MOLECULAR AND CELLULAR BIOLOGY, June 1991, p. 3247–3258
0270-7306/91/063247-12$02.00/0

Vol. 11, No. 6

Functional Interaction of Hybrid Response Elements with Wild-Type and Mutant Steroid Hormone Receptors

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Received 20 September 1990/Accepted 25 March 1991

Steroid hormone receptors can be divided into two subfamilies according to the structure of their DNA binding domains and the nucleotide sequences which they recognize. The glucocorticoid receptor and the progesterone receptor (PR) recognize an imperfect palindrome (glucocorticoid responsive element/progesterone responsive element [GRE/PRE]) with the conserved half-sequence TGTYCY, whereas the estrogen receptor (ER) recognizes a palindrome (estrogen responsive element) with the half-sequence TGACC. A series of symmetric and asymmetric variants of these hormone responsive elements (HREs) have been tested for receptor binding and for the ability to mediate induction in vivo. High-resolution analysis demonstrates that the overall number and distribution of contacts with the N-7 position of guanines and with the phosphate backbone of various HREs are quite similar for PR and ER. However, PR and glucocorticoid receptor, but not ER, are able to contact the 5'-methyl group of thymines found in position 3 of HREs, as shown by potassium permanganate interference. The ER mutant HE84, which contains a single amino acid exchange, Glu-203 to Gly, in the knuckle of ER, creates a promiscuous ER that is able to bind to GRE/PREs by contacting this thymine. Elements with the sequence GGTCAcagTGTYCT that represent hybrids between an estrogen response element and a GRE/PRE respond to estrogens, glucocorticoids, and progestins in vivo and bind all three wild-type receptors in vitro. These hybrid HREs could serve to confer promiscuous gene regulation.

Steroid hormone receptors mediate induction of gene expression by their corresponding hormones through an interaction with DNA sequences located near the regulated promoter. These hormone responsive elements (HREs) have an imperfect palindromic structure and can be classified into two main subgroups: the glucocorticoid responsive element/progesterone responsive element (GRE/PRE)-like group that mediates induction by glucocorticoids, progestins, androgens, and mineralocorticoids, and the estrogen responsive element (ERE)-like group that mediates induction by estrogens, thyroid hormones, vitamin D_3, and retinoic acid (2). The classical sequence of a GRE/PRE is 5'-GGTACAnnnTG TYCT-3', with the right half of the palindrome better conserved among different GRE/PREs (2). The sequence of a classical ERE is the perfect palindrome 5'-GGTCAnnnTG ACC-3' (25–30, 35), but we have recently identified a functional ERE with the sequence 5'-GGTCAccaTGCCC-3' (52) in the promoter of the rabbit uteroglobin gene. An ERE with the half-palindrome sequence 5'-TGGCC-3' has been detected in the promoter of the estrogen-inducible gene pS2 in MCF-7 cells (6, 40). Therefore, the conserved half-site motifs of the two HRE subgroups share positions 1, 2, and 5, underlying the similarities in the DNA recognition mechanisms of the receptors. The main difference between the two subgroups of regulatory elements resides in position 3: always a T in the GRE/PRE and usually an A in the ERE. In position 4, a C can be found in both elements. The difference here resides in the observed tolerance of the GRE/PREs that can accommodate either a C or a T in this position. In fact, a T is preferred and found in 65% of the GRE/PREs (4), and a direct comparison of the perfect palindromes with either C or T at position 4 in gene transfer experiments revealed a higher activity for the T-containing element (29).

The DNA binding domain of the steroid hormone receptors consists of some 70 amino acids, including 10 conserved cysteines that have the potential to form two so-called zinc fingers (13, 14, 49). A comparison of the amino acid sequences of the receptors for different steroid hormones, particularly in the DNA binding domain, enables their classification into two subfamilies (2). The prototypes of these two subfamilies are the glucocorticoid receptor (GR) and the estrogen receptor (ER). Finger swapping experiments among different receptors suggested that, although both zinc fingers are needed for DNA binding, the first zinc finger is more relevant for discrimination between GRE/PRE and ERE (19). More detailed mutational analyses localized the residues relevant for this discrimination to the "knuckle" of the first finger and more precisely to the residues in between the second pair of cysteines and to a residue two positions further downstream (11, 34, 55). Interestingly, a single Gly-to-Glu change in the same knuckle of human (h) GR (amino acid 439) produces a receptor mutant which is able to recognize both a GRE/PRE and an ERE (55), although the corresponding mutant in the hER (Glu-203 to Gly) confers only a weak inducibility of the mouse mammary tumor virus (MMTV) promoter (34). Substitutions in the GR second finger by the corresponding amino acids of ER also reduce the ability of the receptor to distinguish between a GRE/PRE and an ERE (11). Thus, it is likely that both fingers of the GR contribute to the specificity of transcriptional activation from the HRE, indicating that interactions between the two fingers may be crucial in providing the ultimate structure required for appropriate DNA recognition (5, 11). As with receptor proteins, GRE/PREs and EREs can be converted easily into one another (29, 35), but cross-reactivity between

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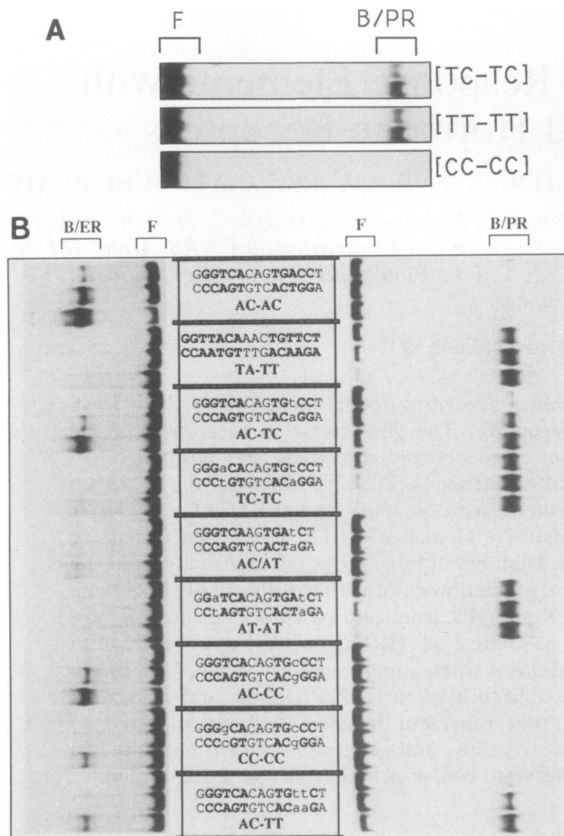
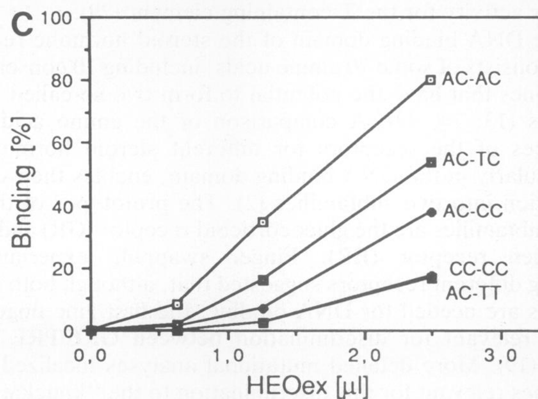
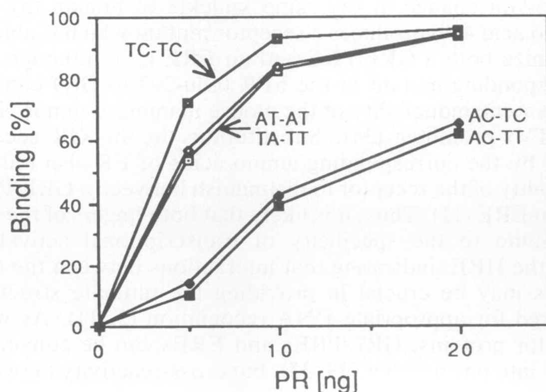






a particular HRE and members of both receptor subfamilies has not been observed.

