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Synergistic action of the glucocorticoid receptor with transcription factors

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Steroid responsive elements (SRE) have been mapped at variable positions relative to the transcription start site and are often adjacent to binding sites of transcription regulatory proteins. In order to define the role of these transcriptional control sequences in the induction process, we inserted the previously defined 15-bp glucocorticoid response element (GRE) or 15-bp estrogen response element (ERE) immediately upstream of the TATA box of the thymidine kinase promoter, deleting all distal promoter elements. Both ERE and GRE confer inducibility by the respective hormone to the truncated promoter. These data suggest that the steroid receptor protein, possibly in conjunction with the TATA box binding protein, is able to form an active transcription complex. In contrast, the GRE when inserted 351 bp upstream of the start site of transcription of the tyrosine aminotransferase gene (TAT) is not capable of mediating hormone inducibility. Inducibility can be attained at this position by either two GREs or a single GRE in combination with a CCAAT motif. A cluster of point mutations in the CCAAT box abolishes hormone inducibility, strongly suggesting a synergistic action between the glucocorticoid receptor and the factor recognizing the CCAAT motif. The CCAAT box can be replaced by a CACCC box, an NF I and an SP1 binding site, thus demonstrating that synergistic action is not restricted to the CCAAT box binding protein. These combinations of a GRE with different transcription factor binding sites show a pronounced cell-type-dependent glucocorticoid induction of expression.

Key words: estradiol/glucocorticoid/synergism/TATA box/transcription factors

Introduction

Control of gene transcription by steroid hormones is mediated by receptor proteins which, upon binding the ligand, interact with regulatory sequences of responsive genes (Ringold, 1985; Yamamoto, 1985; Becker *et al.*, 1986). The sequence elements recognized by different steroid receptors constitute a family of identical (Strähle *et al.*, 1987) or closely related sequences (Klock *et al.*, 1987; Martinez *et al.*, 1987, Klein-Hitpass *et al.*, 1988a). The partially palindromic 15-bp element TGTACAGGATGTTCT is sufficient to render a non-regulated promoter inducible by glucocorticoids. The same oligonucleotide is also recognized by the progesterone receptor, and mutations of known contact sites affect induction by either steroid suggesting that this element is recognized by both receptors similarly (Strähle *et al.*, 1987). The related but distinct oligonucleotide AGGTCACAGTGACCT confers inducibility by estradiol (Klock *et al.*, 1987).

The location of the steroid responsive elements (SREs) within regulated genes is very variable, ranging from positions several kilobase pairs upstream of a promoter (Jantzen *et al.*, 1987) to positions within the first few hundred base pairs upstream (Karin *et al.*, 1984; Buetti and Diggelmann, 1986; Kühnel *et al.*, 1986) or downstream of the cap site (Slater *et al.*, 1985). For some genes these elements were demonstrated to behave as steroid-inducible enhancer sequences (Chandler *et al.*, 1983; Miksicek *et al.*, 1986; Jantzen *et al.*, 1987). Whereas the transcriptional activity of many regulated genes is only modulated by the steroid, the transcriptional activity of other genes, e.g. the proviral genome of MMTV and the egg-white protein genes, is entirely dependent on the presence of hormone, suggesting that the receptors play a crucial role in activation of these genes (Palmiter *et al.*, 1978; Hynes *et al.*, 1979; Yamamoto, 1985).

The mechanism by which hormone receptors increase transcriptional activity is largely unknown. Several observations indicate that the presence of an SRE alone is not sufficient for hormone inducibility but that, in addition, other regulatory elements are required. First, mutation of the nuclear factor I binding site (NF I) in the MMTV LTR destroys the ability of this promoter to respond to glucocorticoids (Buetti and Diggelmann, 1986; Miksicek *et al.*, 1987). Second, exonuclease footprinting experiments suggest that glucocorticoid induction of transcription from the LTR promoter results from recruitment of transcription factors, presumably NF I and a TATA box binding factor(s), at the promoter, indicating that receptor binding promotes the formation of a transcriptionally active complex (Cordingley *et al.*, 1987). Third, glucocorticoid induction leads to changes in DMS reactivity within the intact cell at the two GREs of the tyrosine aminotransferase (TAT) gene as well as at a CACCC box in immediate vicinity of the GRE (Becker *et al.*, 1986), possibly resulting from co-operative interaction between the bound receptor and the CACCC box binding factor. Fourth, removal of sequences with a strong similarity to the CACCC element of the β-globin promoter (Myers *et al.*, 1986), adjacent to the proximal glucocorticoid receptor binding site of the tryptophan oxygenase gene, abolishes glucocorticoid induction, implying the importance of this element for the activity of the GRE (Danesch *et al.*, 1987). Finally, placing a CACCC element in close proximity to a GRE upstream of the thymidine kinase (Tk) promoter enhances inducibility of expression by glucocorticoids (Schüle *et al.*, 1988).

