12. IDE (200 μg) was purified at 4°C from 12 liters of packed human red blood cells in batches of 1.6 liters each, as follows. Cells were centrifuged at 1,500*g*, washed with cold phosphate-buffered saline (PBS), lysed in cold distilled-deionized water containing 10 m*M* EDTA and 1 m*M* phenylmethylsulfonyl fluoride (PMSF), and centrifuged at 13,000*g* for 45 min. The supernatant was filtered, mixed with 550 ml of DEAE A-50 ion-exchange resin (equilibrated in 10 m*M* sodium phosphate, *p*H 7.4), and stirred for 90 min. The supernatant was removed by filtration and, the resin was washed with 1 liter of cold sodium phosphate, *p*H 7.4. Resin was eluted by stirring in 550 ml of 100 m*M* sodium phosphate, *p*H 7.4, and 0.5*M* KCl at 4°C. The supernatant was filtered and loaded onto the immunoaffinity column (5 ml). The column was washed with 50 m*M* Hepes, *p*H 7.6, 150 m*M* NaCl [Hepes-buffered saline (HBS)] and 50 m*M* Hepes, *p*H 7.6, and 500 m*M* NaCl. Elution was with 6*M* urea in HBS. Twenty 2-ml fractions were dialyzed against 3 liters of HBS overnight and assayed by electrophoresis through a 7.5% SDS-polyacrylamide gel. Peak fractions were pooled and concentrated through Centricon 30 (Amicon) filtration membranes. The IDE peak was then applied to preparative SDS-polyacrylamide gels, and the 110-kD band was selectively eluted. Attempts at NH_2-terminal sequencing were negative; therefore the protein was exhaustively digested with trypsin. The resulting tryptic peptides were isolated by HPLC and sequenced by automated Edman degradation on a gas-phase sequencer [Y. Yarden *et al.*, *Nature* **323**, 226 (1986)]. One of the tryptic peptides (WGEIIS QQYNFD, in single-letter code) was used to design the 35-base oligonucleotide probe IDE-5 (TCAAAGTT A/G TACTG CTGG G/C A/T GATGAT T/C TC G/C CCCCA) with the use of the codon usage table of Lathe (*13*).
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Many Transcription Factors Interact Synergistically with Steroid Receptors

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Progesterone (PRE) or glucocorticoid receptor (GRE) DNA binding sites are often found clustered with binding sites for other transcription factors. Individual protein binding sites were tested without the influence of adjacent factors by analyzing isolated combinations of several transcription factor binding sites with PREs or GREs. All show strong synergistic effects on steroid induction. The degree of synergism is inversely related to the strength of the GRE. Thus, a steroid responsive unit can be composed of several modules that, if positioned correctly, act synergistically.

ONE OF THE FIRST SYSTEMS IN which regulatory sequences and the corresponding trans-acting factors were characterized was that of steroid-inducible gene expression (*1*). Active steroid-receptor complexes bind with high affinity to sites in the chromatin and, by an unknown process, regulate transcription of specific genes. Progesterone or glucocorticoid receptor binding sites (PRE/GRE) in several cases are tightly clustered with other regulatory sequences: upstream of the chicken lysozyme gene (*2, 3*) and the human metallothionein II_A gene (*4*) and in the long terminal repeats (LTRs) of murine mammary tumor virus (MMTV) (*5, 6*), murine sarcoma virus (MSV) (7), and Moloney murine leukemia virus (Mo-MuLV) (*8*). A computer search revealed many more similarities to transcription factor binding sites in the direct vicinity of PREs and GREs (*9*). We thus hypothesized that the DNA-bound progesterone or glucocorticoid receptor can cooperate with other transcription factors. This idea is supported by the fact that some receptor binding sites seem to be nonfunctional when analyzed individually (*5, 10–12*). Because single interactions between pairs of bound transcription factors cannot be analyzed within a complex cluster of binding sites, we have tested combinations of several transcription factor binding sites with binding sites for the progesterone or

