An N-Terminal Deletion Mutant of Estrogen Receptor Exhibits Increased Synergism with Upstream Activators and Enhanced Binding to the Estrogen Response Element†

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ABSTRACT: To study the role of the N-terminal region of the estrogen receptor (ER) in transcription activation and in DNA binding, we constructed a mutant of the *Xenopus laevis* ER which lacks amino acids 1−159 (XER160/586). In transient transfections, XER160/586 exhibited <10% of the activity of wild-type XER on a synthetic promoter containing two estrogen response elements (EREs). To examine transcriptional synergism by XER and by XER160/586, we determined the activity of promoters containing EREs and binding sites for either the vitellogenin activator, NF1, or AP1 upstream activator protein. For the three promoters transcription by XER was 2.8-fold greater than expected for additive activities, and transcription by XER160/586 was 6.2-fold greater. These data demonstrate that an upstream activator protein bound near the promoter can partially compensate for the loss of the internal N-terminal (AF1) transactivation domain in XER160/586. Using a promoter interference assay to study the intracellular interaction between ER and the estrogen response element, we found that XER160/586 exhibited a significant increase in affinity for the ERE. Its low basal activity and enhanced affinity for the ERE make XER160/586 an effective dominant negative mutant. When co-expressed with wild-type XER at 1:1 and 5:1 ratios, XER160/586 suppressed the activity of wild-type XER by 57% and >80%, respectively.

The effects of estrogen and other steroid hormones are mediated by intracellular receptors which are members of the steroid/nuclear receptor gene superfamily [reviewed in Evans (1988), Beato (1989), Gorski *et al.* (1993), and Tsai and O'Malley (1994)]. The steroid hormone receptors and other members of this gene superfamily share a common domain structure with distinct regions responsible for hormone binding, DNA binding, dimerization, transcription activation, and nuclear localization. The estrogen receptor (ER)[1] functions as a ligand-dependent transcription activator through the concerted actions of two transcription activation domains, termed AF1 and AF2 or TAF1 and TAF2 (Tora *et al.*, 1989). AF1 appears to be a ligand-independent transactivation domain located in the N-terminal region of the estrogen receptor (Tora *et al.*, 1989; Lees *et al.*, 1989; Berry *et al.*, 1990), containing a long proline-rich hydrophobic domain (Imakado *et al.*, 1991). Sequences essential for the functioning of the ligand-dependent AF2 transactivation domain are located at the C-terminus of the hormone binding domain, and may have the structure of an imperfect amphipathic α-helix (Danielian *et al.*, 1992). The relative contributions of AF1 and AF2 to transcription activation appear to differ on different promoters and in different types of cells (Bocquel *et al.*, 1989; Berry *et al.*, 1990; Metzger *et al.*, 1992; Scull *et al.*, 1992; Tzukerman *et al.*, 1994). In this work we examined the question of whether transactivation domains located in other gene regulatory proteins could act synergistically with the ligand-dependent AF2 transactivation domain to replace the ligand-independent AF1 transactivation domain in the N-terminal A/B region of the ER.

Recent studies indicate that sequences outside of the DNA binding domain can exert a strong influence on DNA binding (Mader *et al.*, 1993; Xing & Shapiro, 1993). Because it can be readily expressed at substantial levels in prokaryotic cells, we have examined the interaction of the ER DNA binding domain with the ERE (Nardulli *et al.*, 1991; Chang *et al.*, 1992; Nardulli & Shapiro, 1992). The isolated ER DNA binding domain binds to the ERE with much lower affinity than the full-length ER (Nardulli *et al.*, 1993). Little is known of the effect of mutation or deletion of other regions of the ER on DNA binding. In this work, we use a sensitive promoter interference assay (McDonnell et al., 1991; Reese & Katzenellenbogen, 1992; Xing & Shapiro, 1993) to investigate the effect of deletion of the N-terminal region of the *Xenopus laevis* estrogen receptor on DNA binding. We report the surprising finding that deletion of the N-terminal 159 amino acids of the XER results in a substantial increase in its ability to bind to the ERE in the *in vivo* promoter interference assay.