We have previously demonstrated that a 15-bp GRE as well as a 15-bp ERE when inserted immediately upstream (position −105) of the well-characterized Tk promoter of

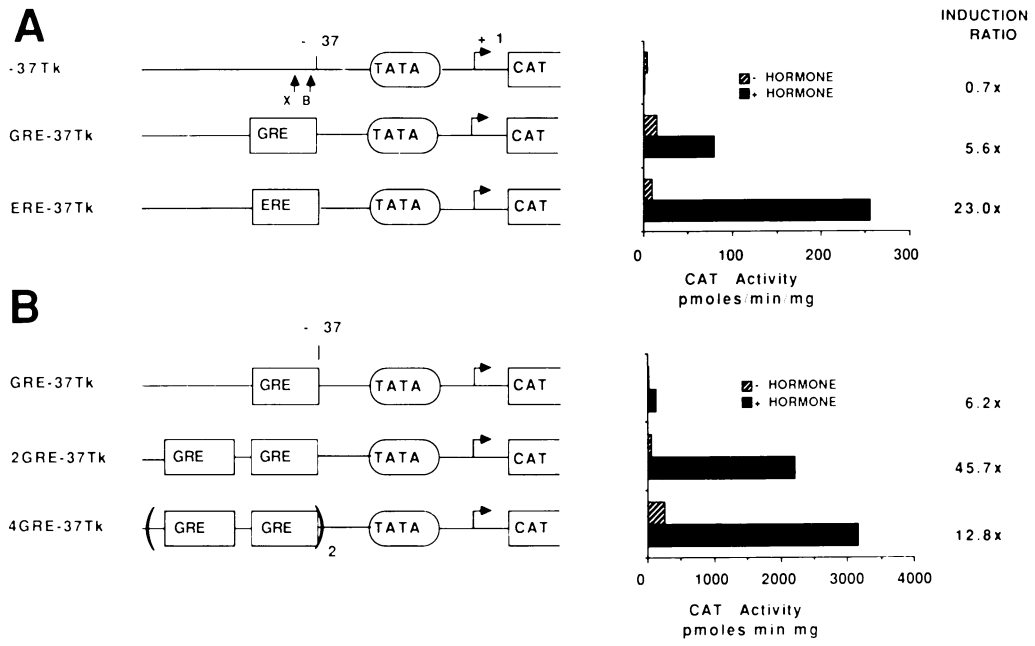

Fig. 1. A glucocorticoid response element (GRE) or an estrogen response element (ERE) upstream of a TATA box is sufficient for steroid induction. (**A**) MCF-7 cells were transfected with plasmids harboring either the 15-bp GRE (TGTACAGGATGTTCT) or the 15-bp ERE (AGGTCACAGTGACCT) upstream of the TATA box of the thymidine kinase promoter. Expression of chloramphenicol acetyltransferase activity (CAT) was compared in extracts from cells treated with and without 10^{-7} M dexamethasone, or in the case of the plasmid ERE-37Tk with 10^{-8} M 17β-estradiol. CAT activity was plotted as pmol chloramphenicol converted per min per mg protein. Induction ratios were calculated by dividing CAT activity in extracts of hormone-treated cells by the CAT activity in extracts from untreated control cells. The 21-bp oligonucleotides containing the hormone response elements were inserted into the *Xba*I (X) site of the construct −37Tk. The center of the palindromes in ERE-37Tk and GRE-37Tk reside 50 bp upstream of the cap site indicated by +1 above the horizontal arrow. (**B**) Plasmids carrying either one, two or four copies of the 15-bp GRE upstream of the TATA box were transfected into MCF-7 cells. Hormonal stimulation of expression was monitored by determining CAT enzymatic activity in extracts from transfected cells treated with or without 10^{-7} M dexamethasone. (Note: a different scale is used in the diagram of panel B compared to panel A.) In constructs 2GRE-37Tk and 4GRE-37Tk the oligonucleotides were inserted into the *Xba*I (X) or the *Bam*HI site (B) respectively upstream of the TATA box in construct −37Tk. The center to center distance between the palindromes in 2GRE-37Tk and 4GRE-37Tk is 27 bp, the center of the first GRE upstream of the TATA box is located at position −59 in 2GRE-37Tk or position −53 in 4GRE-37Tk relative to the cap site (+1). The four GREs in 4GRE-37Tk are in opposite orientation relative to the GREs in constructs GRE-37Tk and 2GRE-37Tk.

the herpes simplex virus (McKnight *et al.*, 1982, 1984; Jones *et al.*, 1985) is sufficient to confer inducibility by the respective steroid (Klock *et al.*, 1987; Strähle *et al.*, 1987). In order to define the role of other transcription elements in the induction process, we asked whether SREs positioned immediately upstream of a TATA box would allow formation of an inducible transcription complex. The results presented here clearly demonstrate that the glucocorticoid and the estrogen receptor function independently of additional upstream factors. However, when inserted at a position further upstream, 351 bp in front of the TAT promoter, a single 15-bp-long GRE on its own does not mediate induction. At this position, either two copies of the GRE or the combination of a GRE with a CCAAT, CACCC, NF I or SP1 motif allow induction. The synergistic function of these elements shows a pronounced cell type specificity, suggesting a possible role of transcription factors in a cell-type-dependent modulation of hormone induction.