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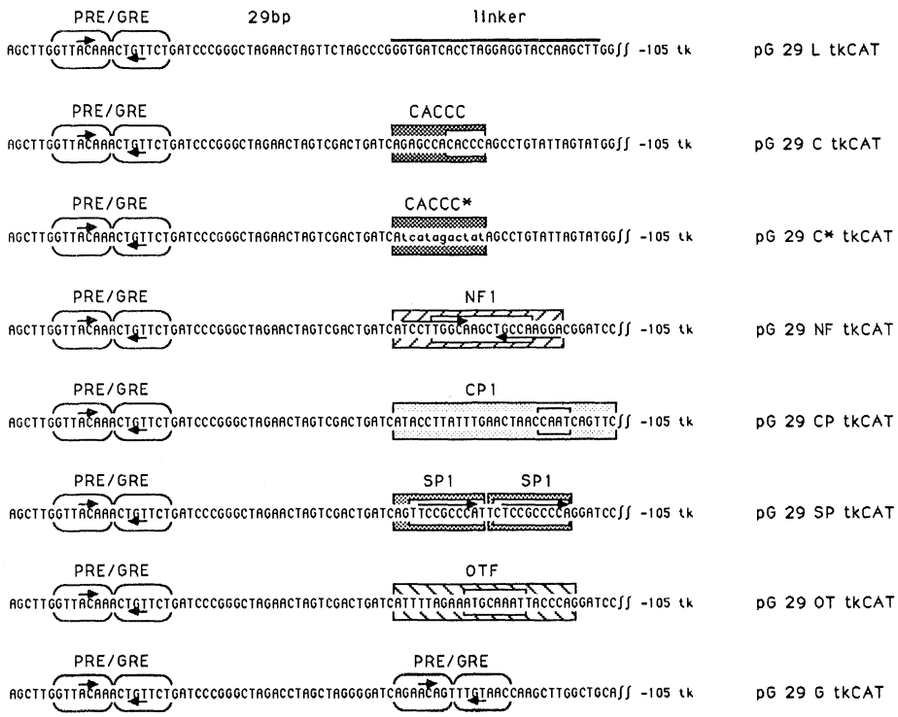

Fig. 1. Different combinations of PRE/GRE transcription factor binding sites were cloned in front of the fusion gene tkCAT. Binding sites for CACCC-factor, NF1, CP1, Sp1, and OTF were synthesized and cloned at a distance of 29 bp to the PRE/GRE. As a control the mutagenized CACCC-box (CACCC*) and an unrelated linker sequence were also cloned at the same distance.

Table 1. Specificity of the progesterone response. pC29GtkCAT (*29*) was transfected into T47D cells and incubated with the indicated steroid hormones. Experiments were done in duplicate.

Steroid	CAT activity [pmol mg^{-1} min^{-1} (± SD)]
$10^{-10}M$ R5020	10 ± 2
$10^{-9}M$ R5020	60 ± 4
$10^{-9}M$ R5020 + $10^{-9}M$ RU38486	<0.5
$10^{-9}M$ R5020 + $5 \times 10^{-6}M$ Cyproterone	90 ± 19
$10^{-9}M$ dexamethasone	<0.5
$10^{-7}M$ dexamethasone	<0.5

the glucocorticoid receptor in the same well-defined sequence environment. We show that a progesterone and a glucocorticoid receptor binding site act synergistically in combination with binding sites for CAAT-box factor, Sp1, octamer transcription factor, CACCC-box factor, nuclear factor I (NF1), and a second progesterone or glucocorticoid receptor.