Some nuclear receptor mutants that have little or no ability to activate transcription function as dominant negative mutants and repress the activity of the wild-type protein when they are co-expressed in the same cells. Perhaps the most effective of these mutants is the *v-erbA* oncogene, which is a mutant form of the thyroid hormone receptor (Damm *et*

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[1] Abbreviations: E_2, 17β-estradiol; ER, estrogen receptor; XER, *Xenopus* estrogen receptor; hER, human estrogen receptor; ERE, estrogen response element; CAT, chloramphenicol acetyltransferase; VA, vitellogenin activator.

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al., 1989; Sap *et al.*, 1989), and three recently described estrogen receptor mutants which contain deletions or mutations in the AF2 transactivation domain (Ince *et al.*, 1993). Since the XER160/586 mutant exhibited little intrinsic ability to activate transcription of our estrogen responsive reporter gene, we investigated its ability to function as a dominant negative mutant. XER160/586 is a powerful dominant negative mutant, which effectively and specifically suppresses the activity of the wild-type XER.

EXPERIMENTAL PROCEDURES

Plasmid Constructions. The TKXER plasmid contains the entire protein-coding region of the *Xenopus* estrogen receptor cDNA (Weiler *et al.*, 1987) fused to the thymidine kinase promoter (Chang & Shapiro, 1990). Oligonucleotides used in mutagenesis were synthesized at the University of Illinois Biotechnology Center and purified by passage through oligonucleotide purification cartridges (Applied BioSystems, Foster City, CA). The N-terminal deletion mutant XER160/586 was constructed by site-directed mutagenesis so that the new N-terminus contained the efficient translation initiation sequence ACCATGG. The mutant was sequenced to confirm the accuracy of the junctions.

The construction of the reporter plasmids 2ERE-TATA-CAT and ERE-VIT-CAT has been described previously (Chang *et al.*, 1992). 2ERE-TATA-CAT contains two copies of the consensus ERE from the *Xenopus* vitellogenin A2 gene (**AGGTCACAGTGACCT**) 38 base pairs upstream of the TATA box of the *Xenopus* vitellogenin B1 gene (−41/+14). ERE-VIT-CAT contains a 618-bp (−596/+21) DNA fragment from the vitellogenin B1 gene. An additional consensus ERE was added 20 nucleotides upstream of the two imperfect EREs. The plasmids VA-TATA-CAT, NF1-TATA-CAT, AP1-TATA-CAT, 2ERE-VA-TATA-CAT, 2ERE-NF1-TATA-CAT, and 2ERE-AP1-TATA-CAT were prepared as we have described (Chang & Shapiro, 1990; Chang *et al.*, 1992). The reporter plasmid CMV-$(ERE)_2$-CAT (Reese & Katzenellenbogen, 1992) used in the promoter interference assays was provided by Dr. J. Reese and Prof. B. Katzenellenbogen (University of Illinois, Urbana, IL). It was constructed by inserting two copies of the consensus ERE into the *Sac*I site of CMV-CAT. The glucocorticoid receptor expression vector $RSVGR_a$ and the reporter pMTV-CAT were provided by Dr. R. Evans (The Salk Institute, La Jolla, CA). The progesterone receptor expression vector CMV-PR_B was provided by Prof. B. W. O'Malley (Baylor College of Medicine, Houston, TX).

Plasmids used for transfections were purified either twice by 6 h of CsCl step gradient centrifugation or once by 6 h of CsCl step gradient centrifugation or once by 6 h of CsCl step gradient centrifugation and once by 36 h of CsCl linear gradient centrifugation. RSVGRa and pMTVCAT were purified on QIAGEN columns (QIAGEN Corp., Chatsworth, CA).