Results

A steroid response element upstream of a TATA box is sufficient for steroid-dependent activation of transcription

In order to assess whether the upstream elements of the Tk promoter are required for hormonal inducibility we deleted both distal elements of the Tk promoter (McKnight *et al.*, 1984) placing the GRE (TGTACAGGATGTTCT) or the ERE (AGGTCACAGTGACCT) directly upstream of the TATA box. The structures of the constructs, GRE-37Tk and ERE-37Tk, are outlined in Figure 1A. Since it has been shown that multiple hormone elements lead to synergistic induction (Jantzen *et al.*, 1987), we also constructed the plasmids 2GRE-37Tk and 4GRE-37Tk, harboring two or four GREs respectively, immediately upstream of the TATA box (Figure 1B). After transfection of these plasmids into MCF7 cells, a human mammary carcinoma cell line expressing glucocorticoid and estrogen receptor (Horwitz *et al.* 1978), hormone dependent expression was analyzed by following chloramphenicol acetyl transferase (CAT) enzymatic activity (Gorman *et al.*, 1982) and Tk CAT mRNA levels using a ribonuclease protection assay (Zinn *et al.*, 1983; Melton *et al.*, 1984).

As demonstrated in Figure 1A, expression of CAT activity from the construct GRE-37Tk, which contains a single GRE, was stimulated 5.6-fold by the synthetic glucocorticoid dexamethasone. Induction ratios varied between 4- and 8-fold in different independent experiments. Putting the GRE 5 bp closer to the TATA box or 26 bp further away by insertion of a synthetic spacer did not alter inducibility (data not shown), indicating that a certain flexibility in the distance between the TATA box and the GRE is allowed. Estradiol also leads to a strong induction of expression from the construct ERE-37Tk, which carries a single ERE upstream

of the TATA box. Expression of CAT activity from the plasmid −37Tk lacking an SRE is ~5−10% of the expression from the intact Tk promoter extending up to position −105 (data not shown), in agreement with published data (McKnight *et al.*, 1981; Pelham and Bienz, 1982). Neither dexamethasone (Figure 1A) nor estradiol (data not shown) have an effect on expression of −37Tk. Thus hormonal inducibility is totally dependent on the presence of an SRE. Recently, Schüle *et al.* (1988) pointed out a sequence with similarity to a CACCC box in pUC18 located immediately upstream of the minimal promoter described here. To exclude the possibility that this element could be functional and therefore could substitute for the deleted distal elements of the Tk promoter we removed a 215-bp *Nde*I−*Hin*dIII fragment containing the CACCC element in construct GRE-37Tk. Induction of CAT expression from construct GRE-37Tk and from its deletion derivative was 8- and 7.5-fold respectively in MCF-7 cells, clearly demonstrating that this sequence with similarity to a CACCC box does not affect inducibility in the cells used (data not shown). These data show that a single SRE is sufficient to stimulate transcription in MCF-7 cells in response to hormone from a promoter which apparently contains no regulatory elements besides the TATA box.

Duplication of the GRE greatly enhances hormone inducibility (Figure 1B). Compared with expression from plasmid GRE-37Tk the levels of hormone-induced CAT activity from the plasmid 2GRE-37Tk is much more than expected for an additive effect of each GRE. Synergistic action of linked GREs has already been demonstrated for the two GREs of the TAT gene (Jantzen *et al.*, 1987). Insertion of four GREs upstream of the TATA box (4GRE-37Tk) when compared with plasmid 2GRE-37Tk does not lead to a significant further increase in hormone-dependent CAT activity; the induction ratio conferred by four GREs is even lower due to elevated levels of CAT expression in the absence of hormone (Figure 1B).

The SREs lead already to a pronounced increase in the level of CAT expression in the absence of hormone (Figure 1). The level of basal activity seems to be correlated with the number of GREs inserted upstream of the TATA box. In order to rule out the possibility that residual hormone in the charcoal-stripped fetal calf serum caused the elevated basal level, incubation of transfected cells was extended after recovery in stripped serum for further 24 h in serum-free medium. Neither the additional incubation in serum-free medium nor administration of the hormone antagonists RU486 or 4-hydroxy-tamoxifen reduced basal levels (data not shown), suggesting that the increase is not caused by residual amounts of hormone.

In order to verify that the induction of CAT expression is based on an increase of faithfully initiated RNA we determined the amount of correctly initiated transcripts from the plasmids GRE-37Tk and 4GRE-37Tk. Total RNA was isolated from cells treated with or without dexamethasone (Chirgwin *et al.*, 1979) and analyzed by the RNase protection procedure (Zinn *et al.*, 1983; Melton *et al.*, 1984). Correctly initiated RNA yields a fragment of 210 nucleotides protected from digestion with RNase A and RNase T1. The results of such an analysis are shown in Figure 2 (note: different exposure times and amounts of RNA were used). Dexamethasone increases the level of correctly initiated RNA from both constructs, confirming that the increases in CAT activity upon administration of the hormone is paralleled by an increase in correctly initiated RNA.