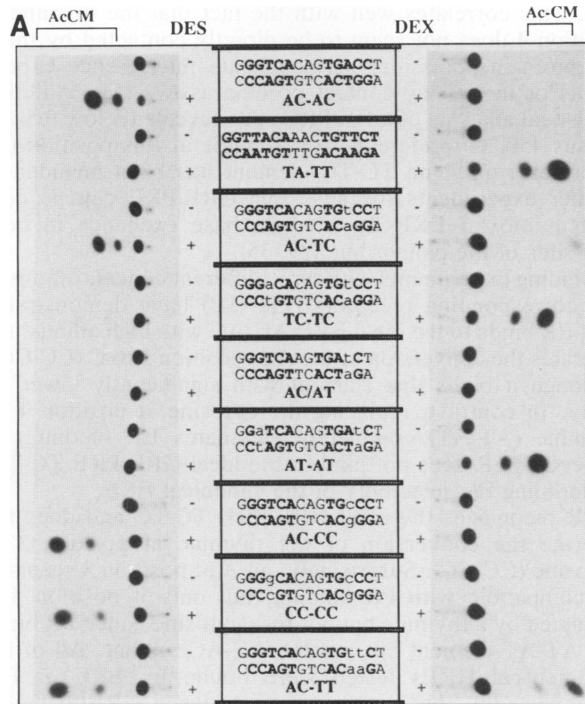
Here we report experiments with a series of symmetrical and asymmetrical variants of GRE/PREs and EREs, as well as combinations thereof, that define more precisely the differences and common features between these two subgroups of regulatory elements. For DNA binding studies we have used the band retardation assay combined with methylation interference techniques to detect contacts with the N7 position of guanine residues. In addition, we have employed a recently developed potassium permanganate ($KMnO_4$) interference method that enables detection of intimate contacts of the receptors with thymines (53). Details of the interactions between the receptors and the HREs have been investigated further by determining the positions of ethylated phosphates that interfere with the formation of specific protein-DNA complexes (50). In this way we have defined the positions of the various HREs that are directly relevant for binding and transactivation by the different steroid receptors and have designed hybrid HREs that are able to respond to estrogens, glucocorticoids, and progestins. In addition to the wild-type receptor we have also employed a mutant estrogen receptor (HE84 [34]) that exhibits a single amino acid exchange, Glu-203 to Gly, in between the two cysteines in the carboxy-terminal end of the first zinc finger. This mutant, HE84, recognizes an ERE and a GRE/PRE (as previously suggested [34]), contacts a thymine at position 3 of a GRE/PRE half-palindrome (like GR and progesterone receptor [PR]), and binds to hybrid HREs with a higher affinity than wild-type receptors.

MATERIALS AND METHODS

Receptors. PR was isolated from uterine cytosol of rabbits treated with estrogen for 1 week and was purified as previously described (56).

Plasmids and DNA fragments. Fragments used for chemical modification interference and band shift experiments were obtained as restriction fragments from cloned synthetic oligonucleotides. The paired synthetic oligonucleotide 5′-AGCTTAGTTTATTGGGTCACAGTGACCTTACCACA AGGATGG (only the 42-nucleotide-long upper strand is shown; the ERE is underlined) containing the consensus ERE (AC-AC) was subcloned into the *Hin*dIII and *Sal*I sites of pTK.CAT.3 (9). Direct cloning of synthetic oligonucleo-

FIG. 1. Sequences and gel mobility shift assay of consensus and mutated HREs with ER and PR. (A and B) The labeled DNA probes (141 bp) used for the binding experiments were obtained from the plasmids, which contain a single copy of the corresponding cloned oligonucleotides, after digestion with *Bam*HI, 5′ end labeling, and redigestion with *Pvu*II. The nucleotide sequence from each wild-type or mutated HRE is boxed in the middle of panel B, with the designation corresponding to positions 3 and 4 on the left palindromic half (lower strand) followed by positions 3 and 4 on the right half (upper strand; for numbering of positions, see Fig. 4). Exchanged nucleotides are indicated in small type, whereas the nucleotides in bold type match the remaining consensus motifs. Three different amounts of the purified PR or WCE of COS-7 cells with the overexpressed ER were used for binding assays in a total volume of 10 µl. First concentration, 0.6 µl of WCE or 5 ng of PR; second concentration, 1.2 µl of WCE or 10 ng of PR; third concentration, 2.5 µl of WCE or 20 ng of PR. F, free DNA; B/ER and B/PR, probe bound to ER and PR, respectively. In experiment A, only the first two concentrations of PR were used. (C) Graphic representation of the above results.



B

CONSTRUCT	ACTIVATION (%)		
	DES	DEX	R5020
AC-AC	100	<0.5	<0.5
TA-TT	<0.5	90.8	100
AC-TC	40.5	4.8	<0.5
TC-TC	<0.5	84	63
AC/AT	<0.5	<0.5	<0.5
AT-AT	<0.5	100	78
AC-CC	41.3	<0.5	<0.5
CC-CC	29.3	<0.5	<0.5
AC-TT	23	17	22

FIG. 2. Sequences and phenotypes of different HREs in gene transfer experiments. (A) Reporter plasmids contain the CAT gene driven by the thymidine kinase promoter (−105/+57) containing at the 5′ end two direct repeated copies of the HREs shown in the boxes in the middle of the figure. The enhancement activities of the different HREs were assayed in HeLa cells transiently cotransfected with the reporter plasmids and the effector plasmids which express GR (RSV-GR [39]), ER (HEO [31]), or PR (hPRO [CAT reaction not shown]) together with the RSV-βGAL expression vector and propagated in the absence (−) or presence (+) of 10^{-7} M dexamethasone (DEX), 10^{-8} M DES, or 10^{-8} M R5020, respectively. AcCM, acetylated chloramphenicol. (B) Quantitation of the enhancement activity of different HREs on ER, GR, and PR action assayed in HeLa cells. The assays were performed in triplicate and standard error was <10%. Average CAT activities were normalized to the activity conferred by each receptor on the maximal induced construct, which was assigned a value of 100.

tides to create the plasmids containing the mutated HREs was also performed. The exchanged nucleotides are indicated in Fig. 1A and 2A in lowercase type within the sequence. A double-stranded oligonucleotide was also synthesized to correspond to the promoter distal binding site from MMTV (−190/−160), 5′-AGCTTAGTTTATGGTTACAAACTGTTCTTAAAACAAGGATGG, and cloned into the same sites of the polylinker of pTK.CAT.3 as the ERE oligonucleotides. Plasmids with the subcloned oligonucleotides were digested with *Hin*dIII and treated with Klenow polymerase, and the 195-bp insert excised with *Bgl*II was subcloned into the same vector after the *Sal*I (blunt-ended)-*Bgl*II fragment was removed in order to produce two directly repeated copies of the corresponding oligonucleotide fragments.

Preparation of WCE. COS-7 cells were grown in Dulbecco modified Eagle medium (GIBCO) with 5% fetal calf serum (FCS; GIBCO) which had been stripped of hormones by a charcoal treatment (12) in 90-mm tissue culture dishes and transfected with 10 μg of the expression vector HEO or HE84 (34) with DEAE-dextran (9). After 48 h, 10^{-8} M diethystilbestrol (DES) was added, and cells were harvested after 1 h. The cellular pellet was dissolved in 200 μl of extraction buffer containing 20 mM Tris (pH 7.5), 2 mM dithiothreitol, 400 mM NaCl, 20% glycerol, and a mixture of the protease inhibitors phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (0.5 μg/ml), and aprotinin (1 μg/ml). After sonication with a Branson Cell Disruptor (small tip; output control 3), the extracts were cleared by centrifugation in an Eppendorf centrifuge (47). Whole-cell extracts (WCE) were frozen and stored at −80°C.