Cell Culture, Transfections, and CAT Assays. The hepatocyte-derived *Xenopus laevis* cell line (XL110 cells) does not contain detectable levels of estrogen receptor (Nielsen & Shapiro, 1990; Chang & Shapiro, 1990). The cells were maintained at 20 °C in phenol red-free 0.6× Higuchi's medium (Higuchi, 1969) supplemented with 10% charcoal−dextran stripped fetal bovine serum. Transfections were carried out by a calcium−phosphate−DNA coprecipitation method with glycerol shock. Briefly, 1 day prior to transfection, 1×10^6 cells were seeded in a 9 cm culture dish. Transfections employed a total of 20 μg of DNA and included as an internal control 1 μg of pT109 luciferase vector (a gift from Prof. S. Nordeen, University of Colorado Health Sciences Center, Denver, CO), expression plasmids encoding either wild-type or mutant XER, the chloramphenicol acetyltransferase (CAT) reporter, and carrier DNA (pTZ18U). After 20−24 h, the medium was aspirated off the cells, and the cells were subjected to a 3-min shock in 15% glycerol/0.6× Higuchi's medium. The cells were then washed and fed with fresh medium either containing or lacking 1×10^{-7} M 17β-estradiol (E_2). Cells were harvested 48−60 h after glycerol shock and broken by three rounds of freezing and thawing in 80 μL of 250 mM Tris, pH 7.8, and 5 mM EDTA. Cell debris was sedimented by centrifugation, and the supernatant was assayed for luciferase activity in a Monolight 2010 luminometer (Applied Luminescence Laboratory, La Jolla, CA). CAT activity was determined by our quantitative mixed-phase assay (Nielsen *et al.*, 1989). Protein concentrations were determined using Coomassie Blue reagent (Bio-Rad Laboratories, Richmond, CA). The data represents the average of at least three separate transfections ± SEM. Statistical analysis was by Student's *T* test for unpaired samples with two-tailed *P* values < 0.05 considered significant.

Whole Cell Estrogen Receptor Assay. Estrogen receptor assays were carried out essentially as we have recently described (Xing & Shapiro, 1993).

RESULTS

XER160/586 Is a Weak Hormone-Dependent Transactivator. To examine the role of the N-terminal AF1 transactivation domain, we prepared an XER deletion lacking the A and B domains (Figure 1, panel A). We employed this large deletion because the N-terminal AF1 transactivation domain of the XER has not been precisely localized. The activity of this XER mutant was determined in transient transfections of a homologous *Xenopus* liver cell line, XL110 cells, which do not contain detectable levels of endogenous ER (Chang & Shapiro, 1990; Nielsen *et al.*, 1989). We initially examined the activity of XER160/586 on a standard synthetic promoter containing two copies of the consensus ERE linked to a vitellogenin TATA box and a CAT reporter gene (Chang *et al.*, 1992). Since this promoter lacks known upstream activator sequences, the XER and the XER mutant should act directly on the formation of the basal transcription complex. When estrogen was present, the wild-type XER exhibited a strong hormone-dependent transactivation, while XER160/586 exhibited $<10\%$ the activity of wild-type XER (Figure 1, panel B, 2ERE-TATA-CAT). We also examined the ability of XER160/586 to activate a complex vitellogenin-derived promoter, ERE-VIT-CAT. This promoter contains binding sites for several upstream activator proteins, including CAAT, NF1, and vitellogenin activator. XER160/586 displayed a reduced, but significant, ability to activate transcription from the complex ERE-VIT-CAT promoter. The overall activity of XER160/586 was approximately 30% that of wild-type ER (Figure 1, ERE-VIT-CAT).