We cloned and tested several factor binding sites ligated to a chloramphenicol acetyltransferase (tkCAT) gene that was driven by the thymidine kinase promoter (*5*) in a vector devoid of fortuitous binding sites in the vicinity of the inserted sequence (*12*). The factor binding sites were the NF1 binding site functional consensus sequence (*5*), the CAAT box [recognized by the CCAAT–binding protein (CP1)] as found in the MSV-LTR (*13*), the Sp1 box I and II of the SV40 enhancer (*14*), the CACCC-box as found in the tryptophan oxygenase (TO) gene (*10*), the binding site for the octamer transcription factor (OTF) as found in the immunoglobulin heavy chain (IgH) enhancer (*15*), and the glucocorticoid receptor binding site at position −186 to −170 of the MMTV-LTR (*16*). We cloned these sequences individually or in combination with the MMTV-GRE upstream of tkCAT (Fig. 1). As a control we also cloned the mutated inactive version of the CACCC sequence (*17*; CACCC*; Fig. 1), as well as an unrelated linker sequence.

We transfected the set of DNA recombinants (Fig. 1) into HeLa cells, because all of the transcription factors to be tested are present in this cell line. The glucocorticoid response was increased by cotransfection of pRSVcGR, a cDNA for the rat glucocorticoid receptor (*18*). The transient transfections were done in triplicate and repeated at least twice. Ribonuclease (RNase) maps of the transcripts agreed with the measured CAT activities (*19*). The MMTV-GRE alone, or in combination with the mutated CACCC-box (CACCC*), had little transcriptional activity when induced with the synthetic glucocorticoid dexamethasone, whereas when all other GRE combinations (with binding sites for NF1, CP1, Sp1, OTF, with the CACCC-box, or a second GRE) were induced with dexamethasone, there was a strong synergistic response. Basal transcriptional activities of all constructs and the induced activities of plasmids without a GRE (C, NF, CP, SP, and OT) were similar. When we transfected these recombinant plasmids into mouse L cell fibroblasts, we obtained similar results (*20*). Glucocorticoid and progesterone responsive elements are at least highly overlapping (*2, 21*) and GRE sequences can mediate a progesterone response (*22*), so we used the recombinant plasmids described above to determine whether the cooperativity can also be seen with the progesterone receptor. As recipient cells we chose the human T47D cell line, which contains high amounts of progesterone receptor, and used the synthetic progesterone R5020. The control plasmids carrying the MMTV-GRE (which acts in this case as a PRE) alone, or in combination with a mutated CACCC-box (G29C*), showed identical basal and induced CAT activities (Fig. 2B). All other combinations

