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Synergistic action of glucocorticoid and estradiol responsive elements

(steroid hormone action/cooperativity)

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ABSTRACT **Modulation of gene expression by steroid hormones is mediated by receptor proteins that associate with regulatory elements of responsive genes upon binding the hormone ligand. The finding that two glucocorticoid responsive elements act cooperatively to stimulate transcription of the tyrosine aminotransferase gene prompted us to explore whether synergistic effects also occur when two different steroid hormone receptors are involved. A region of the chicken vitellogenin II gene that displays homologies to glucocorticoid and estradiol responsive elements was tested for its capability to confer estradiol and glucocorticoid inducibility to a heterologous promoter. When positioned immediately upstream of the thymidine kinase gene promoter, this element enhances expression by either steroid. Combination of both hormones results in a synergistic increase of transcription. Mutational analysis shows that sequences that show similarities of glucocorticoid and estradiol responsive elements are absolutely required for hormone induction. Analysis of the dose dependence of induction by both steroids demonstrates that half-maximal activity is observed at lower hormone concentrations when the other steroid is present in saturating amounts, which suggests that the synergistic induction observed with the combination of hormones is based on a functional interaction of the two hormone receptors.**

Control of gene expression involves specific interactions of transcription factors with regulatory DNA sequences. Although the mechanisms of activation of eukaryotic genes are poorly understood, it is assumed that cooperative action of transcription factors may be involved as has been demonstrated with prokaryotic systems (1, 2). Activation of transcription by steroid hormones is mediated by receptor proteins that bind to specific sequences of inducible genes after binding of the hormone (3–6). Transcription of the tyrosine aminotransferase gene is controlled by two glucocorticoid responsive elements (GRE) in a cooperative manner (5). This finding prompted us to explore whether synergistic effects also occur when two different steroid hormone receptors are involved.

The region between positions −627 and −586 of the chicken vitellogenin II gene contains binding sites for the glucocorticoid and estradiol receptors in close proximity (6, 7). The glucocorticoid receptor binding site contains a sequence that has 10 of 12 base pairs homologous to the consensus proposed for glucocorticoid responsive genes (5, 6). A 15-base-pair oligonucleotide derived from this consensus sequence has been shown to be sufficient for glucocorticoid responsiveness (8). A candidate for an estrogen responsive element (ERE) was first pointed out by sequence comparisons of the vitellogenin genes of frog and chicken (9). This sequence is contained in fragments of the frog vitellogenin A2 and B1 genes and in oligonucleotides that render the thymidine kinase (TK) promoter estrogen-inducible (10–14).

We analyzed whether the sequence element of the chicken vitellogenin II gene confers estradiol and glucocorticoid inducibility to a heterologous promoter and, if so, whether the effects of the inducers are synergistic or additive. We reasoned that cooperative activity, if based on facilitated interaction of two receptors, should be detected by analyzing the dose required for one hormone given alone or in combination with the other.

MATERIALS AND METHODS

Construction of Plasmids. Oligonucleotides were prepared with an Applied Biosystems 380 A synthesizer (Foster City, CA), purified as recommended by the manufacturer, annealed, and inserted into the *Hin*dIII site of pBLCAT2 (15). pchvit contains an oligonucleotide that was synthesized according to the region between positions −627 and −586 of the chicken vitellogenin gene. pcvb harbors mutations in the chicken vitellogenin GRE; pcvc, in the chicken vitellogenin ERE. In the mutant pcvd, the chicken vitellogenin GRE is replaced by a palindromic GRE (8). pcc1 contains the chicken GRE twice as a head-to-head dimer. In pcvb, pcvc, and pcvd, one of the two *Hin*dIII sites was destroyed to facilitate isolation of recombinant clones. In all constructs, the centers of the receptor binding sites have the same positions relative to the TK gene promoter. In pcc1, the distal element is shifted further upstream; the center-to-center distance in this construct is 25 base pairs.