Since XER160/586 retains the hormone binding domain and the ligand-dependent C-terminal AF2 transactivation domain, we examined the question of whether it retained

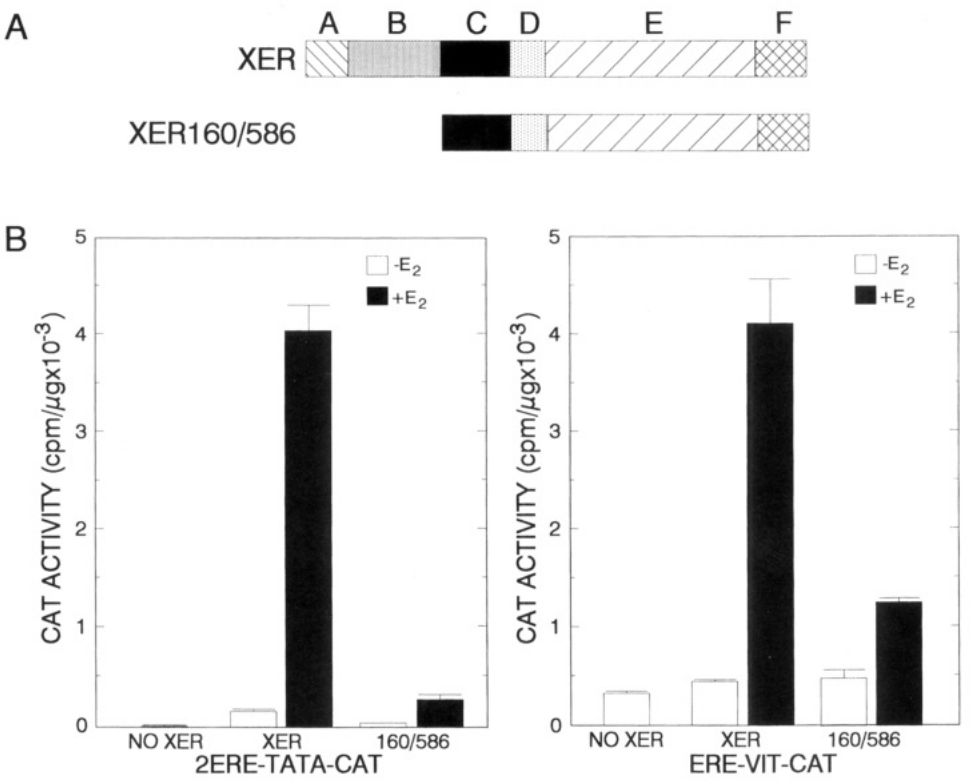

FIGURE 1: Hormone-dependent transactivation by XER and by XER160/586. Panel A contains a schematic representation of wild-type XER and the XER160/586 mutant, which lacks the A and B domains of XER. For simplicity, XER160/586 is sometimes referred to in figures by its constituent amino acids (160/586). In panel B the ability of wild-type XER and of XER160/586 to activate transcription was compared in cotransfections with two estrogen responsive reporter genes. In the left section of panel B, XL110 cells were cotransfected with 5 μg of 2ERE-TATA-CAT and 5 μg of the expression plasmid encoding either wild-type XER or XER160/586. After glycerol shock, the cells were maintained for 48 h in medium either containing 100 nM 17β-estradiol (solid bars) or lacking 17β-estradiol (open bars). The activity of both the unliganded and liganded forms of XER160/586 differed from the activities of the corresponding form of wild-type XER ($P < 0.05$). The transfections shown in the right section of panel B were carried out as described for the left section except that the 2ERE-TATA-CAT reporter plasmid was replaced by the vitellogenin-derived ERE-VIT-CAT reporter plasmid. The activities of liganded wild-type XER and XER160/586 were different, while there was no statistically significant difference in the activities of the unliganded forms of the two receptors using the ERE-VIT-CAT reporter plasmid. For both reporter plasmids there was a statistically significant ($P<0.05$) estrogen induction of transcription by XER160/586. The data represent the mean $\pm$ SEM for three separate transfections.