DNA binding assays. For mobility shift DNA binding assays, purified PR and GR or WCE from COS-7 cells was incubated in a 10- to 20-μl reaction mixture containing 15 mM Tris (pH 7.5), 10% glycerol, 90 mM NaCl, 1 mM dithiothreitol, 3 mg of bovine serum albumin (BSA) per ml, and 0.1 ng (10,000 cpm) of end-labeled DNA fragments. In the incubation assays with PR and GR 100 ng of calf thymus DNA was added, and in the binding reactions with WCE 500 ng of poly(dI-dC) was used as a nonspecific competitor.

Free DNA and DNA-protein complexes were resolved on a 4% polyacrylamide gel as described by Schauer et al. (43). The relative intensities of the individual bands were analyzed by densitometric scanning of the films with a Desaga Quick-Scan densitometer coupled to a Hewlett-Packard recorder-intergrator.

Cell culture and DNA transfection. HeLa cells were propagated in Dulbecco modified Eagle medium supplemented with 5% FCS and transferred to RPMI 1640 (GIBCO) with 5% charcoal-stripped FCS in the absence of phenol red for transfections. Cells were transfected at 50 to 70% confluency on 60-mm petri dishes with a total of 3.5 μg of DNA by using the DEAE-dextran method. In each case 1 μg of the β-galactosidase expression vector RSV-βGAL, 1.5 μg of the receptor expression vector (pRSV-GR [39] or hPRO or HEO [31]) and 1 μg of chloramphenicol acetyltransferase (CAT) reporter plasmids were cotransfected into the cells. Cells were treated (or not treated) with hormone 48 h before harvesting. CAT assays were performed as previously described (18), and CAT activity was calculated relative to β-galactosidase activity (21).

Methylation interference assay. Interference with the binding of GR, PR, or ER caused by methylation of target DNA was measured essentially as previously described (23). Briefly, end-labeled DNA fragments were treated with dimethyl sulfate for 3 min as described by Maxam and Gilbert (37). The methylated fragments were included in preparative binding reactions with the receptor proteins and subsequently electrophoresed through a 4% polyacrylamide gel. Following electrophoresis, DNA was electroblotted over-

night onto Whatman DE81 paper in 0.5× Tris-borate-EDTA (TBE) buffer at 100 mA and 18 V (Bio-Rad Trans-Blot System). Radioactive bands were localized by autoradiography of the wet DE81 paper. The DNA was excised from the paper and eluted in 300 to 500 μl of 10 mM Tris (pH 7.5)–1 mM EDTA–1.5 M NaCl at 37°C for 2 h. After chloroform-isoamylalcohol (24:1) extraction and ethanol precipitation, the DNA was cleaved at the guanines and subsequently electrophoresed on 15% acrylamide–8 M urea gels.

T-specific modification by $KMnO_4$. End-labeled DNA fragments were dissolved in 5 μl of 30 mM Tris (pH 8.0) buffer and heated at 95°C. After 2 min, samples were cooled on ice and thymine-specific modification was performed by adding 20 μl of a 2.5×10^{-4} M potassium permanganate solution for 10 min at 20°C. Reactions were stopped with 225 μl of 0.33 M sodium acetate (pH 7.0)–0.22 M β-mercaptoethanol and 750 μl of ethanol. DNA was precipitated for 10 min at −80°C and, after a second precipitation, was dissolved in 10 μl of 10 mM Tris (pH 8.0)–1 mM EDTA–30 mM NaCl, heated at 60°C for 5 min, and slowly cooled to room temperature (42, 53). The premodified DNA fragments were subsequently added to a preparative scale binding reaction with the receptors and analyzed as described above for methylation interference, including piperidine cleavage of the modified thymines.

Ethylation interference. Ethylation interference experiments were performed as described by Siebenlist and Gilbert (50). About 10 to 20 ng of $5'$-^{32}P-labeled single-stranded synthetic oligonucleotide was resuspended in 100 μl of sodium cacodylate (pH 8.0) and mixed with an equal volume of ethanol saturated with ethylnitrosourea. The reaction was incubated for 30 min at 50°C and stopped by the addition of 20 μl of 3 M sodium acetate at −20°C. The DNA was resuspended, reprecipitated, and dissolved in 5 μl of 10 mM Tris (pH 7.5)–1 mM EDTA–30 mM NaCl and a threefold molar excess of the complementary nonlabeled strand. The mixture was then heated at 60°C for 5 min, slowly cooled to room temperature, and subsequently used in the binding assay.

Computer graphics. Computer graphic analysis of the receptor contact sites on the DNA double helix was performed with an Evans and Southerland PS 390 system and a Digital Equipment VAX 3500 computer. For display and manipulation of the B-DNA double helices, the UCSF MIDAS molecular modeling software was used (1, 32).

RESULTS

Binding of ER and PR to variant HREs. To examine the significance of relevant positions within the HRE for binding of steroid hormone receptors from different subgroups, we synthesized a series of ideal GRE/PREs or EREs and variants thereof with exchanges at either position 3 or 4 of one or both half-palindromes. We have introduced a nomenclature which refers to these bases of each half-palindrome (Fig. 1), with the left half read from the antisense strand and the right half read from the sense strand, so the canonical ERE, TGACC (26, 29), is called AC-AC and the canonical PRE/GRE, TGTTCT (29), is called TT-TT. The PR receptor used in the binding experiments was purified from rabbit uterus (56), and the ER was derived from WCE (see Materials and Methods) of COS-7 cells transfected with HEO expression vector (31). First, we examined the binding affinity of steroid hormone receptors for the symmetrical HREs by the band shift assay (15). A comparison of the binding of PR to TT-TT and TC-TC revealed that binding affinity for the TC-TC element is slightly lower for PR (Fig. 1A). This correlates well with the fact that the thymine at position 4 does not seem to be directly contacted by these receptors in potassium permanganate interference experiments or in missing contact probing assays (53). Addition statistical analysis of GRE/PREs has revealed a low (65% T versus 45% C) preference for thymine at this position (4). Therefore, only the TC-TC element has been included in further experiments, because this GRE/PRE can be converted into an ERE by a single base exchange in both half-sites of the palindrome (29, 35).

Binding experiments with three different concentrations of the corresponding receptors (Fig. 1B) have demonstrated that ER binds to the ideal ERE AC-AC with high affinity and tolerates the conversion of the A at position 3 to C (CC-CC), although it binds this element with significantly lower affinity. In contrast, replacing the cytosine at position 4 by thymine (AT-AT) completely eliminates ER binding. As expected, ER does not bind to the ideal GRE/PRE TC-TC, underlining the selectivity of the canonical ERE.

PR recognizes the ideal GRE/PRE TC-TC and does not tolerate the conversion of the thymine at position 3 to cytosine (CC-CC). Surprisingly, an A at position 3 seems to be compatible with PR binding, but only if position 4 is occupied by a thymine but not by a cytosine, since PR binds the AT-AT element but not the AC-AC variant. All of the symmetrical HREs tested were bound by ERE or PR. Unexpectedly, the discrimination between GRE/PRE and ERE can occur exclusively either at position 3 (AC-AC/TC-TC) or at position 4 (AC-AC/AT-AT) of the half-palindromes.

