

# A DNA sequence of 15 base pairs is sufficient to mediate both glucocorticoid and progesterone induction of gene expression

(steroid hormone action/steroid receptor/transcription regulation)

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**ABSTRACT** To define the recognition sequence of the glucocorticoid receptor and its relationship with that of the progesterone receptor, oligonucleotides derived from the glucocorticoid response element of the tyrosine aminotransferase gene were tested upstream of a heterologous promoter for their capacity to mediate effects of these two steroids. We show that a 15-base-pair sequence with partial symmetry is sufficient to confer glucocorticoid inducibility on the promoter of the herpes simplex virus thymidine kinase gene. The same 15-base-pair sequence mediates induction by progesterone. Point mutations in the recognition sequence affect inducibility by glucocorticoids and progesterone similarly. Together with the strong conservation of the sequence of the DNA-binding domain of the two receptors, these data suggest that both proteins recognize a sequence that is similar, if not the same.

The effect of steroid hormones on specific gene transcription is mediated by receptors to which the hormones bind with high selectivity and affinity. Activation of transcription results from binding of the hormone-receptor complex to specific sequences of inducible genes (1, 2). Surprisingly, it has been observed that fragments of the chicken lysozyme gene and of the long terminal repeat of mouse mammary tumor virus that confer glucocorticoid inducibility also allow progesterone induction (3, 4). The glucocorticoid and progesterone receptors bind to similar sequences within these fragments, suggesting that the binding sites may be closely related, but distinct (5, 6). Both types of receptors also bind to partially overlapping sequences in the rabbit uteroglobin gene (7, 8). These sequences, however, have not been functionally characterized. To define the sequence requirements for specific binding of the glucocorticoid receptor and to determine the degree to which this sequence overlaps with the recognition sequence for the progesterone receptor, a minimal glucocorticoid response element (GRE) 15 base pairs (bp) in length was defined. This 15-bp sequence is part of the GRE region of the tyrosine aminotransferase (TAT) gene protected against DNase I digestion by the purified glucocorticoid receptor (9). We demonstrate here that the same 15-mer which confers responsiveness to glucocorticoids also mediates progesterone induction. Since point mutations in this 15-bp sequence affect inducibility by both steroids similarly, we suggest that the recognition sequences of the glucocorticoid and progesterone receptors are similar, if not identical.

## MATERIALS AND METHODS

**Plasmid Constructions.** Oligonucleotides were prepared with an Applied Biosystems 380A DNA synthesizer and purified as recommended by the manufacturer. Annealing of

the complementary strands, which generated *Bam*HI or *Xba*I cohesive ends, and cloning upstream of the herpes simplex virus thymidine kinase (TK) promoter in pBLCAT2 (10) were done by standard procedures (11). pBLCAT2 contains the complete TK promoter (-105 to +62) linked to the chloramphenicol acetyltransferase (CAT) coding region (12). In the plasmids pGRE22, pGREPal, and pGRE16, the binding sites were synthesized as 27-bp oligonucleotides that were inserted into the *Bam*HI site of the polylinker immediately upstream of the TK promoter in pBLCAT2. In pGRE15A and pGRE15B and in the plasmids containing single or double point mutations, the binding sites were synthesized as 21-bp oligonucleotides and cloned into the *Xba*I site of the polylinker. By inserting the 21-bp oligonucleotides into the *Xba*I site and the 27-bp oligonucleotides into the *Bam*HI site of the polylinker, the centers of the pentadecamer recognition sequences were put into the same position relative to the TK promoter in all the plasmid constructs.

**Cell Culture and Transfection.** MCF7 and T-47D human breast cancer cells were cultured as described (4). Transfection of MCF7 and T-47D cells by the DEAE-dextran procedure was performed as described (4), with two modifications: (i) the plasmid concentration in the DEAE-dextran mixture was reduced to 1  $\mu$ g/ml and (ii) after treatment with chloroquine diphosphate, the cells were kept in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% charcoal-stripped fetal bovine serum. After 14-18 hr the medium was replaced by serum-free DMEM and the incubation was continued for another 12-14 hr before harvesting. Immediately after chloroquine diphosphate treatment, hormone alone or hormone and antagonist were administered in ethanol solution to a final concentration of 0.1% ethanol. Unless otherwise stated, 0.1  $\mu$ M dexamethasone or 0.1  $\mu$ M R 5020 (a synthetic gestagen; ref. 4) was applied (see also legends to figures). Control plates contained 0.1% ethanol.