the capacity for ligand-dependent transactivation. In the absence of exogenous E_2, full-length XER exhibits some basal activity on the synthetic promoter containing two EREs (Figure 1, 2ERE-TATA-CAT, $-E_2$). It is not clear whether this represents a genuine ligand-independent ability to activate transcription mediated by the AF1 domain (Tzukerman *et al.*, 1990) or is due to the presence of traces of E_2 in the charcoal-stripped serum used in the transfections (Webster *et al.*, 1988; Berry *et al.*, 1990; Chang *et al.*, 1992). The XER160/586 mutant exhibited a statistically significant ($P < 0.05$) decrease in basal activity relative to wild-type XER (Figure 1, panel A, $-E_2$, compare XER and 160/586). In agreement with our earlier reports (Chang *et al.*, 1992; Xing & Shapiro, 1993), using the synthetic 2ERE promoter and the wild-type XER, estrogen elicited a 20−30 fold increase in CAT activity. CAT activity with the XER160/586 mutant exhibited a much smaller, but statistically significant ($P < 0.05$), 7-fold estrogen induction (Figure 2, 2ERE-TATA-CAT). Although the XER160/586 mutant contains the complete hormone binding domain, we carried out these experiments in 100 nM 17β-estradiol, far above the 0.1−1 nM level of 17β-estradiol at which the wild-type XER yields maximal CAT activity in this system (Xing & Shapiro, 1993). It is therefore unlikely that the reduced estrogen induction we observed with the XER160/586 mutant is due to failure to saturate the mutant receptor with estrogen. When we compared the activity of XER and the XER160/586 mutant on the complex ERE-VIT-CAT promoter containing the natural vitellogenin B1 promoter, the wild-type XER exhibited a 9-fold increase in activity on this promoter in the presence of E_2, while XER160/586 exhibited an increase of approximately 2.5-fold (Figure 1).

Upstream Activator Proteins Can Partially Substitute for the N-Terminal Transactivation Domain of XER. XER160/586 had minimal ability to activate transcription from the simple 2ERE promoter, but exhibited significant ability to activate transcription from the complex ERE-VIT-CAT promoter, which contains multiple transcription factor binding sites (Figure 1). This data raised the question of whether exogenous transactivation domains in transcription factors bound to their recognition sequences might partially replace the AF1 region deleted in XER160/586. To test this possibility, we employed reporter genes in which a binding site for either the NF1, AP1, or vitellogenin activator upstream binding proteins was located near the 2EREs. In agreement with our previous work (Chang *et al.*, 1992), the wild-type XER exhibited significant synergism with all three promoters (Figure 2, XER). For the three promoters, the activity averaged 2.8 times the level we would observe if the activity of XER on the 2ERE plasmid and the activity of each of the activator sequences were additive. The 2.8-fold average synergism exhibited by wild-type XER on the