Transfection Experiments. Human MCF7 cells were transfected with the DEAE-dextran transfection procedure as described (16, 17), with the modification that the plasmid concentration in the DEAE-dextran transfection mixture was reduced to 1 μg/μl. Cells were cultured for 48 hr in phenol red-free Dulbecco's modified Eagle's medium containing 10% (vol/vol) charcoal-stripped fetal calf serum in the absence of hormone or in the presence of 0.1 μM dexamethasone, 10 nM estradiol, or both hormones. After the cells were harvested, the extracts were assayed for chloramphenicol acetyltransferase (CAT) activity as described by Gorman *et al.* (18). To determine the dose dependence of induction, transfection was performed on 150-mm culture dishes. Fifteen hours after transfection, cells were trypsinized, split, and plated on 50-mm culture dishes. Hormones were added to the final concentrations as indicated in Fig. 3, and incubation was continued for 40 hr.

Electroporation was performed by using a Gene Pulser in combination with a capacitance extender from Bio-Rad (Richmond, CA). Plasmid DNA (20 μg) was added to 10^7 cells suspended in 0.8 ml of phosphate-buffered saline. Electroporation was performed at room temperature with a capac-

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Abbreviations: TK, thymidine kinase; GRE, glucocorticoid responsive element; ERE, estrogen responsive element; CAT, chloramphenicol acetyltransferase.

itance of 960 μF and 300 V. After the pulse, cells were split, plated onto 50-mm dishes, and cultured in the absence or presence of hormones. Cells were harvested and lysed 48 hr after electroporation, and extracts were tested for CAT activity.

Analysis of the Transcription Start Point. The plasmid pchvit was transfected into MCF7 human breast adenocarcinoma cells by the DEAE-dextran transfection procedure. RNA was prepared 24 hr later by the guanidinium isothiocyanate method (19). Aliquots (20 μg) of total RNA were hybridized to a uniformly labeled SP6 transcript overlapping the transcription start in pBLCAT2 and further processed as described (16).

RESULTS

An oligonucleotide was synthesized according to the sequence between positions −627 and −586 of the chicken vitellogenin II gene (20) and was inserted upstream of the TK gene promoter driving the expression of the bacterial CAT gene (Fig. 1*A*). This construct, referred to as pchvit, was transiently transfected into the human breast cancer cell line MCF7, which contains receptors for estrogens and glucocorticoid hormones (21). Hormone-dependent expression of this plasmid was analyzed by assaying CAT activity (18) and TK–CAT mRNA levels (16, 17).

The 41-base-pair-long fragment of the chicken vitellogenin gene conferred estradiol and glucocorticoid inducibility to the TK gene promoter (Fig. 1*B*). Expression of pchvit was stimulated by 0.1 μM dexamethasone, a synthetic glucocorticoid, and by 10 nM estradiol. Combination of both hormones resulted in an increase in CAT activity that was 3-fold higher than the level expected for a mere additive effect of the hormones. In several independent experiments, this cooperative effect was 2.5- to 5-fold.

To verify that the synergistic action of dexamethasone and estradiol is exerted at the level of transcription, we analyzed the amount of TK–CAT mRNA synthesized. An RNase protection experiment using an RNA probe specific for the TK–CAT fusion gene (17) showed that administration of dexamethasone, estradiol, and the combination of both hormones increases the amount of mRNA initiating from the correct start site in the TK gene promoter (Fig. 2). The pattern of induction was similar to that obtained by testing CAT enzymatic activity, again showing that estradiol and dexamethasone induce cooperatively the transcription of pchvit.

Since the centers of the two presumptive hormone responsive elements are separated by 23 base pairs, which would

