varies slightly, but none of them appears determinant on its own. It is the sum of these contributions that is responsible for the amplitude of the response, as shown by the very low activity of the GRU mutated in both Ets- and HNF3-binding sites. This mutated GRU still possesses all of the binding sites for the GR, the C/EBP family, and the CACCC box binding factors (22), showing that these factors are not sufficient for the function of this GRU.

Is little participation of each factor the general case for this GRU, or is there a particular situation in which the contribution of one factor (in addition to the GR) is more determinant than that of the others? This may occur, for example, at a specific developmental stage or in response to a specific stimulus. Differentiated hepatoma cells are a convenient experimental system for mechanistic analyses but are largely different from liver cells in tissue, and many of the events occurring in a liver cannot be reproduced. Particularly, the balance of both transcription factor levels and signal transduction cascades is significantly perturbed by immortalization and in vitro culture conditions (e.g., see reference 27).

Variations in the balance of the transcription factors should lead to different behaviors of the GRU. No systematic study of the Ets members expressed in hepatocytes has been performed. We have shown that GABP is responsible for the major DNA-binding activity found in hepatoma cells, but it is not the only one known to be expressed in liver cells (6, 7, 32, 46). Although we have not detected a DNA-binding activity corresponding to Ets-2 in liver or hepatoma cell nuclear extract by EMSAs and a specific antibody (data not shown), Northern (RNA) blot analysis of hepatoma cell RNA using an *ets* probe revealed the *ets-2* RNA pattern (data not shown). The lack of detectable Ets-2 DNA-binding activity in crude nuclear extract is likely to be due to the effect of the internal inhibitory domain that has been described for both Ets-1 and -2 (26, 34, 62). It is presently not known if this inhibition can be relieved following a conformational change induced in response to a specific stimulus, but this is an attractive possibility. Although Ets-1 is not expressed in liver cells (7), it appears to participate in the function of the GRU in a manner similar to that of the Ets-related activities present in hepatoma cells. Moreover, cotransfection experiments using an Ets-2 expression vector gave rise to similar results (data not shown). Therefore, the exact identity and role of the participating Ets members are not clear, but it is likely that there is some functional redundancy between the factors interacting with these sites.

The signal transduction cascades are particularly important in the function of Ets members that are at the endpoints of PKC, Ca^{2+}, and Ras, Raf, and mitogen-activated protein (MAP) kinase pathways (11, 17, 18, 37, 45). This endpoint position is certainly important for cell growth control, as assessed by the oncogenic properties of some members of the family (for reviews, see references 25, 36, and 58). The Ets-binding sites present in the GRU may allow the integration of the glucorticoid response of the TAT gene with the control of cell growth. However, our attempts to link these Ets sites to the regulation of cell growth in hepatoma cells have been unsuccessful. We have not observed any extensive modulation of the Ets DNA-binding activity upon serum or phorbol ester stimulation (data not shown), as described for another cell line (60). However, since this control is lost upon cell transformation (60), this lack of control may be due to the oncogenic event(s) that occurred in the hepatoma cell line used.

Occupancy of Ets-binding sites and relationship with chromatin structure. The differences in activity between the Ets-

binding sites of the −2,500 GRU can be correlated with the in vivo footprinting analysis of the TAT gene in hepatoma cells. This study reveals that the active site is largely occupied independently of the presence of glucocorticoids, whereas the inactive site is not detectably occupied under the same experimental conditions. This is in contrast to in vitro analyses, which reveal similar levels of occupancy of the two sites both when crude hepatoma cell nuclear extract and when recombinant Ets-1 are used. Chromatin structural features are often responsible for differences between in vitro and in vivo occupancy levels (see, for example, references 49 and 63) and could therefore be involved in the different relative occupancy levels of the two sites. However, this cannot be simply correlated with what is known of the nucleosomal phasing of the −2,500 GRU (13, 47). An array of phased nucleosomes is present over the region in hepatoma cells that is not treated with glucocorticoids. According to the nucleosome positions described elsewhere (13, 47), the upstream Ets site would sit on a nucleosome, whereas the downstream one would reside in the linker region. Upon hormonal stimulation, the structure of the two nucleosomes that surround this linker region is specifically altered (13, 47), and this alteration is presumably responsible for the glucocorticoid-dependent interaction of the liver-specific factor HNF3 (49). However, Ets interaction with the upstream site does not seem to be affected by the presence of a structurally intact nucleosome, and it does not appear to be modified upon hormonally induced nucleosomal alteration, even though this alteration greatly increases the accessibility of surrounding sequences to restriction enzyme cleavage (*Sty*I and *Xba*I [47]). In contrast, the Ets site in the linker region, which would have been expected to be more accessible, is not occupied, even after the chromatin structural alteration. Whether it is chromatin structural features or other *trans*-acting factors that inhibit the interaction of Ets-related factors with that site is therefore unclear.

Ets-related activities and cell-type-specific chromatin dynamics. The in vivo footprinting analysis also shows that the upstream site, which is largely occupied in hepatoma cells in a glucocorticoid-independent manner, is not detectably occupied under the same experimental conditions in a fibroblast cell line that does not express the TAT gene. This absence of interaction is correlated neither with a different level or composition of Ets-related DNA-binding activities, as judged from an EMSA analysis (data not shown), nor with variations in the cytosine methylation status of the corresponding sites (data not shown; this is expected since there is no CpG that could be methylated in this region [42]). Therefore, the interaction of Ets-related activities is likely to be inhibited by chromatin structural features, as has been proposed for other cases (see reference 63, for example). The features involved are at a hierarchical level above the nucleosome, since, in hepatoma cells, the nucleosome alone is not sufficient to prevent the interaction of Ets-related activities with the upstream site. This absence of interaction is correlated with a general locking of the chromatin structure of the entire gene; none of the DNase I-hypersensitive sites that are present on the 5′-flanking sequences of the gene in hepatoma cells (21) can be detected in this fibroblast cell line, irrespective of the presence of glucocorticoid hormones (48a). Presumably, there is a higher-order chromatin structural transition that increases the accessibility of the entire genomic domain (for a review, see reference 8) and that allows the interaction of Ets-related activities with the upstream site.

Our observation is the first evidence of a regulatory factor(s) interacting with the −2,500 GRU of the TAT gene in the absence of hormone, and it reveals that, in TAT-expressing cells, the GRU is in a pre-activated state prior to glucocorticoid stimulation. The Ets-related factor(s) that occupies the upstream site could therefore play an important permissive role for subsequent hormonal induction either through classical cooperativity with the GR or via an effect on chromatin structure, i.e., allowing the establishment of a conformational state favorable to the glucocorticoid-dependent nucleosomal alteration. Such a possibility is suggested by our observation that Ets-1 can introduce a dramatic bend in its target DNA (unpublished results). If Ets-related activities participate in the cell-type-specific chromatin dynamics, their role might have been underestimated by the transient expression assay used here. Indeed, some aspects of the glucocorticoid regulation of the mouse mammary tumor virus GRU that involve chromatin structural alteration are not reproduced in transient expression experiments (2). Clearly, definitive assessment of the contribution of Ets-binding sites to the function of the GRU requires a true in vivo analysis using, for example, a transgenic mouse model.

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