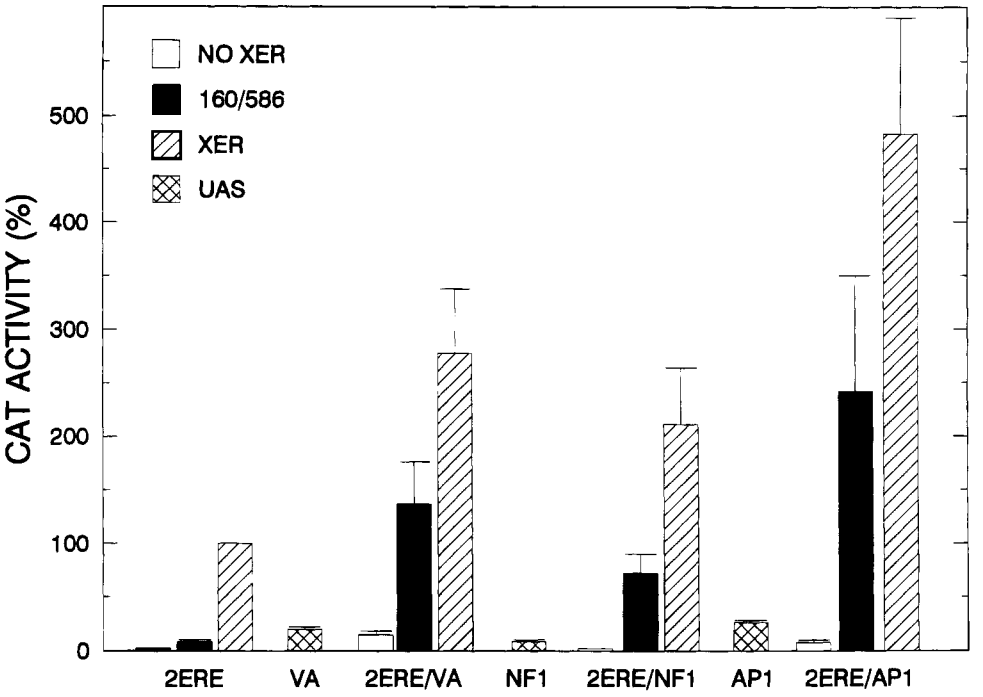

FIGURE 2: Synergistic interactions between the XER and XER160/586 proteins and upstream activators. XL110 cells were cotransfected with 1 μg of XER expression plasmid (hatched bars), 1 μg of XER160/586 (black bars), no expression plasmid (open bars), or 5 μg of reporter plasmids containing either single copies of the synthetic activator sequences VA, NF1, and AP1, or single copies of the VA, NF1, and AP1 upstream activators and two EREs (cross-hatched bars). Cells were maintained in 100 nM E_2 for 48 h after glycerol shock. The data represent the mean ± SEM for three separate transfections. For all four reporter genes there was a statistically significant ($P < 0.05$) induction of activity by liganded XER and by liganded XER160/586. Although there was a significant difference in the activity of XER and of XER160/586 on the simple 2ERE reporter plasmid, because of the increased fold synergism exhibited by XER160/586, the differences in activity between XER and XER 160/586 were not significant at $P < 0.05$ for the 2ERE/VA, 2ERE/NF1, and 2ERE/AP1 reporter plasmids.

three promoters was approximately 2-fold less than the average 6.2-fold synergism exhibited by XER160/586 on the same promoters. (The difference in fold synergism between XER and XER160/586 was significant with P values < 0.05). These results indicated that while both the AF1 and AF2 transactivation domains are required to obtain a fully active estrogen receptor, the presence of other transcription factors on the promoter can partially compensate for the absence of the internal N-terminal AF1 transactivation domain.

The XER160/586 Mutant Exhibits Increased Binding to the ERE. To investigate the influence of flanking sequences on the interaction of the ER DNA binding domain with the ERE, we employed a recently developed promoter interference assay. This assay measures the interaction of the ER with the ERE in intact cells. In this assay, ER bound to two EREs near the transcription initiation site of a strong cytomegalovirus (CMV) promoter competes for binding with basal transcription factors. The extent of interference with transcription provides a measure of the interaction of the ER with the ERE (McDonnell *et al.*, 1991; Reese & Katzenellenbogen, 1992; Xing & Shapiro, 1993). We cotransfected 10 μg of the expression plasmid encoding wild-type XER, or XER160/586, and 100 ng of the CMV-$(ERE)_2$-CAT promoter interference plasmid into the *Xenopus* liver cells (Figure 4). In a preliminary study liganded wild-type XER reduced CAT activity by 52% (data not shown), a value similar to that obtained in our recent study (Xing & Shapiro, 1993). In order to study this phenomenon in detail, we carried out promoter interference studies at a wide range of concentrations of transfected DNA encoding the wild-type XER and the XER160/586 mutant. Although there was some