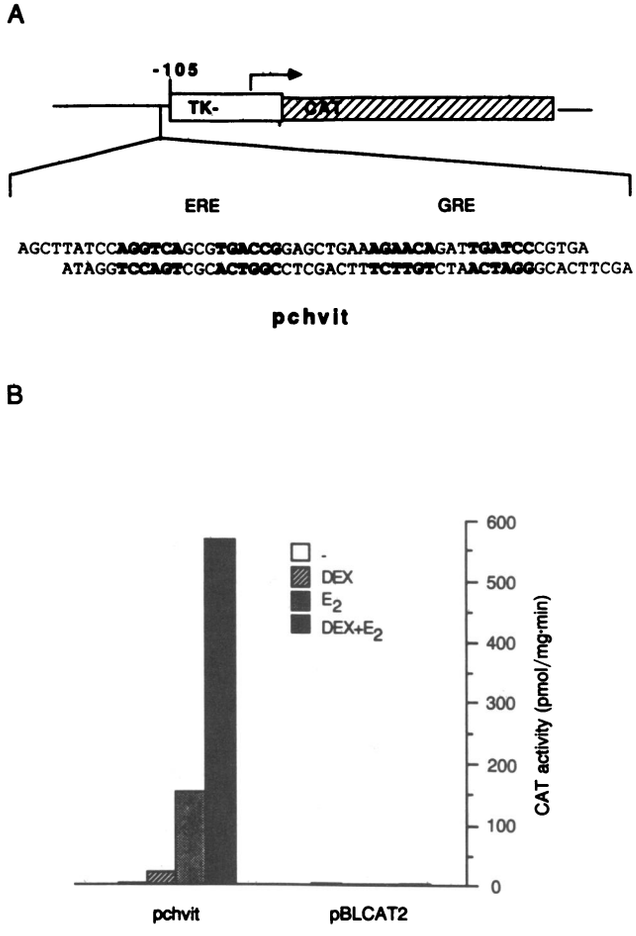

FIG. 1. Estradiol and dexamethasone induce transcription of the plasmid pchvit synergistically. (*A*) pchvit contains the sequence between positions −627 and −586 of the 5′ flanking region of the chicken vitellogenin II gene linked to the TK gene promoter driving the CAT gene in pBLCAT2 (15). Sequence similarities with previously defined EREs and GREs are indicated by boldface letters (5, 10). (*B*) Induction of CAT expression of pchvit by dexamethasone, estradiol, and the combination of both hormones. pchvit and pBLCAT2 were transiently transfected into MCF7 cells by the DEAE-dextran procedure. The results are shown in bars, each representing an average of duplicate transfections. Cells were cultured without hormone (−), with 0.1 μM dexamethasone (DEX), 10 nM estradiol (E_2), or the combination of both hormones (DEX + E_2). CAT activity is in pmol · mg^{-1} · min^{-1}.

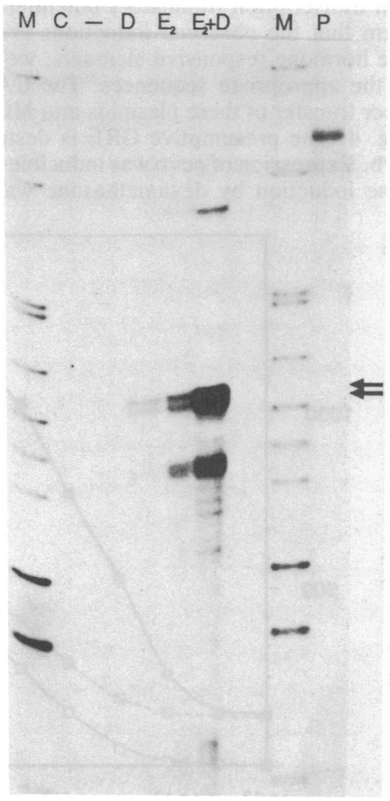

FIG. 2. Hormone inducibility of CAT mRNA levels parallels increased CAT activity. The 5′ ends of the transcripts derived from pchvit after transfection into MCF7 cells were mapped by the RNase protection procedure. Cells were treated without hormone (lane −) or with 0.1 μM dexamethasone (lane D), 10 nM estradiol (lane E_2), or the combination of both hormones (lane E_2 + D). (Other lanes: M, pBR322 cleaved with *Hpa* II and endlabeled; C, probe hybridized to 20 μg of RNA from untransfected cells; P, probe). Arrows point to the bands derived from correctly initiated TK–CAT transcripts (210 nucleotides).