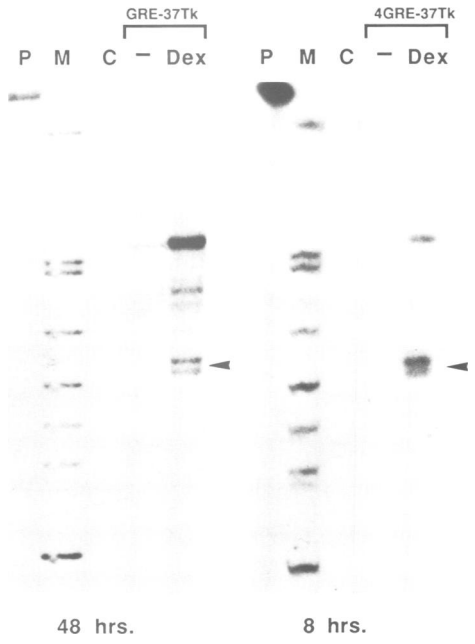

Fig. 2. Induction of CAT activity is based on an increase of correctly initiated transcripts. MCF-7 cells were transfected with either plasmid GRE-37Tk or with plasmid 4GRE-37Tk and treated without (−) or with 10^{-7} M dexamethasone (Dex). Total RNA (40 μg, 4GRE-37Tk; 100 μg, GRE-37Tk) from transfected cells was hybridized to a uniformly labeled, antisense SP6 transcript and digested with RNase A and RNase T1. The protected RNA fragments were separated on a 6% sequencing gel. Fragments resulting from correctly initiated transcription are indicated by arrows. The two autoradiographs shown result from different exposure times (8 h for 4GRE-37Tk; 48 h for GRE-37Tk). P, probe; M, *Hpa*II-digested and end-labeled pBR322; C, RNA from untransfected cells.

Either two copies of a GRE or combination of a single GRE with a CCAAT motif is required for induction from an upstream position

In several cases, GREs have been shown to display properties of hormone-inducible enhancers (Yamamoto, 1985; Jantzen *et al.*, 1987). To address the question whether a 15-bp-long GRE can also behave as an enhancer and can confer hormone induction from a promoter-distant position we inserted it 351 bp upstream of the tyrosine aminotransferase gene promoter in the plasmid −351TAT (see Figure 3A). The plasmid −351TAT contains the promoter with 5′-flanking sequences (−351 to +62) of the TAT gene driving the expression of the CAT coding region. The plasmids were introduced by the DEAE-Dextran procedure into Ltk^- cells. This cell line has been used previously to map the GREs of the TAT gene (Jantzen *et al.*, 1987). Hormone-dependent stimulation of expression was analyzed by determining CAT activity in extracts from transfected cells treated with or without 10^{-7} M dexamethasone.

As shown in Figure 3A and B, expression from plasmid GRE-351TAT could not be induced by administration of dexamethasone. Thus, when positioned −351 bp upstream, the 15-bp-long GRE is not sufficient to render expression from the TAT promoter hormone inducible. Since glucocorticoid-dependent expression of the TAT gene is exerted by two co-operating GREs from far upstream (Jantzen *et al.*,