The fact that most of the naturally occurring HREs have an imperfect palindromic structure (4) prompted us to analyze whether a half-GRE/PRE can replace the degenerated half of an imperfect palindromic ERE and vice versa. In addition to the two asymmetric hybrid constructs AC-TC and AC-TT, we have included the asymmetric promoter distal GRE/PRE of MMTV TA-TT (45), a recently discovered asymmetric ERE from rabbit uteroglobin promoter AC-CC, and an AC/AT element that in addition to a C-to-T conversion at position 3 of one half of the ideal ERE AC-AC lacks a nucleotide in the spacer between the two half-sites, to prove whether contacts in both half-sites contribute to binding.

A comparison of the binding curves obtained with the two hybrid constructions shows that their affinity for the PR is similar, whereas the ER binds better to AC-TC than to AC-TT, suggesting that a T in position 4 is less favorable for ER binding. The AC/AT element binds neither ER nor PR (Fig. 1B). This finding underlines the need for correct spacing of the half-palindromes and indicates that contacts in both half-sites contribute to binding. The asymmetric AC-CC element binds the ER rather efficiently but shows no detectable binding to PR, whereas the asymmetric GRE/PRE from MMTV TA-TT binds PR but not ER (Fig. 1B and C). In similar experiments, the binding behavior of the GR prepared from rat liver (17) was found to be indistinguishable from that of PR (53a).

We conclude from these results that hybrid HREs with a T or C in position 3 bind the ER equally efficiently, whereas only those with a T in position 3 reveal significant binding to PR and GR. In contrast, symmetric HREs with a T in position 3 or 4 cannot bind the ER but efficiently bind PR and GR (see TC-TC and AT-AT elements). The ability of hybrid HREs to bind ER, PR, and GR suggests that essential nucleotide contacts within the binding sequence are common to both types of half-site motifs (ERE and GRE/PRE).

Functional activity of variant HREs. To evaluate the abilities of the different HREs to mediate induction by different steroid hormones, we cloned two copies of the corresponding synthetic oligonucleotides upstream of the thymidine kinase-CAT hybrid gene (tkCAT) and analyzed CAT activity after cotransfection with receptor cDNAs into HeLa cells. As an internal control the β-galactosidase expression vector, RSV-βGAL, was used (see Materials and Methods). A comparison of the results obtained with the synthetic estrogen diethylstilbestrol (DES) and with the synthetic glucocorticoid dexamethasone is shown in Fig. 2A. The results from several experiments are listed in Fig. 2B. As expected, the canonical ERE AC-AC only responded to estrogens and the symmetric mutation of the element CC-CC decreases the efficiency of estrogen-dependent induction, but the CC-CC element still remains a functional ERE. The GRE/PRE TC-TC responded only to glucocorticoids or progestins and not to estrogen. The symmetric C-to-T substitution in position 4 generated the variant AT-AT, which responds very efficiently to both glucocorticoids and progestins but not to estrogens (Fig. 2). In addition to the behavior of symmetrical HREs, we found that the hybrid HREs, AC-TC and AC-TT, do indeed respond to both estrogens and glucocorticoids. In parallel experiments we found that the hybrid variant AC-TT also responds to the synthetic progestin R5020 (Fig. 2B), but the AC-TC variant did not respond under our experimental conditions, although the binding affinity of these two elements for the PR (Fig. 1B) is similar. The ability of the hybrid element AC-TT to respond to all three steroid hormones is about 20% of that found with the corresponding ideal HREs (Fig. 2B).

The double mutant AC/AT does not respond to any of the three hormones, whereas the asymmetric variant corresponding to the ERE of the uteroglobin promoter (AC-CC) does not respond to glucocorticoids or progestins but is an efficient mediator of estrogen induction.

With the exception of the lack of response of AC-TC to progestins, the results of the gene transfer experiments confirm the DNA binding studies, in that those HRE variants able to bind a particular receptor are also effective in mediating induction by the corresponding hormone in vivo.

Fine analysis of the contacts between receptors and various HREs. To analyze similarities and differences in the mechanisms by which various steroid hormone receptors recognize and distinguish different HREs, we used binding interference techniques. The inclusion of hybrid elements in these studies allows, for the first time, the analysis of the contacts made by receptors that productively interact with half-sites of palindromes to which they normally do not bind. Methylation interference was used to detect contacts with the N-7 position of guanines (41), and the $KMnO_4$ interference method served to map contacts with thymines (53). $KMnO_4$ attacks the C-5–C-6 double bond of thymines in single-stranded DNA (42). After reannealing, the double-stranded DNA can be used for binding interference experiments. This method yields information concerning the contribution of the methyl groups of thymine bases to the specific protein-DNA interaction (53). Because of the symmetric nature of the perfect palindromic elements to which the receptors bind as dimers, we have only analzed one of both strands of elements AC-AC, AT-AT, and TC-TC for interference experiments.

As previously reported, the ER contacts the three guanine residues within the half-ERE, 5'-TGACC-3', one in the sense strand and two in the antisense strand (28, 38, 52, 52a). In terms of thymines, only the first T in the top strand (+1) is contacted, whereas the T in the bottom strand opposite to the A in position +3 can be modified by $KMnO_4$ without interfering with ER binding (Fig. 3A, AC-AC). Since position 3 can also accommodate a C, a T, and even a G in one half of the palindrome and at least a C in both halves, it seems that this position is not in intimate contact with the ER.

The GR and the PR contact two guanines within the half-GRE/PRE, 5'-TGTTC-3', the G in position +2 in the sense strand, and the G in the antisense strand opposite to position +5 (10, 44, 54). When a C is found in position +4, the opposite G is hypermethylated in the presence of GR (24) or PR (51, 52a) in methylation protection experiments. In interference experiments, methylation of the G opposite to the C in position 4 of the TC-TC element interferes with binding of the PR (Fig. 3D). The hybrid HRE AC-TC shows a similar methylation interference pattern with PR (Fig. 3B and D and 4A). Binding of ER to the AC-TC element is inhibited by modification of any of the guanines within the left or the right half of this HRE. Therefore, with respect to contacts with the N-7 positions of guanines, there is no basic difference between ER and GR or PR binding to their conserved HREs. In addition, the $KMnO_4$ interference method detects contacts of the GR (53) and the PR with the thymine in position +1 that is also contacted by the ER in the ERE (AC-AC in Fig. 3A). In this respect again all three receptors behave similarly. However, modification of the thymine in position +3 in the promoter distal HRE of MMTV (TA-TT [53]) and in the TC-TC variant (Fig. 3D) interferes with the binding of GR and PR. This is the main difference in the recognition mechanisms of the two subgroups of hormone receptors; the ER cannot accommodate a T in position 3 on both sides of the HRE palindrome.

We also have analyzed directly the thymine contacts of ER and PR on the sense strand of the hybrid element AC-TT by the $KMnO_4$ interference method (Fig. 3E). Modification of the first T on the right half of the palindrome (+1) interferes with ER and PR binding. In addition, whereas the PR contacts the T in position +3 in the right half, the ER contacts the T in position −3 in the left half of the HRE (Fig. 3E and 4). It is surprising that modification of the T in position −3 in the left half interferes with the binding of ER, since modification of the equivalent position in the consensus ERE AC-AC does not influence ER binding (Fig. 3A and 4). This could be due to the lower affinity of ER for the AC-TT element that requires an intact left half to form stable complexes. However, the differential interference pattern between PR and ER in position +3 again underlines the significance of position 3 for discrimination between GRE/PRE and ERE.