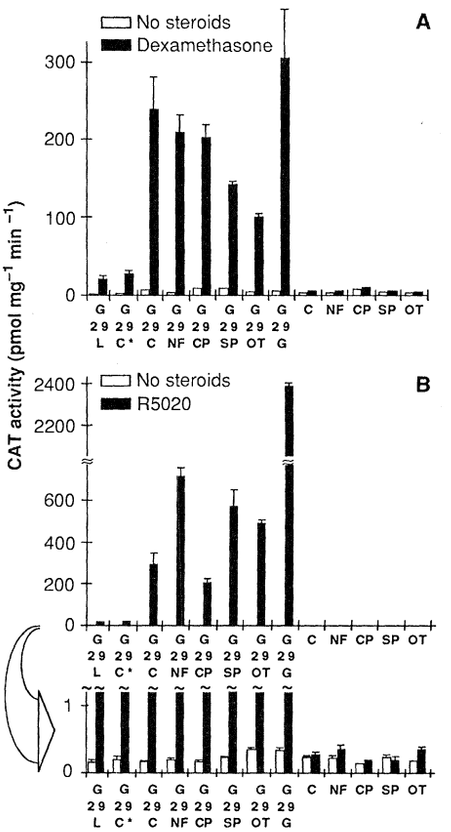

Fig. 2. Progesterone or glucocorticoid induced activity of several binding site combinations. Different plasmids with or without PRE/GRE were transfected into recipient cells and analyzed for CAT activity upon incubation with either dexamethasone or the progestin R5020. The plasmids used were derivatives of pG29LtkCAT (G29L) (see Fig. 1), and the corresponding deletions without a PRE/GRE (C, NF, CP, SP, OT). (**A**) Transfection into HeLa cells (*30*) and transient expression with $5 \times 10^{-7}M$ dexamethasone (filled bars) or without steroids (open bars). All test plasmids (1.5 p*M*) were cotransfected with 2 μg of pRSVcGR (*18*). Cells were harvested and tested for CAT activity 40 hours after transfection (*31*). All transfections were done in two triplets (standard deviations are indicated): one triplet was treated with the steroid. Complete series of transfections were done at least twice. Cell extracts were heat treated (10 min, 60°C) and replenished with acetyl coenzyme A for long enzyme incubations (>4 hours). (**B**) Transfection into T47D cells and transient expression with $10^{-9}M$ R5020 (filled bars) or without steroids (open bars). To visualize the basal activity and the activity of the PRE-free constructs, the low CAT activity range is shown with a magnified scale (lower part of Figure). T47D cells were transfected as described (*32*).

Table 2. The distance effect of the CACCC-box and PRE on progesterone induction. Linkers of different lengths were placed between the CACCC-box and the MMTV-PRE (*29*). Upon transfection of these plasmids into T47D cells, triplicate cultures were incubated without or with $10^{-9}M$ R5020. CAT activities were determined and the induction calculated for each of the transfected plasmids.

Plasmids	CAT activity		Induction*
	−R5020 [pmol mg^{-1} min^{-1} (± SD)]	+R5020 [pmol mg^{-1} min^{-1} (± SD)]	
pC29LtkCAT	0.34 ± 0.09	0.58 ± 0.06	2
pC8GtkCAT	0.33 ± 0.03	138 ± 17	416
pC12GtkCAT	0.56 ± 0.08	396 ± 31	713
pC15GtkCAT	0.79 ± 0.08	614 ± 126	874
pC18GtkCAT	0.98 ± 0.13	491 ± 73	500
pC20GtkCAT	0.78 ± 0.21	354 ± 46	454
pC23GtkCAT	0.71 ± 0.04	276 ± 31	387
pC26GtkCAT	0.56 ± 0.01	361 ± 18	645
pC29GtkCAT	0.62 ± 0.15	768 ± 38	1246
pC30GtkCAT	1.01 ± 0.06	699 ± 19	694
pC33GtkCAT	0.64 ± 0.03	189 ± 50	297
pC36GtkCAT	0.81 ± 0.18	437 ± 54	537
pC39GtkCAT	0.76 ± 0.12	402 ± 27	530

*Induction was calculated as the ratio of the induced to the uninduced values.

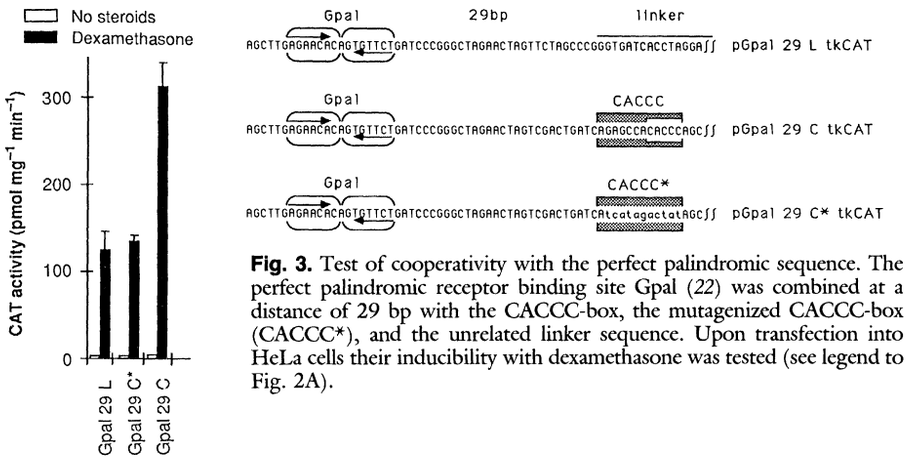

Fig. 3. Test of cooperativity with the perfect palindromic sequence. The perfect palindromic receptor binding site Gpal (*22*) was combined at a distance of 29 bp with the CACCC-box, the mutagenized CACCC-box (CACCC*), and the unrelated linker sequence. Upon transfection into HeLa cells their inducibility with dexamethasone was tested (see legend to Fig. 2A).