position the receptors on the same side of the DNA helix, we investigated the possibility that the observed cooperativity is based on facilitated interaction of the respective receptors. Therefore, the dose dependence of induction by each steroid alone or in the presence of saturating concentrations of the second hormone was determined. We reasoned that an increase in the affinity of one receptor to its DNA target in the presence of the other would be reflected in an alteration of the dose–response curve. Half-maximal activity of the hormone combination should be observed at a lower concentration than with one hormone alone. The dose–response curves also should reveal whether the hormones act additively or synergistically. In the case of additive effects, the curves should be parallel. If the hormones act synergistically, the maximal activity for the hormone combination should be higher than expected for a mere additive effect. The results of this experiment are shown in Fig. 3. Half-maximal estradiol-dependent induction was reached at 15 pM; it was shifted to less than half of this concentration when saturating amounts of dexamethasone were present (Fig. 3). In a second experiment, half-maximal induction by estradiol was observed at 23 pM and was shifted in the presence of 0.1 μM dexamethasone to 6.2 pM. Dexamethasone induced transcription of pchvit half-maximally at 19 nM, whereas 8 nM was required when saturating doses of estradiol were present (Fig. 3*B*). In a second experiment, dexamethasone induced transcription half-maximally at 22 nM in the presence of estradiol at 7 nM.

The shifts in both dose–response curves suggest that either of the two receptors is able to influence the DNA-binding properties or transcription stimulatory potential of the other.

To confirm that the observed inductions are due to the presumptive hormone responsive elements, we constructed mutants in the appropriate sequences. The CAT activities obtained after transfer of these plasmids into MCF7 cells are given in Fig. 4. The presumptive GRE is destroyed in the plasmid pcvb. Expression of pcvb was inducible by estrogen; however, the induction by dexamethasone was abolished. The plasmid pcvc, which contains mutations in the presumptive ERE was inducible by dexamethasone but not by estradiol. Since induction of pchvit by glucocorticoids was lower than with previously tested GREs (8, 12), we were interested to see how a construct in which the chicken vitellogenin GRE is replaced by a more potent element (pcvd) would respond to the hormones. Transcription of pcvd was inducible by both hormones. Induction by dexamethasone was much greater than that found with the sequence derived from the chicken vitellogenin II gene. It is interesting that the combination of both steroids resulted in an additive but not synergistic increase of CAT activity from this plasmid containing a "strong" GRE. The constructs pcvb and pcvd showed additional interesting but puzzling effects. Upon mutation of the GRE in pcvb, the basal activity was increased about 20-fold. Whether we by chance created a positive element cannot be excluded at present, since we did not analyze additional mutants. Repression of basal and estradiol-stimulated activity by dexamethasone must not be a specific effect because the transcription of pBLCAT2 was also inhibited by dexamethasone (data not shown). The same effect was also observed by Martinez *et al.* (13). Furthermore, an interaction of the glucocorticoid receptor with the estrogen receptor binding site *in vivo* cannot be excluded, even though an interaction of the glucocorticoid receptor with the ERE could not be detected by *in vitro* cleavage inhibition pattern experiments (W. Schmid, personal communication). Construct pcvd showed a high level of expression before and after hormone addition. The increase in basal activity upon insertion of a strong GRE in close proximity to a strong ERE may be due to binding of receptors even in the absence of hormone.

Since we have previously observed that duplication of a GRE that allows strong induction did not result in a cooperative increase in expression (U.S., unpublished results), we wanted to probe for cooperativity with the duplicated weak GRE derived from the chicken vitellogenin gene. Expression