The antisense strand of the variant HRE AT-AT, which responds to glucocorticoids and progestins, also was analyzed by interference techniques (Fig. 3C). In addition to the expected contacts at the guanine residues, only modification of the first T (−1) interferes with binding of the PR, whereas again there is no contact with the T in the antisense strand opposite to the A in position +3 (Fig. 3C and 4). Thus, the PR interacts with the 5-methyl group of the T-A base pair in position 3 but cannot interact with the 5-methyl group of an A-T base pair in this position. In position 4 the 5-methyl group of the T-A base pair is not contacted. Also in the TGTTC motif, modification of the thymine in position +4 does not interfere with the PR or GR binding (53) (variant AC-TT in Fig. 3E).

Ethylation interference. To detect contacts between ER or PR and the DNA backbone, we used the method of ethyla-

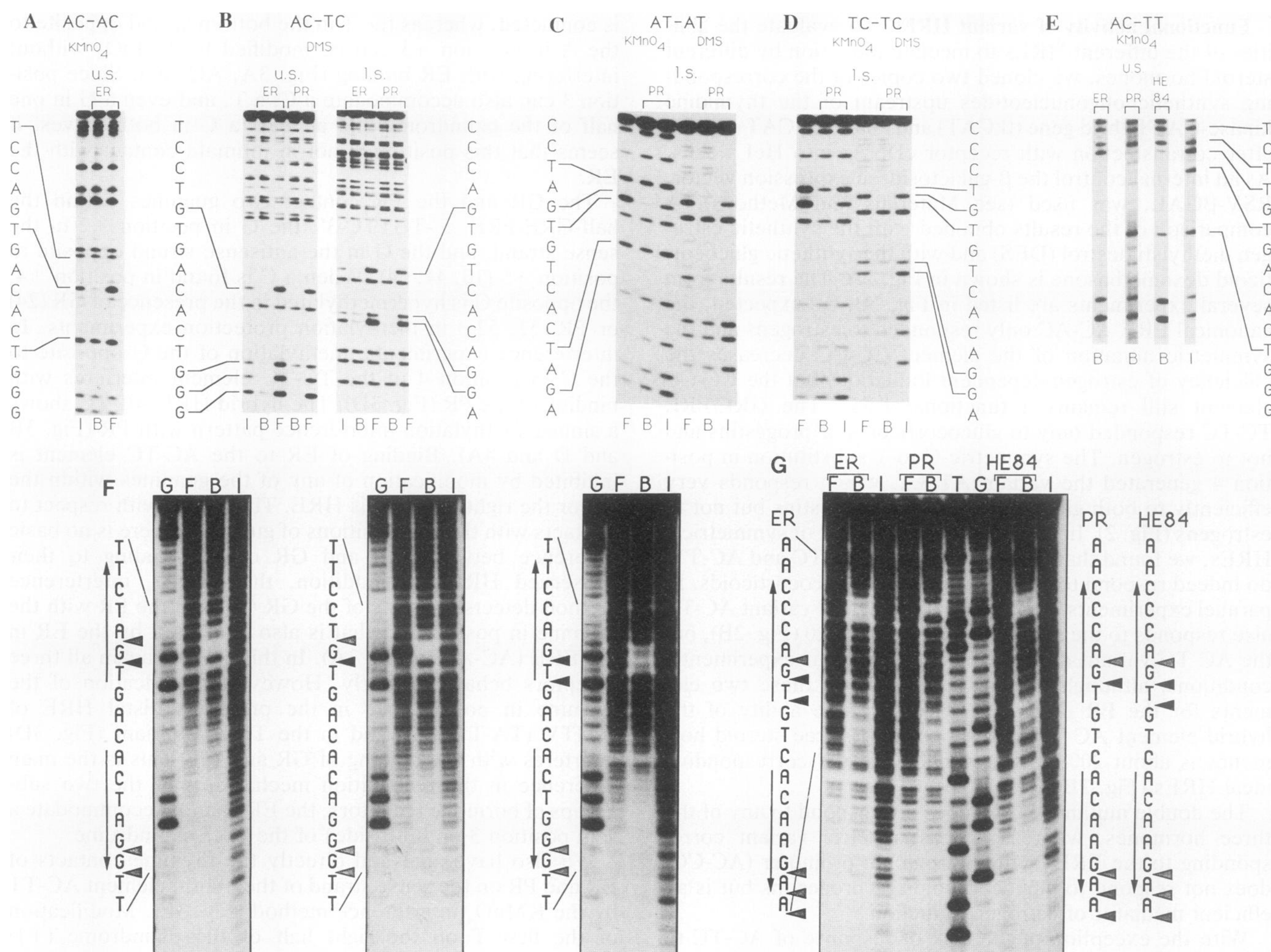


FIG. 3. Chemical modification interference experiments. (A through E) All fragments used for the methylation and thymine-specific interference experiments were 5′ end labeled and were isolated from the vectors which contain a single cloned copy of the corresponding oligonucleotide (see Materials and Methods). The upper strand (u.s.)-labeled fragments carrying the AC-AC, AC-TC, and AC-TT elements were isolated as 81-bp-long *Hin*dIII-*Eco*RI fragments, and the lower strand (l.s.; 141 bp)-labeled fragments were produced by *Bam*HI and *Pvu*II digestion. From the TC-TC and AT-AT elements only the lower strand, which represents the *Bam*HI-*Hin*dIII fragment (48 bp) of the corresponding vectors, was analyzed. Premodified DNA fragments were then used in a preparative gel retardation assay with partially purified PR or WCE from COS-7 cells transfected with HEO or the ER mutant HE84. Free probes (F) and probes comigrating with the identified complexes (B) were recovered and, together with the input DNA (I), cleaved with piperidine at modified thymines ($KMnO_4$) or guanines (dimethyl sulfate [DMS]). The cleavage products were displayed by electrophoresis through 15% denaturing polyacrylamide gels. (F and G) Synthetic oligonucleotides labeled on the 5′ end (42 nucleotides long) were modified by ethylnitrosourea and then used in interference experiments as described above. Ethylated phosphates were cleaved with alkali. (F) From left to right, upper strands of AT-AT, TC-TC (with PR), and AC-AC (with ER). (G) From left to right, ER, PR, HE84, with the lower strand of AC-TT.

tion interference (50), which in the case of the 434 repressor yields excellent agreement with the results of X-ray crystallography (7). Cleavage of 5′-labeled ethylated DNA by alkali gives rise to three labeled products, namely, DNA fragments which terminate with 3′-OH, 3′-ethylated phosphate, or 3′-phosphate. In our experiments we used short DNA fragments (42 bp), and therefore all of these products of alkaline cleavage are separated in the lower part of the sequencing gel (Fig. 3F and G). Bands that are underrepresented in the receptor-bound DNA are indicated by filled triangles. Black triangles correspond to strong (more than 10 times) interference, and grey triangles correspond to significant but weaker effects (Fig. 3F, AT-AT, TC-TC, and AC-AC on the upper strand; and 3G, AC-TT on the lower strand). Ethylation of DNA in these positions inhibits binding of the receptors. A summary of the ethylation interference pattern observed with the ER, the PR, and the HE84 mutant (see below) and several HREs is shown in Fig. 4B. The interaction of the ER and PR with the phosphate backbone of their binding sites is similar, but not identical. Ethylation of the phosphate 5′ to symmetric position 1 does not inhibit binding of PR but prevents binding of ER; the opposite is true for the ethylated phosphate 5′ to position 3. Ethylation of the phosphate 5′ to position 2 generally inhibits binding of both receptors. The phosphate contacts of PR, ER, and HE84 5′ of positions 5 and 6 are indistinguishable.