**CAT Assays.** CAT assays were performed as described (12). After exposure to x-ray film, radioactive spots on the thin-layer chromatogram were cut out for quantitation by liquid scintillation counting. All numbers given, with the exception of the data for the dose-response curves, represent the average of CAT activity (pmol per min per mg of protein) from two parallel transfections.

**RNA 5'-End Mapping.** Total RNA was prepared by extraction with guanidinium thiocyanate followed by centrifugation through a CsCl cushion (13). Transcription start sites were determined by the RNase-protection procedure, using uniformly labeled antisense RNA probes (4, 14, 15). Twenty-microgram aliquots of total cellular RNA were hybridized with the probe and further processed as described elsewhere (4), with the modification that the concentration of RNase T1 was reduced to 20 units/ml.

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Abbreviations: GRE, glucocorticoid-response element; CAT, chloramphenicol acetyltransferase; TAT, tyrosine aminotransferase; TK, thymidine kinase.

## RESULTS

**The Glucocorticoid Receptor Binding Site Is Sufficient to Confer Inducibility on a Transcriptionally Competent Promoter.** To define the sequence required to constitute a GRE, we placed oligonucleotides modeled after a receptor binding site of the TAT gene (9) upstream of the TK promoter. The promoter activity was followed in recombinant plasmids in which this promoter was fused to the bacterial CAT gene (12). Expression of these plasmids was studied after transient introduction into the human breast cancer cell lines MCF7 and T-47D, which have glucocorticoid and progesterone receptors (16), respectively. Glucocorticoid- and progesterone-dependent expression of these plasmids was analyzed by following CAT enzymatic activity (Figs. 1, 3, and 4) and by measuring TK-CAT mRNA levels (Fig. 2) with a RNase-protection assay (14, 15).

As seen in Fig. 1A, a 22-bp fragment derived from the TAT

gene is sufficient to confer glucocorticoid inducibility on the TK promoter in MCF7 cells. This oligonucleotide corresponds to the sequence that is protected by the purified glucocorticoid receptor against digestion by DNase I and displays strong similarity with the GREs of other glucocorticoid-inducible genes (9). A characteristic of this sequence is a 15-bp motif with partial dyad symmetry (indicated by arrows in Fig. 1). We also tested whether the symmetrical portion of this sequence alone is capable of rendering the TK promoter glucocorticoid-inducible. The 22-bp oligonucleotide in which most of the nucleotides outside the partial palindrome have been modified (pGRE16) maintains the capacity to make the TK promoter glucocorticoid-inducible (Fig. 1A). To test whether the GRE consensus sequence (9) itself is sufficient for glucocorticoid control, a 15-bp-long sequence was examined. The oligomer comprising only 15 bp of GREII (pGRE15A) of the TAT gene leads to a strong