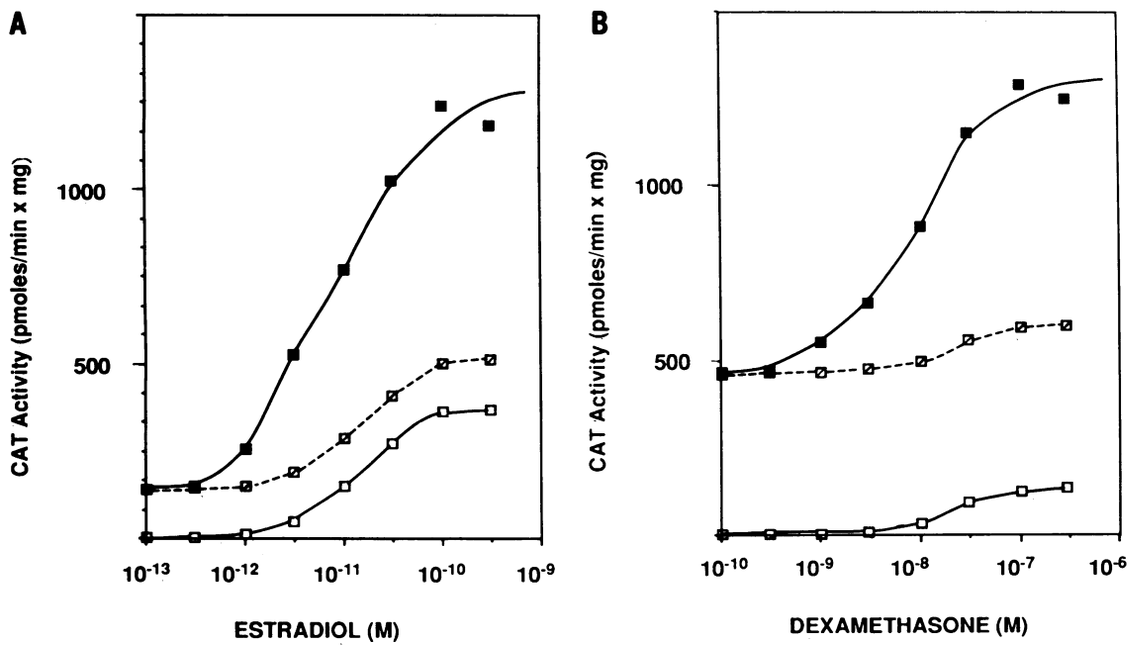

FIG. 3. Dose dependence of induction by estradiol and dexamethasone. MCF7 cells were transfected with pchvit DNA by the DEAE-dextran procedure, split 15 hr later, and then treated with the indicated concentrations of hormones for 40 hr. (*A*) Induction of CAT activity by increasing concentrations of estradiol (□) and by increasing concentrations of estradiol in the presence of 0.1 μM dexamethasone (■). The curve expected for an additive effect of the hormones was calculated by adding the value obtained by 0.1 μM dexamethasone to the values obtained with increasing concentrations of estradiol (□). (*B*) Effect of increasing concentrations of dexamethasone in the absence (□) and in the presence (■) of 10 nM estradiol on the transcription of pchvit. The curve expected for additive hormone induction was calculated as in *A* (□). Activity is expressed as pmol · mg^{-1} · min^{-1}.