Mutation of individual thymines to desoxyuridine. We have previously shown for the GR and the PR that interference by modification with $KMnO_4$ is due to intimate contact of the proteins with thymines (53). To demonstrate that a similar

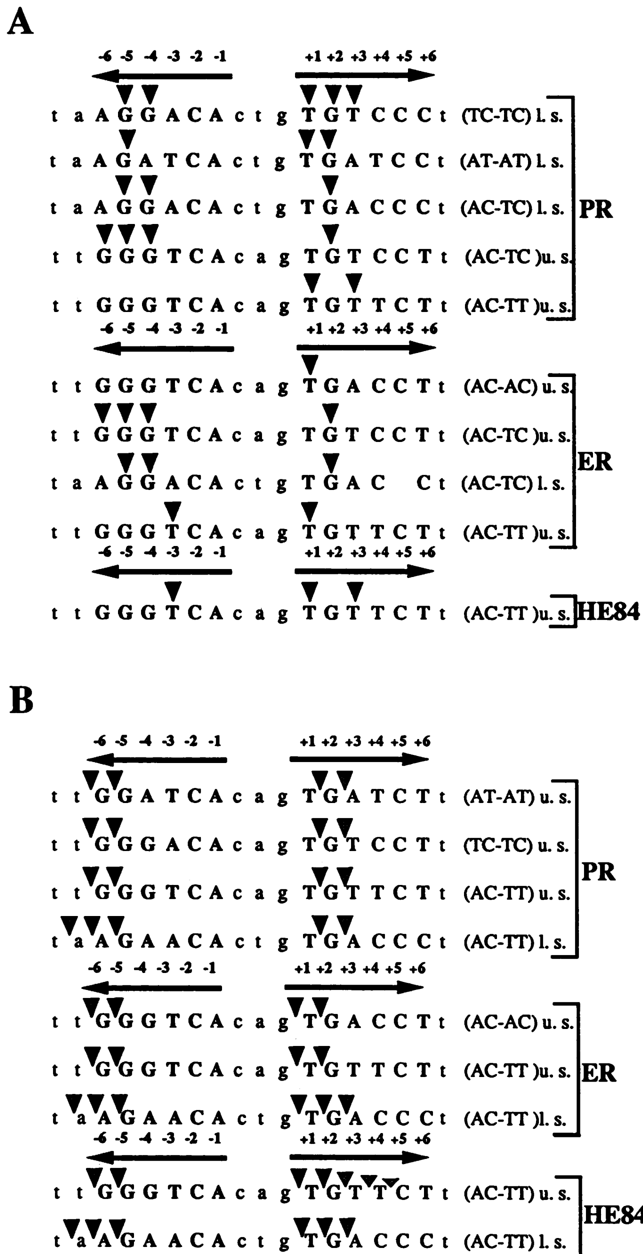


FIG. 4. Summary of the chemical modification interference patterns. Modifications of guanines and thymines (A) or phosphates (B) that interfere with receptor binding are indicated by black triangles. Positions that, when modified, weakly inhibit receptor binding are indicated by grey triangles. Lowercase letters within the sequence indicate nucleotides that are not part of the half-palindromes. l. s., lower strand; u. s., upper strand.

mechanism applies for the ER, we compared the affinity of the ER for synthetic oligonucleotides containing desoxyuridine instead of thymine in the appropriate symmetric positions within the ERE AC-AC (Fig. 5). It is clear that whereas introduction of desoxyuridine in position 1 interferes with ER binding, the same symmetric replacement in position 3 has no influence on binding. This result again confirms the close correlation between $KMnO_4$ interference and contacted thymine methyl groups and supports the hypothesis

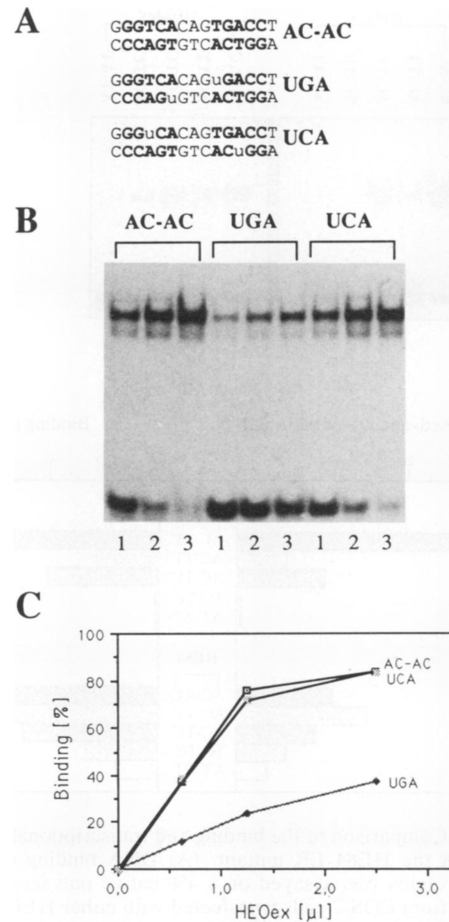


FIG. 5. Effects of the substitution of thymines by desoxyuridine on the binding of ER. (A) Nucleotide sequence of the consensus ERE (AC-AC) and the oligonucleotides (42 bp) carrying the desoxyuridine substitutions (u) which were used for the gel retardation experiments. (B) Gel retardation assay with WCE from COS-7 cells with overexpressed ER (see Materials and Methods). Synthetic single-stranded oligonucleotides (42 nucleotides) were labeled on the 5′ end and annealed with the nonradiolabeled complementary strand, and double-stranded fragments were then isolated from an 8% native polyacrylamide gel. Lanes 1, 0.6 µl of WCE; lanes 2, 1.2 µl of WCE; lanes 3, 2.5 µl of WCE. (C) Quantitative representation of the binding results described above.

that the ER does not contact the DNA double helix in this position.

A point mutant of the ER interacts with an ideal GRE/PRE. It has been reported recently that a few amino acid exchanges in the first zinc finger of the ER are sufficient to generate hybrid receptors able to confer estrogen inducibility to the HRE of MMTV (34). It is also known that exchanging Gly-439 with a Glu in the knuckle of the first zinc finger of hGR generates a mutated receptor able to confer dexamethasone inducibility to the MMTV HRE and to an ERE (55). The corresponding mutated ER (HE84 [34]) reduces the estrogen inducibility of an ERE and confers only very weak inducibility to the MMTV HRE.

To better understand the role of individual amino acids in interaction between the steroid hormone receptors and the HREs, we analyzed the DNA binding specificity and inducibility of the HE84 ER mutant with different HREs and

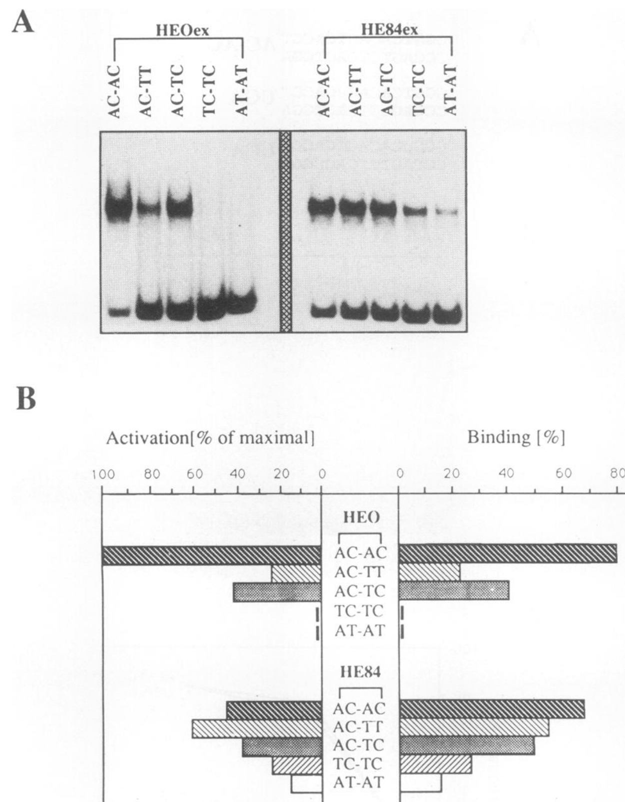


FIG. 6. Comparison of the binding and transcriptional activation of ER with the HE84 ER mutant. (A) DNA binding of the two receptor proteins was assayed on a 4% native polyacrylamide gel with WCE from COS-7 cells transfected with either HEO or HE84. The DNA fragments (141 bp) tested are identical to those used for the experiments shown in Fig. 1. Complexes were formed (or not formed) in the presence of 2.5 μl of WCE in a standard binding reaction. (B) Quantitation of the binding and transcriptional activity of ER and the HE84 mutant. The binding results represent the values obtained from the experiment shown in panel A, and transcriptional activation is shown in bars as the mean value from two or three independent transfections in HeLa cells. Since the basal activity was very low and similar for different reporter plasmids, only the hormone (10^{-8} M DES)-induced values are shown.