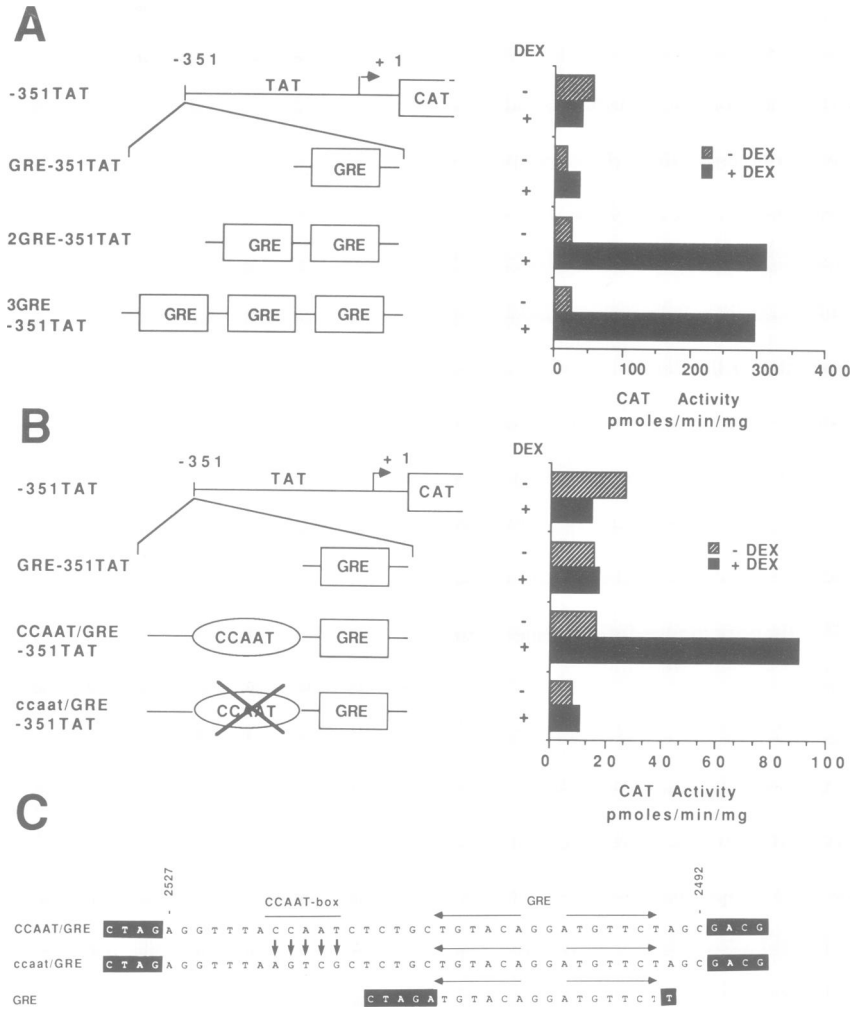

Fig. 3. Either two glucocorticoid response elements (GREs) or combination of a GRE with a CCAAT motif are required for induction from a promoter distant position. (**A**) Constructs containing either one (GRE-351TAT), two (2GRE-351TAT) or three (3GRE-351TAT) copies of the 15-bp GRE (sequence depicted at the bottom) at position −351 upstream of the tyrosine aminotransferase promoter (TAT) were transfected into Ltk$^-$ cells and analyzed for hormone-dependent expression of the linked chloramphenicol acetyltransferase gene (CAT). CAT activity was determined in transfected cells treated with or without 10^{-7} M dexamethasone. Results are expressed as pmol of chloramphenicol acetylated per min per mg of protein. The center to center distance of the GREs is 21 bp. The promoter-proximal copy of the GRE in 3GRE-351TAT is in the opposite orientation with respect to the GREs in construct 2GRE-351TAT. (**B**) Ltk$^-$ cells were transfected with plasmids carrying the oligonucleotides depicted in panel C upstream of the TAT promoter at position −351. Expression of CAT activity was determined in transfected cells treated with or without 10^{-7} M dexamethasone. Construct CCAAT/GRE-351TAT harbors a fragment (−2527 to −2492) of the glucocorticoid-dependent enhancer of the TAT gene which contains a CCAAT motif 6 bp away from the 15-bp GRE. In plasmid ccaat/GRE-351TAT the CCAAT motif is destroyed by a cluster of transversions. (**C**) Sequence of oligonucleotides which have been inserted into the *Xba*I site upstream of the TAT promoter in construct −351TAT. Sequences not derived from the GREII of the TAT gene are indicated by black boxes. Palindromic structures of the GREs are indicated by horizontal arrows above the sequence. Short vertical arrows above the oligonucleotide ccaat/GRE indicate the transversions by which the CCAAT box has been destroyed.

1987) we inserted two or three copies of the 15-bp GRE upstream of the TAT promoter, generating plasmids 2GRE-351TAT and 3GRE-351TAT respectively. As shown in Figure 3A, the presence of two GREs upstream of the TAT promoter results in a >10-fold stimulation of CAT expression by dexamethasone. Again, as already observed in the experiments analyzing multiple copies of GREs upstream of the TATA box, insertion of more than two GREs does not further increase hormone inducibility (Figure 3A). In contrast to the observation that the 15-bp GRE does not allow hormone induction when positioned 351 bp upstream of the TAT promoter, we previously demonstrated that the 15-bp-long GRE when flanked by additional sequences of the glucocorticoid-inducible enhancer of the TAT gene does confer inducibility on the TAT promoter at this position (Jantzen *et al.*, 1987). Closer inspection of this 35-bp fragment revealed a CCAAT motif 6 bp upstream of the GRE (see Figure 3C). The CCAAT motif has been found in many class II promoters and its integrity is required for full promoter function (McKnight and Kingsbury, 1982; Jones *et al.*, 1985). To test the functional significance of the CCAAT motif we destroyed the CCAAT motif by a cluster of five point mutations generating plasmid ccaat/GRE-351TAT (Figure 3B). Indeed, mutation of the CCAAT-box entirely abolishes induction of CAT expression by dexamethasone, suggesting that a CCAAT-box binding protein functionally co-operates with the glucocorticoid receptor.

These results demonstrate that for induction from an upstream position a combination of elements, either two GREs or a GRE and a CCAAT motif, is required.