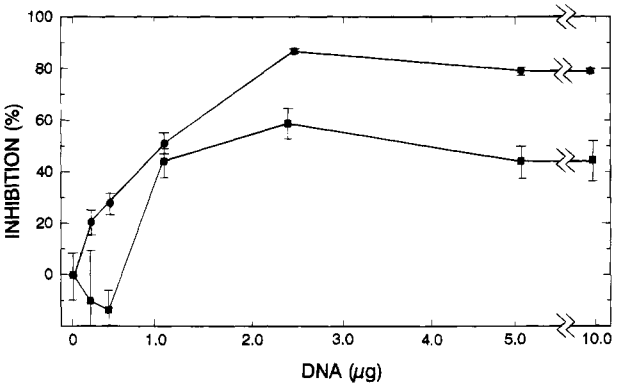

FIGURE 3: XER160/586 exhibits enhanced affinity for the ERE in promoter interference assays. XL110 cells were cotranfected with 100 ng of CMV-$(ERE)_2$-CAT reporter plasmid and increasing amounts (0.075−10 μg) of the expression plasmids encoding wild-type XER (■) or the XER160/586 mutant (●). CAT activity in the absence of 17β-estradiol and any XER expression plasmid was set equal to 0% inhibition. Cells were maintained in medium with 100 nM 17β-estradiol. The data represent the mean ± SEM for three separate transfections.

variation at individual DNA concentrations, the XER160/586 mutant was clearly more effective in inhibiting the activity of the promoter than the wild-type XER (Figure 3). While XER160/586 was more effective than wild-type XER in interfering with the activity of the promoter at every DNA concentration tested, the different levels of promoter interference by XER160/586 and by wild-type XER were most striking at 0.15, 2.5, and 5 μg of transfected DNA ($P < 0.05$) and at 10 μg of DNA ($P = 0.07$). At 2.5−10 μg of transfected DNA, interference with the promoter by wild-type XER reached a plateau at approximately 50% (Figure

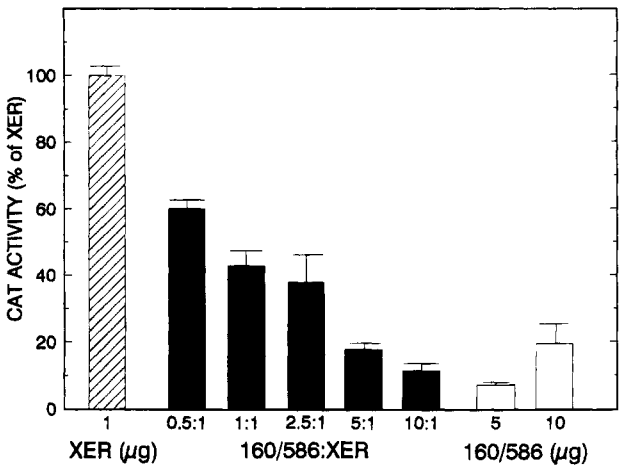

FIGURE 4: XER160/586 is a powerful dominant negative mutant. XL110 cells were cotransfected with 5 μg of the 2ERE-TATA-CAT reporter plasmid and either the wild-type XER (hatched bar) or the XER160/586 mutant (open bars). To test for the dominant negative phenotype, the cells were transfected with 1 μg of DNA encoding wild-type XER and with increasing amounts of DNA encoding the 160/586 mutant. CAT activity in transfections carried out with increasing 160/586:XER DNA ratios was determined (black bars). The data are not corrected for our observation that, after transfection of equal amounts of DNA, the level of XER160/586 is approximately one-third lower than the level of wild-type XER. Cells were maintained in medium containing 100 nM E_2 for 48 h after glycerol shock. The differences in activity between XER alone and 160/586:XER were statistically significant ($P < 0.05$) at all transfection ratios. The activities of 5 and 10 μg of XER160/586 alone were not significantly different from the activities of the 5:1 and 10:1 160/586:XER ratios. The data represent the mean ± SEM for three separate transfections.