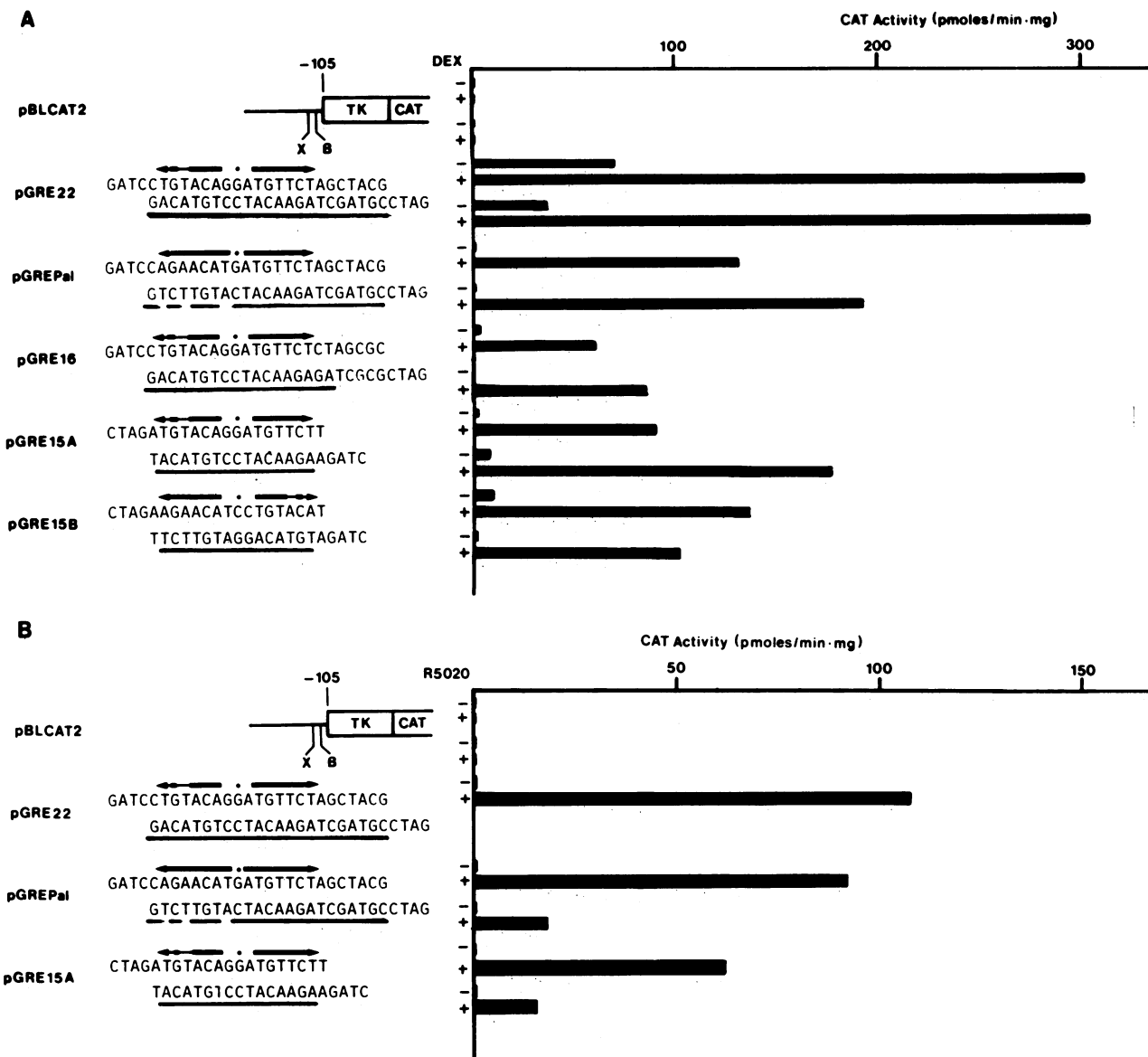


FIG. 1. Induction of CAT expression of TK-CAT constructs containing GREs by dexamethasone and R 5020 in MCF7 (A) and T-47D (B) cells. The oligonucleotides were inserted upstream of the TK-CAT fusion gene in pBLCAT2. After transient introduction into MCF7 and T-47D by the DEAE-dextran procedure, CAT activity was measured without hormone added (-) or in the presence (+) of 0.1  $\mu$ M dexamethasone (A) or 0.1  $\mu$ M R 5020 (B). The results of two independent experiments are shown. Each bar represents the average of CAT activity obtained from duplicate transfections. Sequences underlined are identical to GREII of the TAT gene. Numbers in the plasmid designations indicate the number of base pairs identical to the GREII of TAT. pGREPal contains an oligonucleotide with a perfect palindromic sequence. Arrows overlining the oligonucleotides indicate the palindromic structure of the response elements; thick parts indicate those base pairs that fit into the palindromic symmetry.

increase in dexamethasone-dependent expression of CAT activity and, furthermore, is equally effective in the inverse orientation (pGRE15B) (Fig. 1A). The induction of expression of these plasmids is prevented by administration of the antiglucocorticoid RU 486 (17, 18) (data not shown). The antihormone alone does not affect the basal level seen after introduction of pGRE22, indicating that this level of expression is not due to residual glucocorticoids. These experiments show that a 15-bp sequence with partial symmetry is sufficient to confer glucocorticoid regulation when linked in cis to a normally unresponsive promoter.