characterized its molecular contacts on the hybrid HRE AC-TT. The mutant receptor, HE84, was expressed in COS-7 cells, and its DNA binding behavior was compared with that of the wild-type ER expressed under identical conditions (Fig. 6). It is clear that whereas the wild-type ER binds only to the ERE or to hybrid HREs containing one-half of the canonical ERE, the mutant HE84 recognizes all of these HREs and in addition binds to a conserved GRE/PRE and to the functional GRE/PRE AT-AT. Binding of HE84 to the hybrid HREs is relatively more effective than binding of the wild-type ER (Fig. 6). Note that wild-type ER (HEO) and HE84 were present in about the same amount in the extracts from transfected COS-7 cells as confirmed by Western immunoblotting (not shown) with the ER-specific monoclonal antibody H222 (20). These results were confirmed in gene transfer experiments in which the mutant receptor HE84 was able to confer estrogen inducibility to constructions carrying GRE/PREs (Fig. 6B). Thus, a single amino acid exchange in the knuckle of the first zinc finger confers to the ER the ability to interact specifically with GRE/PREs or with hybrid HREs.

To understand the mechanism of DNA sequence recognition by the mutant receptor HE84 more precisely, we performed modification interference experiments to compare the molecular interaction with the hybrid variant AC-TT to that of ER and PR. Since the contacts of ER and PR with the N-7 positions of guanines are basically similar within the conserved or hybrid HREs, we used ethylation and thymine-specific modification interference for this analysis (Fig. 3E and 3F). The ethylation interference pattern with HE84 (Fig. 3G and 4B) is similar to that found with ER and distinct from that caused by PR on the same variant AC-TT. In the $KMnO_4$ modification experiments the HE84 mutant shows an intermediate interference pattern (Fig. 3E and 4A). Modification of the thymines in position +1 and +3 on the right half of the HRE interferes with the binding of HE84 (as well as the PR), and modification in position −3 in the left half interferes weakly (strongly with ER binding). It seems, therefore, that the mutant receptor HE84 contacts the same phosphate groups as the ER, whereas the major differences can be detected in the thymine contacts which appear to be very similar to those produced by PR (Fig. 3E and 4).

DISCUSSION

Interaction of steroid hormone receptors with symmetric HREs. Most naturally occurring HREs for steroid hormones are imperfect palindromes with the two symmetrical halves separated by 3 nonconserved base pairs (4). Each half contains 5 (EREs) to 6 (GRE/PREs) base pairs, and the degree of conservation of these positions is highly variable. Whereas position 1, 2, and 5 are identical between HREs for vertebrate steroid hormones, positions 3 and 4 differ and form the basis for the discrimination between GRE/PRE and ERE. Position 6 seems to be important exclusively for the GRE/PRE family of steroid hormone receptors. Our in vivo and in vitro data demonstrate that a symmetric ERE with the canonical structure AC-AC or the variant ERE CC-CC is exclusively recognized by the ER but not by GR or PR. On the other hand, the symmetric ideal GRE/PREs, TT-TT (29) or TC-TC, are exclusively recognized by the GR or the PR but not by the ER. These results confirm the significance of positions 3 and 4 for HRE discrimination. Since the canonical ERE AC-AC differs from the GRE/PRE TC-TC only in position 3, it is clear that this is a key position for specific receptor binding. We know that the 5-methyl group of the T in this position is contacted directly by GR and PR, since its modification by $KMnO_4$ or its replacement by desoxyuridine interferes with binding of these receptors (53). We also know that the ER does not contact the methyl group of the T opposite to the A in position 3 of the ERE AC-AC, because this T can be modified or replaced by desoxyuridine without influencing ER binding. No interference has been detected at A residues within an ERE or HRE (28, 54) by modification with dimethyl sulfate, but it remains to be established whether the adenines contribute to specific protein-DNA interactions through the major groove. In fact, position 3 of EREs also accommodates a C, a T, or even a G in one-half of the palindromes (6, 52) (Fig. 1B, AC-TC) or even a C in both sides of the palindrome (Fig. 1B), and therefore neither base at this position is likely to be contacted by the ER. Even more significant is the fact that symmetric HREs with a T in this position are unable to bind the ER or to respond to estrogens in gene transfer experiments. The 5′-methyl

group of thymine that is positioned in the major groove through which steroid hormone receptors are believed to recognize their HREs is one of the most striking features distinguishing a CG from an AT base pair. It may be that the methyl group of position 3 that is contacted by PR and GR (53) causes a steric hindrance and so may be incompatible with ER binding. A similar steric hindrance may also occur when a T is located in position 4. This may explain why the symmetric HRE AT-AT, which only differs from a canonical ERE in position 4, does not bind ER nor respond to estrogens in gene transfer experiments. Again, no symmetric HRE with a T in position 4 is recognized by ER.

The symmetric HRE AT-AT binds PR and GR with affinities similar to that of an ideal GRE/PRE and mediates efficient induction by glucocorticoids and progestins in gene transfer experiments. This finding is unexpected, because most GRE/PREs carry a T in position 3 (2), and a single copy of the AT-AT construction had been reported previously to be unable to confer glucocorticoid responsiveness to the thymidine kinase promoter in MCF-7 cells (29). Since we have prepared DNA for most DNA binding and all gene transfer experiments from Dam$^+$ bacteria, a possible explanation could be that the A at position 3 is part of a *dam* methylation site (GATC) and is therefore methylated at position 6 (16). It has been shown that the methyl group of thymines, the base usually found at this position, significantly contributes to binding of GR and PR (53). Even if the thymine gets replaced by cytosine, its methylation to 5-methyl cytosine enhances binding of GR and PR (53). Therefore, it could be possible that this methylated adenine functionally replaces the thymine. However, the ethylation interference experiment with the AT-AT element has been performed with unmethylated synthetic oligonucleotides, and this demonstrates that PR can recognize an unmethylated AT-AT element. Whether the *dam* methylation leads to an increase in the affinity of GR and PR to this binding site remains to be established.

All of these findings suggest that GR and PR require contact with the methyl group of the T in position 3, whereas the thymine in position 4 contributes to the binding stabilization via a different mechanism, because its modification does not interfere with PR binding to AT-AT (Fig. 3C). In any case, the efficient function of the AT-AT HRE as GRE/PRE and its complete lack of activity as an ERE suggest that the discrimination may also take place exclusively in position 4.