with binding sites for NF1, CP1, Sp1, OTF, a wild-type CACCC-box, or another GRE/PRE showed synergism. The highest induction (several thousand times) occurred with the two GRE/PRE elements (G29G). The basal activities of all constructs tested did not vary significantly, and the plasmids containing transcription factor binding sites without a GRE/PRE were not inducible (Fig. 2B, expanded scale). Measurement of basal activities is crucial to distinguish additive effects of two elements from cooperative effects. To prove that we were analyzing the progesterone receptor we tested several steroids and found that the effect of 1 n*M* R5020 was completely abolished by the antiprogesterone RU38486 (*23*), whereas the antitestosterone (Cyproterone) had no significant effect, and 10^{-7}*M* dexamethasone did not induce these cells (Table 1).

To analyze a possible interaction between the bound factors, we inserted segments of 8 to 39 bp between the progesterone receptor binding site and the CACCC-box. We chose this order of sequence elements (CACCC, PRE, tk-promoter) to keep the distance of PRE to the promoter constant, changing only the position of the CACCC-box. The size of the inserted space affected transcriptional activities; the most effective spacing was 15 bp, 29 bp, and 36/39 bp (Table 2). This is different from the induction pattern of the glucocorticoid receptor–CACCC combination (*12*). In particular, 20-bp spacing resulted in little cooperativity when induced with progesterone (Table 2), but it showed a peak of cooperativity when induced by dexamethasone (*12*). Such a difference is probably caused by a difference in size, binding geometries, or both of the two receptors. The distance dependence is not absolute, and can best be explained by flexibility of the proteins, looping out of the spacing DNA, or both. Such a loop mechanism is proposed for several transcription systems (*24*).

We determined whether the observed synergism depends on the type of GRE sequence used. The proximal GRE of the TO gene is a very weak GRE that requires the presence of a CACCC-box (*10*). The GRE of MMTV confers a measurable inducibility to the tk promoter when tested without other transcription factors (Fig. 2A). This induction can be further increased by the presence of CACCC or other sequences recognized by transcription factors (Fig. 2A). We tested a perfect palindromic GRE sequence [Gpal, (*22*)] that has a strong inducibility without other transcription factors (Fig. 3). As expected, the addition of a mutant CACCC-box (CACCC*) did not change the induction when compared to the Gpal activities, but a wild-type CACCC-box did lead to a further increase (Fig. 3). The magnitude of this increase was not as great as is seen with the MMTV-GRE (compare G29C* and G29C in Fig. 2A with Gpal29C* and Gpal29C in Fig. 3). Therefore, the inducibility of weak GREs can be greatly increased, whereas activity of strong GREs can only be weakly increased, by another transcription factor.

In this study we tested combinations of DNA binding sites for steroid receptors with the DNA binding sites of different transcription factors in identical DNA sequence environmnents. We found cooperativity of the progesterone and glucocorticoid receptors with binding sites for CP1, Sp1, OTF, NF1, the CACCC-box, or a second steroid receptor binding site. This may explain the clustered presence of these sequences in the vicinity of steroid inducible genes. Our finding that every transcription factor tested cooperates with steroid receptors is in agreement with the demonstration (*25*) that the yeast GAL4 protein can cooperate with the glucocorticoid receptor in mammalian cells. This synergism suggests either a direct protein-protein interaction of these factors or the involvement of an additional protein that binds to both factors and that may also contact other proteins of the transcriptional machinery. The DNA binding and nonbinding factors can cooperate: the chicken ovalbumin DNA binding factor COUP must interact with the S300-II factor for efficient in vitro transcription (*26*). Cooperativity between steroid receptors in induction has been shown in some cases (*5, 6, 11, 27*), as well as receptor-receptor binding (*28*). We use the term "steroid hormone responsive unit" (HRU; *27*) for such a modular structure consisting of one or several hormone responsive elements combined with other transcription factor elements.

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