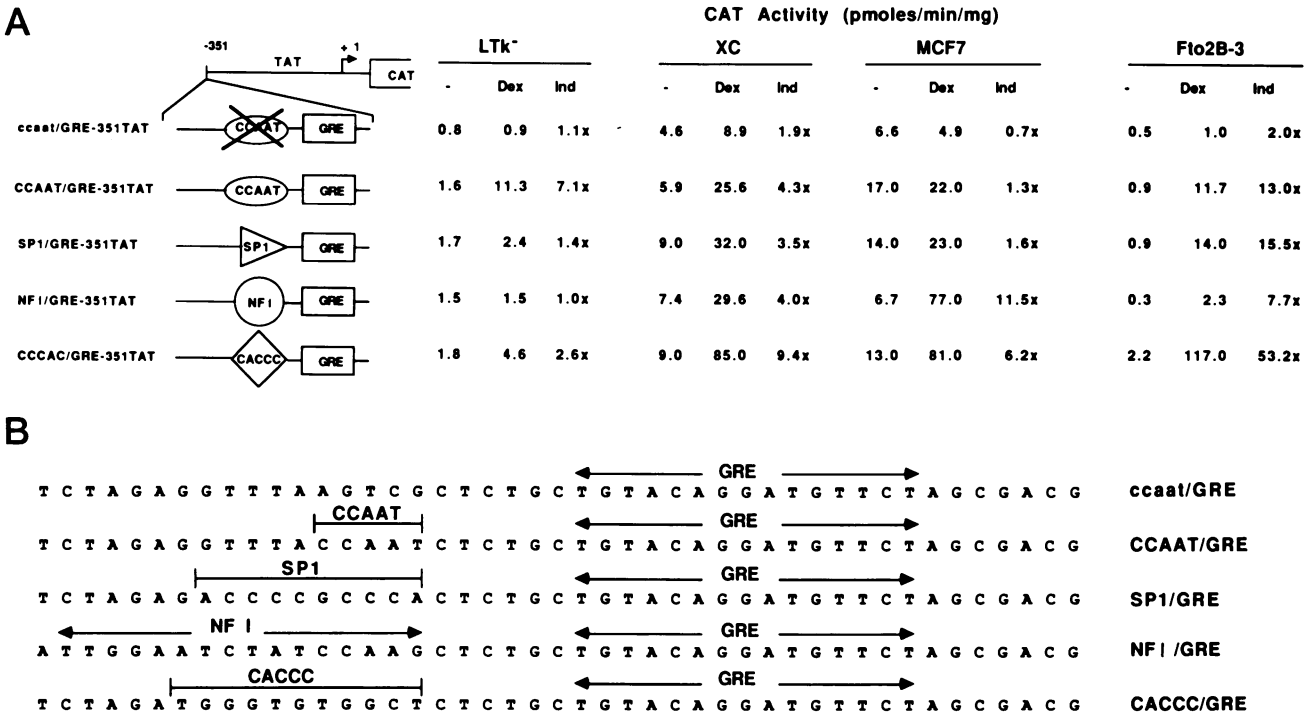

Fig. 4. Transcription factors co-operate with the glucocorticoid receptor. (**A**) Oligonucleotides containing a single GRE linked to either a CCAAT motif (CCAAT/GRE), the recognition sequence of transcription factor SP1 (SP1/GRE), the recognition sequence of nuclear factor I (NF I/GRE) or the CACCC element of the β-globin promoter (CACCC/GRE) were inserted 351 bp upstream of the tyrosine aminotransferase promoter (TAT). The plasmids were transfected into Ltk$^-$ cells, XC cells, MCF-7 cells or Fto2B-3 cells and inducibility of expression was monitored by determining CAT activity in transfected cells treated without (−) or with 10^{-7} M dexamethasone (Dex). Numbers represent pmol of chloramphenicol acetylated per min per mg of protein. Induction ratios (Ind) were calculated by dividing CAT activity determined in extracts from hormone-treated cells by the CAT activity in untreated control cells. One representative experiment with each cell line is shown. (**B**) Sequence of oligonucleotides inserted upstream of the TAT promoter.

Several transcription factors act synergistically with the glucocorticoid receptor in a cell-line-dependent manner

The observation that a GRE and a CCAAT motif functionally co-operate prompted us to ask whether this interdependence is restricted to the CCAAT motif or whether other motifs recognized by transcription factors also lead to hormone induction when combined with a GRE. We therefore tested constructs which contain upstream of the TAT promoter at −351 a 15-bp GRE together (see Figure 4B) with either the NF I recognition sequence from the LTR of MMTV (NF I/GRE-351TAT; Miksicek *et al.*, 1987), with the CACCC element of the β-globin promoter (CACCC/GRE-351TAT; Myers *et al.*, 1986) or the SP1 recognition sequence from the second distal signal of the Tk promoter (SP1/GRE-351TAT; McKnight *et al.*, 1984). These plasmids were transfected into different cell lines and analyzed for inducibility of CAT expression. The results are presented in Figure 4A. Combination of the GRE with the CACCC element led reproducibly to a 2- to 3-fold induction of CAT expression by dexamethasone in Ltk$^-$ cells compared to a 7- to 10-fold induction of CAT expression by construct CCAAT/GRE-351TAT. Neither an NF I site nor an SP1 site close to the GRE could restore hormone inducibility in Ltk$^-$ cells. In the rat fibroblast cell line XC a 9-fold induction of CAT expression could be achieved by combining the CACCC element with the GRE. Weaker but still significant effects on inducibility were conferred by a GRE linked to an NF I, CCAAT or SP1 motif upstream of the TAT promoter. In MCF-7 cells, only expression from plasmids NF I/GRE-351TAT (12-fold) and CACCC/GRE-351TAT (6-fold) were inducible by dexamethasone. A very strong induction of CAT expression (53-fold) from CACCC/GRE-351TAT was observed in the hepatoma cell line Fto2B-3. The SP1, CCAAT and NF I motif in combination with a GRE gave 15-fold, 13-fold and 7-fold induction respectively in Fto2B-3 cells. Insertion of these elements upsteam of the TAT promoter had no or only a weak effect on basal level expression in the absence of hormone (Figure 4A). Taken together, these results demonstrate that not only a CCAAT motif but also an NF I, CACCC or SP1 element can act synergistically with a GRE. The activity of these elements is apparently dependent on the cell line in which the constructs were analyzed, most likely reflecting the presence and/or the abundance of a particular factor in the tested cell line.

Discussion

We demonstrate here that a 15-bp ERE or a 15-bp GRE mediates induction of transcription by estradiol or dexamethasone respectively when positioned directly upstream of a TATA box. However, we found that a 15-bp GRE is not sufficient when positioned 351 bp upstream of the TAT gene promoter. At this promoter distant position either two copies of a GRE or combination of a GRE with a CCAAT motif are required for hormonal activation of the TAT promoter. The CCAAT motif can be replaced by motifs recognized by other transcription factors.