Interaction of steroid hormone receptors with asymmetric and hybrid HREs: a promiscuous HRE. Since many of the naturally occurring HREs are not completely symmetrical and it has been shown that some asymmetric HREs can give a greater hormone induction than the perfect palindrome TT-TT (22), we analyzed the interactions of various receptors with HREs asymmetric in position 3. We made the general observation that the three receptors can accommodate a great deal of asymmetry. Provided one-half of the palindrome corresponds to the conserved element, the other half can vary considerably and tolerates bases in this position that are incompatible with binding to symmetric HREs. This observation prompted us to construct hybrid HREs with half corresponding to the canonical ERE and the other half corresponding to a conserved GRE/PRE. The two hybrid HREs tested, AC-TC (see references 35 and 36) and AC-TT, indeed bind the receptors for estrogens, glucocorticoids, and progestins, whereas only the variant AC-TT responds to all three hormones in gene transfer experiments. The discovery of this class of promiscuous HREs raises the possibility of their physiological involvement in the regulation of genes that are known to be inducible by estrogens as well as by progestins and glucocorticoids (3). In the 5′ flanking region of one of those genes, namely, rabbit uteroglobin, we have identified one such hybrid HRE at position −1017 (8) that seems to be a hybrid element composed of a half-GRE/PRE (TGTTCT) and a half-ERE (TGACC) spaced by 3 base pairs; this composition is identical to that of the AC-TT element tested. It remains to be established whether this element is involved in mediating multihormonal regulation of the uteroglobin gene in different tissues of the rabbit.

In good correlation with the DNA binding experiments, AC-TC responds to estrogens at 40% and AC-TT responds at 23% of the level of the canonical ERE, underlining the inhibitory role of a T-A base pair in position 3 or 4 for the ER. Of these two hybrid elements only AC-TT responds to glucocorticoids and progestins, and the extent of the response, 20%, correlates roughly with the relative affinity determined in DNA binding experiments. However, AC-TC does not respond to progestins and shows a very weak response to glucocorticoids. It is interesting to note that genes negatively regulated by glucocorticoids often contain binding sites for GR with the structure TC-AT, the right half of the palindrome being more conserved (4). An analysis of different single mutants in the DNA binding domain of GR has revealed a good correlation between DNA binding and repression activity (46). Thus, asymmetric HREs with the structure AC-TC or TC-AT may bind GR and PR in a nonproductive form. As with the symmetric HRE AT-AT, position 3 can be an A without reducing binding or response to glucocorticoids and progestins, provided that a T is located in position 4. The comparison between the TC-TC and AC-TC HREs is also interesting, since they differ only in position −3. The DNA binding results and, more clearly, the gene transfer experiments demonstrate that a T in this position is essential for GR and PR binding, again indicating the differential tolerance of GRE/PRE and ERE in this position for the binding of the corresponding receptors.

A mutant ER (Glu-203 to Gly) recognizes GRE/PREs as well as EREs. It has been reported that a few amino acid exchanges in and around the knuckle of the first zinc finger of the ER are sufficient to alter the selectivity of its DNA recognition (34). Here we have shown that the mutant ER, HE84 (Glu to Gly in position 203 of the ER), which contains two glycine residues in between the cysteines of the same knuckle, is able to recognize a canonical ERE as well as a conserved GRE/PRE. This mutant exhibits higher affinity than the wild-type ER for hybrid HREs composed of a half-ERE linked to a half-GRE/PRE. In addition HE84 ER is able to bind, albeit with low affinity, to symmetric HREs containing a T in position 3 or 4, a configuration that is incompatible with the binding of wild-type ER. From interference experiments we also know that HE84 ER contacts the T in position +3 of the hybrid HRE AC-TT, as does the PR, and shows more similarity to ER in the phosphate contact pattern on the same HRE. The results of gene transfer experiments confirm the DNA binding studies (Fig. 6B). The weak inducibility of the MMTV promoter by HE84 (34) could be due to the primary sequence and the complicated array of receptor binding sites within the MMTV long terminal repeat.

It has been proposed that the 10 amino acids following the knuckle in the first zinc finger could be part of a short amphipathic helical structure suitable for interaction with DNA (5). The DNA contacts identified with ER, PR, and HE84 in interference experiments with specific bases and

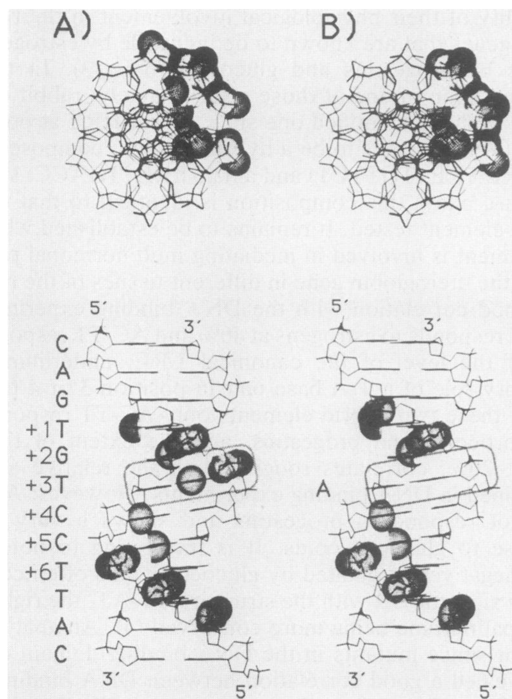


FIG. 7. Computer graphics representation of contacted sites between GR and one-half of a GRE/PRE (A) and ER and one-half of an ERE (B). The van der Waals spheres of the phosphate with their corresponding two oxygen atoms, the N-7 nitrogen atoms of the guanines, and the 5′-methyl groups of the thymines that have been identified by interference experiments are shown. The grey boxes indicate the position of base pair 3 of the half-palindrome.

with the phosphate backbone are made within one turn of the double helix and are all accessible from one face of the helix in the major groove (Fig. 7). Therefore, the distribution and the relative distances of the contacts we determined are fully compatible with this model, in that the contacts could be made by such an alpha-helix (Fig. 7).

The exchange of Glu-203 to glycine results in a loss of DNA binding specificity of ER, because the corresponding mutant HE84 is able to bind to GREs and EREs. This suggests that the side chain of glutamate prevents binding of wild-type ER to GREs, since it gets replaced by a single hydrogen if glutamate is substituted by glycine. This idea is supported by the location of Glu-203 in the DNA binding domain of ER. Together with Gly-204 and Ala-207, two other amino acids that have been identified to be important for DNA binding specificity of ER (34), Glu-203 is exposed to solution at the N-terminal part of the amphipathic helix that is believed to be the DNA recognition helix and therefore probably interacts with the HRE (48). The fact that replacing Glu-203 of ER by Gly only causes a moderate decrease of its affinity for EREs (50% reduction of induction in vivo and 20% reduction of binding affinity in vitro) does not necessarily exclude the possibility that this amino acid is involved in hydrogen bonding with bases of the ERE. It has been shown for other DNA binding proteins that the deletion of single sites for protein-base hydrogen bonding only leads to small energy differentials compared with that of the wild type (33).

The ethylation interference patterns of ER and PR appear to be very similar, but ethylation of the phosphate 5′ of position 1 of the half-palindrome exclusively interferes with ER binding (Fig. 3F and G). Like wild-type ER, HE84 contacts this phosphate but has a changed DNA binding specificity. Thus, the ability to contact this phosphate and the ability to make a hydrophobic interaction with the thymine at position 3 are not interconnected and are probably mediated by different amino acids.

Our results provide insight into the mechanisms by which the nuclear receptors recognize their cognate sequences and will be useful for the interpretation of structural data derived from two-dimension nuclear magnetic resonance or X-ray crystallography.

ACKNOWLEDGMENTS

The contributions of the first and second authors were equivalent. We thank Bernhard Gross, Marburg, Federal Republic of Germany, for the preparations of purified PR. We are especially grateful to P. Chambon for communicating unpublished results and for comments on the manuscript.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Be 759/8-2) and by the Fonds der Chemischen Industrie.

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