A characteristic of the defined response element is its partial symmetry. To test whether a perfectly symmetrical oligonucleotide maintains the capacity to mediate glucocorticoid induction, a synthetic oligonucleotide in which the partially symmetrical sequence has been changed to a perfect palindrome was placed upstream of the TK promoter. This sequence with perfect symmetry (pGREPal) also confers strong glucocorticoid inducibility (Fig. 1A).

**A Single 15-bp Sequence Mediates Both Glucocorticoid and Progesterone Induction.** Gene-transfer experiments using chimeric plasmids containing 5' flanking sequences of the chicken lysozyme gene and the long terminal repeat of mouse mammary tumor virus, combined with *in vitro* binding studies with the purified glucocorticoid and progesterone receptors, have indicated that the effect of both steroids appears to be mediated by overlapping sequences (3–6). Having established that a 15-bp sequence is sufficient for glucocorticoid responsiveness (Fig. 1A), we asked whether the same element can also function as a progesterone response element and how, if at all, the recognition sequences for these two classes of steroid receptors differ. We therefore tested TK–CAT recombinants carrying various GREs for their ability to allow induction by progesterone in T-47D cells (Fig. 1B). Expression of the same plasmids that show regulation by glucocorticoids is strongly dependent on the presence of R 5020, a synthetic gestagen. These observations imply that the sequences required for induction by glucocorticoids and progesterone are very similar or possibly identical (see below).

To verify that the hormone-dependent increases in expression of the recombinant plasmids are due to an increase in correctly initiated transcripts, RNA from transfected MCF7 and T-47D cells was analyzed by a RNase-protection assay (14, 15). The protected fragments expected for correctly initiated RNA and for transcripts originating upstream of the initiation site are indicated in Fig. 2A. Fig. 2B shows an analysis of the expression of a TK–CAT recombinant carrying the 15-bp sequence (pGRE15A) in both cell lines. In the presence of steroids, the plasmid gives rise to a strong increase in correctly initiated TK–CAT mRNA, indicating that the observed steroid-dependent increases in CAT activity are based on faithful transcription initiation.

Fig. 3 shows the steroid-concentration dependence of expression of the TK–CAT recombinant carrying the 15-bp oligonucleotide. Induction by dexamethasone or R 5020 takes place within a range of concentrations expected from the affinity of the receptors for these two ligands. Both the dexamethasone- and the R 5020-dependent expression are abolished by the presence of a 10-fold excess of the antiglucocorticoid and antigestagen RU 486 (17, 18). Interestingly, estradiol has no effect on expression in MCF7 cells, even though this cell line possesses sufficient amounts of the estradiol receptor to allow induction of estrogen-responsive marker genes to occur (10, 16). Plasmids in which the hexanucleotide sequence TGTTCT in both halves of the palindrome has been changed to TGACCT allow induction by estradiol, but not by dexamethasone, suggesting that estradiol regulation requires a related but distinct sequence (24). The absence of any effect by the other steroids—in particu-