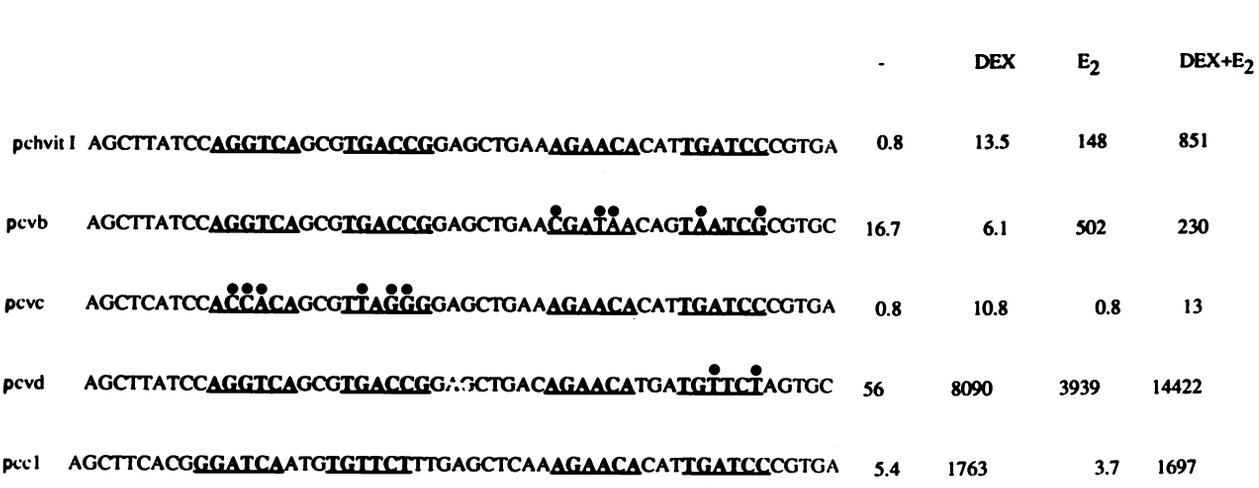

FIG. 4. Analysis of mutations in the steroid hormone responsive elements of the plasmid pchvit. Oligonucleotides harboring mutations (indicated by dots) in the presumptive GRE (pcvb) and ERE (pcvc) were tested in parallel with pchvit for their capability to mediate induction of CAT expression by dexamethasone and estradiol. In the mutant pcvd, the chicken vitellogenin GRE is replaced by a palindromic GRE (8). pcc1 contains the chicken vitellogenin GRE twice. Underlining indicates partial palindromic sequences. DNA transfer was performed by electroporation. CAT expression was measured in cells treated for 48 hr without hormone (column −), with 0.1 μM dexamethasone (DEX), with 10 μM estradiol (E_2), or with both hormones (DEX + E_2). CAT activity is in pmol · mg^{-1} · min^{-1}.

of pcc1, which contains the chicken vitellogenin GRE twice was strongly induced by dexamethasone (Fig. 4). The induction was much higher than one would expect for a mere additive effect (compare pchvit and pcvc with pcc1).

DISCUSSION

In conclusion, our results show that the region between positions −627 and −586 of the chicken vitellogenin II gene is able to confer inducibility by estradiol and dexamethasone to a heterologous promoter. Analysis of mutations in the hormone responsive elements and comparisons with previously defined GREs and EREs shows that the cis-acting elements conferring estradiol and dexamethasone responsiveness in the chicken vitellogenin II gene are the sequences GGTCAGCGTGACC and AGAACACATTGATCC, respectively. Combination of the two hormones stimulates transcription synergistically. However, cooperativity is only observed when the relatively weak GRE of the chicken vitellogenin II gene is linked to the ERE. The palindromic sequence AGAACATGATGTTCT, which is strongly inducible by dexamethasone, acts in combination with the ERE in a merely additive manner. Similarly, strong cooperativity is also seen when the GRE of the chicken vitellogenin gene is duplicated, but not with the duplicated GRE from the gene for tyrosine aminotransferase (U.S., unpublished results). The fact that in the chicken vitellogenin II gene the centers of these hormone responsive elements are separated by approximately two helical turns indicates that the bound proteins are on the same side of the DNA double helix; this is compatible with the idea that the hormone receptors are in direct contact. The shifts in the dose dependence observed when hormone combinations are analyzed support but do not prove the hypothesis that the hormone receptors interact. Whether the observed synergism can be explained by receptor–receptor interaction, which could facilitate formation or stabilization of the hormone receptor–DNA complexes, or by contact with other proteins in a cooperative manner is not yet clear. DNA-bound progesterone receptor molecules have been shown to interact when bound to adjacent binding sites (22). We have not been able to do cleavage protection studies to analyze whether the glucocorticoid and estradiol receptor bind cooperatively because the estradiol receptor is very unstable *in vitro*.

Recent work of Martinez *et al.* (13) and Klein-Hitpass *et al.* (23) shows that two EREs in the vitellogenin B1 and B2 genes act synergistically to achieve high estrogen inducibility. Furthermore, it was shown that spacers of 5–31 base pairs inserted between the elements did not affect synergism. Thus, it is unlikely that the cooperativity is dependent on a strict helix alignment of the response elements. We did not analyze whether different spacings of the GRE and ERE would affect the observed synergy. To get more insights into the mechanisms of cooperative activation of transcription by steroid hormone receptors, additional analysis with receptor mutations and *in vitro* binding studies have to be done.

The combination of the two hormone-dependent regulatory elements in the chicken vitellogenin gene suggests that glucocorticoids may intensify induction of transcription by estradiol. The synergism between these two steriod receptors may indicate that cooperative binding of two transcription regulatory proteins to their target sequences may be a general mechanism to achieve synergistic induction.

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