The observation that a single GRE or an ERE upstream of a TATA box confers hormone inducibility argues strongly in favor of the notion that the glucocorticoid receptor, as well as the estrogen receptor, do not require additional promoter elements besides the TATA box to stimulate faithful transcription in response to hormone. Similar results were obtained from investigating the function of regulatory elements of other genes. The heat shock response element (Pelham and Bienz, 1982), the metal response elements (Searle *et al.*, 1985; Stuart *et al.*, 1985), the serum response element (Mohun *et al.*, 1987) and the octamer motif (Wirth *et al.*, 1987) are functional when placed upstream of a TATA box in the absence of other regulatory elements. The fact that all these elements stimulate transcription when combined with a TATA box points to a mechanism of transcriptional stimulation common to all these factors.

The SREs increase the basal level of expression from constructs containing an SRE immediately upstream of the TATA box in the absence of hormone. Elevated basal expression is correlated with the number of GREs inserted upstream of the TATA box. This increase may be caused by interactions of the hormone-free receptor with the binding site in close proximity to the TATA box leading to stimulation of transcription. At least *in vitro*, receptor has been shown to bind in the absence of hormone (Bailey *et al.*, 1986; Wilman and Beato, 1986). However, the two GREs of the TAT gene are protected *in vivo* against methylation only after administration of glucocorticoids (Becker *et al.*, 1986). Alternatively, another transcription factor may recognize the 15-bp element. We are presently investigating these possibilities by band-shift and footprinting experiments as well as by a detailed mutational analysis.

GREs act synergistically regardless of whether two copies are inserted directly upstream of the TATA box or 351 bp upstream of the TAT promoter. Synergism between two GREs was originally observed in a mutational analysis of the glucocorticoid-inducible enhancer of the TAT gene (Jantzen *et al.*, 1987) and subsequently also shown for the EREs of the vitellogenin genes (Martinez *et al.*, 1987; Klein-Hitpass *et al.*, 1988b). Whereas one GRE is capable of mediating induction when in close proximity to the TATA box, duplication of the GRE is needed for action at a distance. Bienz and Pelham (1986) came to similar conclusions when studying the heat shock response elements of the heat shock protein 70 gene of *Drosophila melanogaster*. Two heat shock response elements are required for activation from a promoter distant position (Bienz and Pelham, 1986). This suggests that a more complex mechanism is involved in activation from further upstream. Interestingly, inserting more than two copies of the GRE does not lead to a further increase in inducibility, suggesting that sites required for mediation of receptor action are already saturated by positioning two GREs either immediately upstream of the TATA box or at a promoter-distant location.

Most interestingly, the second GRE required to allow induction from a promoter-distant position can be replaced by a CCAAT, NF I, CACCC or SP1 motif. The first hints for a functional interdependence of the glucocorticoid receptor with other transcription factors have been the mutational analysis of the MMTV promoter which revealed that inducibility by glucocorticoids is strongly dependent on the integrity of an NF I motif (Buetti and Diggelmann, 1986; Miksicek *et al.*, 1987) and the observation that within intact cells glucocorticoid induction leads to strong changes in dimethylsulfate reactivity at the two GREs and concomitantly at a CACCC element in immediate vicinity of the proximal GRE in the TAT gene (Becker *et al.*, 1986). Occupancy of this CACCC element is entirely dependent on hormone addition, indicating that receptors binding to the GRE is the event triggering the appearance of the hormone-dependent hypersensitive site (Becker *et al.*, 1984; Jantzen *et al.*, 1987). From these observations it is suggested that the hormone leads to recruitment of transcription factors rather than activation of prebound transcription factors. In agreement, exonuclease footprinting experiments suggest that glucocorticoid induction of transcription from the LTR promoter of MMTV is accompanied by the recruitment of transcription factors, presumably NF I and TATA box binding factor(s), at the promoter (Cordingley *et al.*, 1987). However, whether synergism is a direct consequence of protein–protein interactions as shown for the λ repressor (Hochschild and Ptashne, 1986) and heat shock transcription factor (Topol *et al.*, 1985; Cohen and Meselson, 1988) or whether synergism is based on a more indirect mechanism like removal of a nucleosome by the glucocorticoid receptor thereby allowing access of other transcription factors needs to be clarified. We are currently purifying the CCAAT box and CACCC box binding proteins from rat liver and are studying the nucleosomal organization before and after induction to distinguish between the two events. It is possible that both changes in chromatin structure and co-operative interaction of the receptor with transcription factors are involved and required for the steroid response. The fact that this synergism is not restricted to the CCAAT-binding protein points to a more general system of interactions of transcription regulatory proteins with the glucocorticoid receptor. The degree by which the CCAAT, NF I or CACCC elements co-operate with a GRE is strongly dependent on the cell type in which the constructs were tested, which most likely reflects the presence and/or abundance of a particular factor in a given cell line.

The very strong effect of the CACCC element in plasmid CACCC/GRE-351TAT on the induction ratio in the hepatoma cell line Fto2B-3 and the fact that a CACCC element resides in close proximity to GREs of the liver-specific tyrosine aminotransferase and tryptophan oxygenase genes suggests that the glucocorticoid response of a gene could be magnified in a cell-specific fashion by combination of a GRE with an element recognized by a cell-specific factor. A cell-specific protein (GT-IC) has been identified that binds to the CACCC-related element of the SV40 enhancer (GT-I motif) as well as to the CACCC element of the β-globin promoter with high affinity (Xiao *et al.*, 1987). The presence of GT-IC protein in a particular cell line correlates well with the activity of the GT-I motif of the SV40 enhancer when tested in gene transfer experiments (Namiyama *et al.*, 1987).