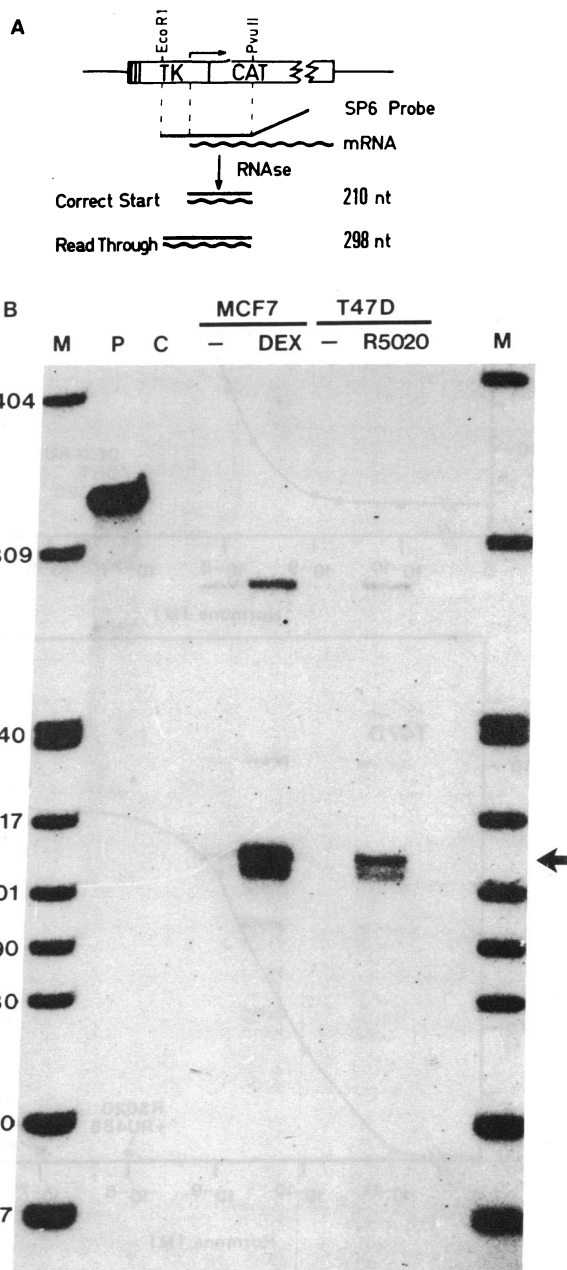


FIG. 2. Mapping of 5' end transcripts derived from pGRE15A transfected into MCF7 and T-47D cells. (A) The probe used for determining the transcription start site by the RNase-protection procedure and the fragments resulting from correctly initiated and read-through transcripts are shown. Lengths are given in nucleotides (nt). (B) Total RNA was isolated from MCF7 and T-47D cells transfected with pGRE15A, and 20- $\mu$ g aliquots were analyzed as described in *Materials and Methods*. Cells were treated with 0.1  $\mu$ M dexamethasone (DEX) or 0.1  $\mu$ M R 5020, respectively. The 210-nt fragment corresponding to the correct initiation site is indicated by the arrow. Lanes M: size markers (pBR322 digested with *Hpa* II and end-labeled with  $^{32}$ P). Lane P: probe. Lane C: MCF7 RNA from a mock transfection.

lar, by progesterone in MCF7 cells and by dexamethasone in T-47D cells—is probably due to the low level of the corresponding receptors (refs. 4 and 16; W. Ankenbauer, personal communication).

**Base Substitutions Affect Induction by Glucocorticoids and Gestagens Similarly.** The data presented thus far demonstrate that an oligomer of 15 bp with partial palindromic structure is sufficient to give glucocorticoid as well as progesterone

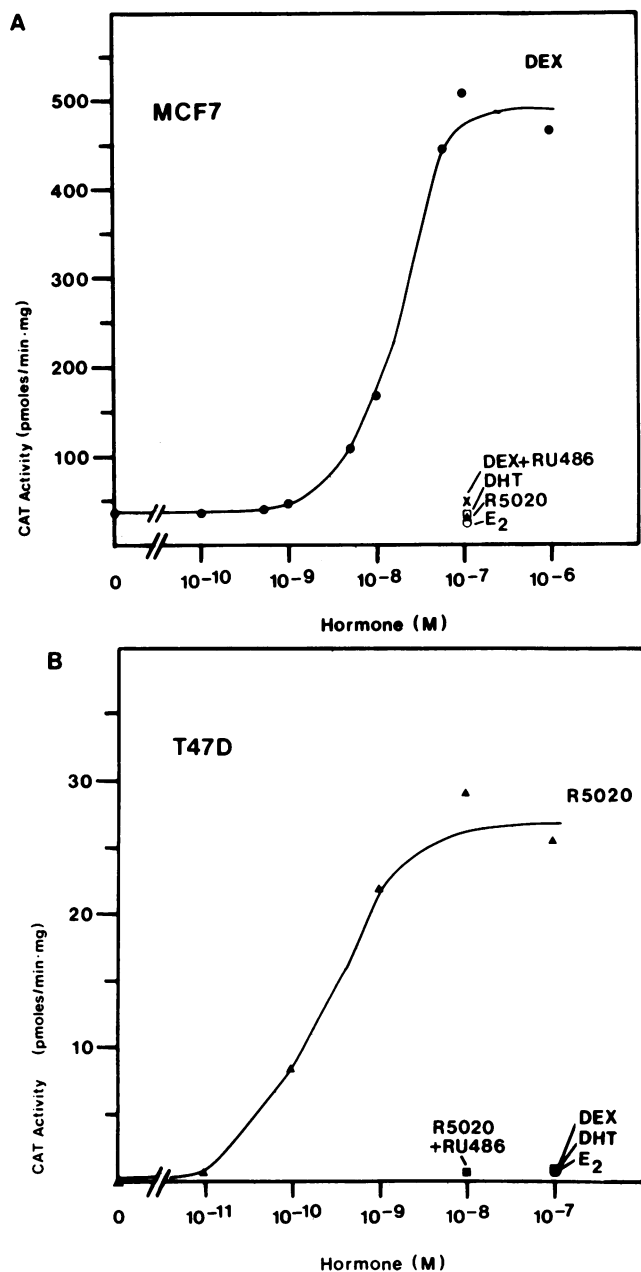


FIG. 3. Dependence of CAT expression on steroid concentration. Cultures of MCF7 (A) or T-47D (B) cells were transfected in parallel with the plasmid pGRE15A, and the induction of CAT expression by various concentrations of dexamethasone (DEX) or R 5020 was monitored. The concentrations of the steroids administered are indicated: ●, DEX; ▲, R 5020; ○, estradiol (E<sub>2</sub>); □, dihydrotestosterone (DHT). A 10-fold excess of RU 486 abolished induction of CAT expression both by dexamethasone (×, 0.1 μM dexamethasone plus 1 μM RU 486) in MCF7 cells (A) and by R 5020 (■, 0.01 μM R 5020 plus 0.1 μM RU 486) in T-47D cells (B).

induction. To obtain additional support for this surprising observation, the 15-bp oligonucleotide was modified at several sites, some of which are known to be contacted by the glucocorticoid receptor (19, 20). The effect of these single or double substitutions upon inducibility by dexamethasone and R 5020 was tested as before. The results (Fig. 4) show that all three modified oligonucleotides have lost, or are strongly impaired in, the ability to confer both glucocorticoid and progesterone inducibility on the TK promoter. These findings suggest that contact sites on both halves of the recognition sequence are required for steroid-induced transcription and that, within the limits of the mutational analysis performed up

to this point, there are no discernible differences between the sequences that govern specific binding by the progesterone receptor and those that govern specific binding by the glucocorticoid receptor.

## DISCUSSION

The data presented above define a partially palindromic sequence of 15 bp as sufficient for transcriptional activation of the TK promoter by glucocorticoids and progesterone. It is surprising that a single response element is sufficient to allow strong control of TK-promoter expression by steroids, since two or more GREs occur in most glucocorticoid-inducible genes analyzed thus far (1, 9, 19). As shown by linker-scanning mutagenesis, several elements in the long terminal repeat of mouse mammary tumor virus contribute to hormonal control (21). Two elements located 2.5 kbp upstream of the TAT gene act in a cooperative fashion, so that both elements together elicit a stronger response than the sum of responses of each element alone (9). Thus, multiple copies of the 15-bp element might be required to allow fine tuning of a physiologically adequate response. We have identified several base substitutions in either half of the binding site that abolish inducibility by glucocorticoids; significantly, the same mutations diminish induction in response to progesterone. A rotationally symmetrical oligonucleotide in which the hexanucleotide TGTTCT is precisely repeated confers both glucocorticoid and progesterone inducibility on the TK promoter. These observations and the finding that the hexanucleotide TGTTCT by itself (22) is not a functional GRE are compatible with the interpretation that this 2-fold symmetry may be important for recognition of this sequence by these receptors. In analogy to many prokaryotic DNA-binding proteins, this suggests, but certainly does not prove, that the receptors may bind to their recognition sites as dimers.

It is interesting that some of the recombinant plasmids, in particular pGRE22, show an increase in activity in the absence of glucocorticoids when compared to pBLCAT2. This increase in basal activity upon insertion of a steroid response element is not, however, seen in T-47D cells. Whether this reflects differences in DNA-binding affinity of the ligand-free glucocorticoid and progesterone receptors in the particular sequence environment of pGRE22 or differences in interaction of the receptors with other factors in MCF7 and T-47D cells is unknown. The generation in this particular plasmid of a constitutively enhancing element that is recognized in MCF7 cells, but not in T-47D cells, cannot be excluded at present.