The fact that a single 15-bp GRE is not sufficient for induction from a distance suggests that multiple GREs or a combination of a GRE with other factor binding sites are required to constitute a hormone-inducible enhancer. In addition, clustering of modules in regulatory regions might thus be a means in evolution to circumvent the problem of spontaneously arising regulatory factor binding sites, a statistically frequent event given the short recognition sequences of regulatory proteins.

Materials and methods

Plasmid constructions

Cloning of plasmids was performed following standard procedures as described (Maniatis *et al.*, 1982). The oligonucleotides were synthesized with an Applied Biosystems 380A DNA synthesizer and purified by gel filtration and gel electrophoresis (only oligonucleotides >40 bases). All plasmids are derivatives of pBLCAT2 (Luckow and Schütz, 1987) or pTATKpn (Jantzen *et al.*, 1987). The plasmid −37Tk was obtained by replacing the 169-bp *Bam*HI/*Bgl*II fragment of pBLCAT2 (Luckow and Schütz, 1987) with the *Bam*HI/*Bgl*II fragment of the TK linker-scanner mutant LS-47/-37 (McKnight and Kingsbury, 1982). Oligonucleotides which contain combinations of a GRE and transcription factor binding sites (see Figure 4 for sequence) were inserted into the *Xba*I site of a derivative of pTATKpn (Jantzen *et al.*, 1987) in which the *Bam*HI site at the *Bam*HI/*Kpn*I fusion site had been reconstituted. Further details on the construction of the plasmids are available on request. All constructs in which synthetic sequences were cloned were sequenced.

Cell culture and transfection

LTk$^-$ (Jantzen *et al.*, 1987), XC (Svoboda, 1960), MCF-7 (Horwitz *et al.*, 1978) and Fto2B-3 (Killary and Fournier, 1984) cells were grown as described previously (Miksicek *et al.*, 1986, 1987; Jantzen *et al.*, 1987). Fto2B-3 cells are a clonal isolate of Fto2B (E.Schmid and G.Schütz, unpublished). Ltk$^-$ cells were transfected using the DEAE-Dextran protocol as described previously (Jantzen *et al.*, 1987) with the modification that cells were incubated with a DNA mix containing 2 μg/ml plasmid DNA, 200 μg/ml DEAE-Dextran (0.5 Md, Sigma) and 0.1 mM chloroquine-diphosphate in Dulbecco's modified Eagle's medium (DMEM) in a CO_2 incubator at 37°C for 5 h. MCF-7, XC and Fto2B-3 cells were transiently transfected by electroporation (Fromm *et al.*, 1985; Chu *et al.*, 1987) using a Biorad Gene Pulser in combination with a Biorad Capacitance extender. Cells were trypsinized, washed off the plates with charcoal-treated medium (Danesch *et al.*, 1987), washed once with PBS and resuspended in PBS at 10^7 cells/ml. Resuspended cells (0.8 ml) were transferred to tubes containing 20−40 μg of plasmid DNA in 100 μl TE (10 mM Tris−HCl, pH 7.5, 1 mM EDTA). The cell/DNA mix was transferred to cuvettes (Biorad, Art.-Nr. 160-285), exposed to a pulse (300 V, 960 μF) and transferred to a tube containing 2 ml of charcoal-treated medium. The whole procedure was performed at room temperature. After 5−10 min the cells were plated onto two 10-cm dishes containing 10 ml of charcoal-treated medium supplemented with 0.1% ethanol alone or hormone (10^{-8} M estradiol, 10^{-7} M dexamethasone in 0.1% ethanol). Cells transfected with ERE-37Tk were kept in medium lacking phenol red to exclude the estrogen effect of phenol red (Berthois *et al.*, 1986). After electroporation cells were grown for 24−30 h at 37°C in a CO_2 incubator before harvesting.

CAT assays and start site mapping of RNA

Extracts were prepared by three cycles of freezing and thawing of cells resuspended in 0.25 M Tris−HCl, pH 7.8 (XC, MCF-7) or 0.25 M Tris−HCl, pH 7.8; 5 mM EDTA (Fto2B-3). Protein concentration was determined by the Bradford assay (Bradford, 1976). Extracts from Fto2B-3 were heat treated (10 min, 60°C) to increase the sensitivity of the assay (Crabb and Dixon, 1987; M.Boshart, personal communication). CAT assays were done as described elsewhere (Gorman *et al.*, 1982) using 50−200 μg of protein. RNase protection experiments were carried out by hybridizing a uniformly labeled SP6 transcript (Miksicek *et al.*, 1986) overlapping the transcription start site of the fusion gene with 40 μg (4GRE-37Tk) or 100 μg (GRE-37Tk) of total RNA from MCF-7 cells transfected with GRE-37Tk or 4GRE-37Tk (for details of the procedure see Zinn *et al.*, 1983; Melton *et al.*, 1984; Strähle *et al.*, 1987).

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