Considering the vastly different physiological effects of glucocorticoids and progesterone, it is surprising that a single oligonucleotide of 15 bp confers inducibility both by glucocorticoids and by progesterone and that base substitutions affect the action of both steroids similarly. However, this observation finds its correspondence in the fact that the DNA-binding domains of the glucocorticoid and progesterone receptors are 91% identical at the amino acid sequence level (ref. 23 and references therein). The structural similarities of the two proteins imply a similar interaction with the 15-bp recognition sequence. The inducibility conferred by the 15-mer derived from the TAT GRE and the perfectly palindromic element is comparable for glucocorticoids and progesterone, suggesting that the glucocorticoid and progesterone receptor have similar affinities for these response elements. However, since T-47D cells have an elevated level of progesterone receptor, it is possible that the weaker affinity of the progesterone receptor for the GRE is overcome by the higher concentration of the progesterone receptor.

The strong conservation both of the protein region that recognizes DNA and of the DNA sequence itself implies that

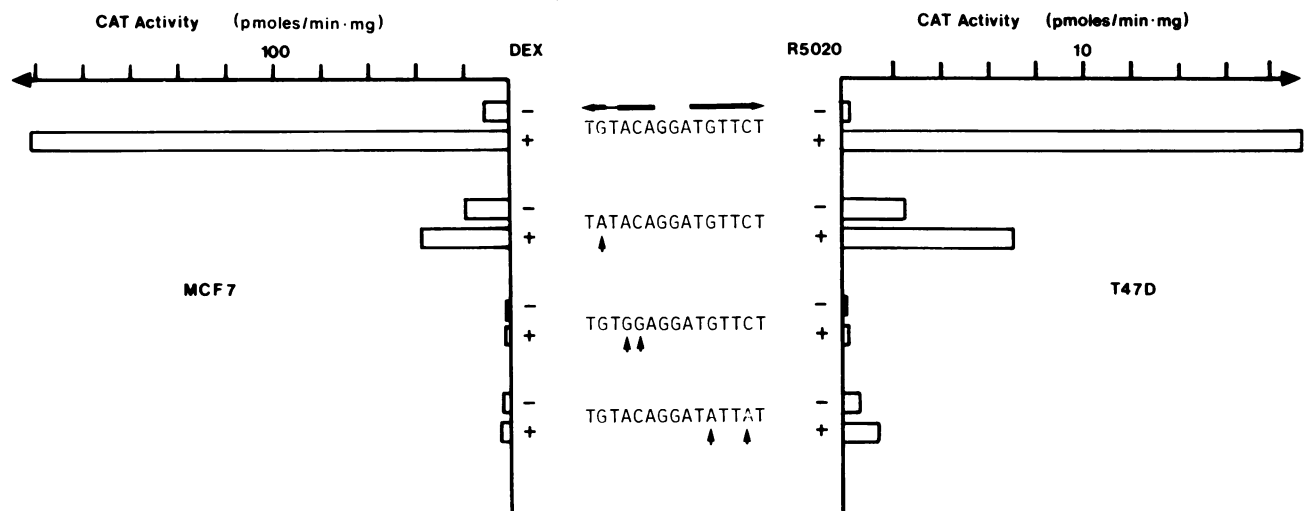


FIG. 4. Mutational analysis of the 15-bp steroid response element. Oligonucleotides harboring single or double point mutations in the pentadecameric sequence were cloned in front of the TK-CAT fusion gene and tested, in parallel with the wild-type construct pGRE15A, in MCF7 and T-47D cells for their ability to mediate induction of CAT expression by dexamethasone (DEX) and R 5020, respectively. The sequence of the wild-type 15-mer is outlined by two arrows indicating the palindromic structure (thick parts of arrows identify bases fitting the palindromic symmetry). Below the wild-type 15-mer, the sequences of the mutants are shown. The mutated bases are indicated by small arrows pointing upward.

other parts of the receptors and/or additional proteins are of importance to evoke steroid-specific effects. Responses of target cells to specific steroids thus must be determined, at least in part, by the specific receptors any given cell expresses, by the level of receptor expression, and by the response to which the gene has been committed.

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