3, XER; Xing & Shapiro, 1993). Interference with the promoter by the significantly smaller XER160/586 was substantially higher and ranged from 79% to 87% (Figure 3, XER160/586). At very low levels of transfected expression plasmid (0.075 μg and 0.15 μg) wild-type XER did not interfere with promoter activity, while XER160/586 exhibited 20−30% interference. Although we would have preferred to confirm these observations with *in vitro* gel mobility shift assays, the low level of expression of transfected XER in both *Xenopus* cells and mammalian cells has made it impossible for us to carry out gel mobility shift assays on cell extracts expressing transfected wild-type XER.

Although the level of interference of wild-type XER with the promoter reaches a plateau at approximately 50%, and was therefore not sensitive to XER level (Figure 3, XER), it remained possible that the enhanced ability of the XER160/586 mutant to interfere with transcription at low levels of transfected DNA was due to the production of a higher level of protein. Using a whole cell assay (Xing & Shapiro, 1993) to determine the levels of XER160/586 and of full-length XER produced from their respective expression plasmids, we found that the level of XER160/586 produced was actually slightly lower than the level of full-length XER. Since the XER160/586 mutant is actually expressed at a lower level than full-length XER, its increased ability to interfere with the promoter represents an intrinsic property of the XER160/586 mutant and is not due to elevated levels of expression.

XER160/586 Is a Powerful Dominant Negative Mutant Effectively Suppressing the Activity of Wild-Type XER. The XER160/586 mutant exhibits <10% the activity of wild-type XER on the standard 2ERE-TATA-CAT reporter gene (Figure 1) and shows an enhanced ability to bind to the ERE (Figure 3). It therefore seemed probable that when co-expressed in the same cells as wild-type XER, XER160/586 would act as a dominant negative mutant, suppressing the activity of wild-type XER. The ability of the XER160/586 mutant to antagonize estrogen-dependent transcription by wild-type XER was evaluated in a series of transfections in which different ratios of the expression plasmids encoding wild-type XER and the XER160/586 mutant were used. XER160/586 proved to be a potent dominant negative mutant at all of the DNA ratios tested. At low 0.5:1 and 1:1 DNA ratios, the XER160/586 mutant exhibited a statistically significant ($P < 0.05$) suppression of XER activity to 61% and 43% of wild-type XER activity (Figure 4, 160/586:XER, 0.5:1, 1:1). At DNA ratios of 5:1 and 10:1 it achieved nearly complete suppression of wild-type XER activity, and the only activity seen was that contributed by the XER160/586 mutant (Figure 4, compare 160/586:XER, 5:1 and 10:1, and 160/586, 5 μg and 10 μg).

XER160/586 Does Not Suppress Glucocorticoid Receptor or Progesterone Receptor-Mediated Transcription. The ability of XER160/586 to suppress transcription by XER was not due to a general suppression of transcription. Our transfection data was corrected by the use of a thymidine kinase control promoter, indicating that the data were not due to a general suppression, or "squelching", of transcription. It remained possible that XER160/586 acted as a dominant negative mutant by suppressing a step in transcription activation common to all steroid hormone receptors. We therefore examined the ability of XER and of the XER160/586 mutant to suppress transcription mediated by the glucocorticoid receptor and by the progesterone receptor. Hydrocortisone and the synthetic progestin R5020 effectively induced transcription by the glucocorticoid receptor and by the progesterone receptor, respectively (Figure 5). Neither XER nor the XER160/586 mutant suppressed transcription by glucocorticoid receptor or progesterone receptor at either 1:1 or 5:1 ratios. XER160/586 is therefore an ER-specific dominant negative mutant, and it does not block steps in transactivation common to other steroid receptors.

DISCUSSION

Both TAF1 and TAF2 Transactivation Domains Are Required for Effective Transactivation by XER. The role of the two transactivation domains in the estrogen receptor has been the subject of several studies. It has been suggested that they act as distinct types of transactivation domains, functioning by different mechanisms (Tora *et al.*, 1989; Tasset *et al.*, 1990; Tzukerman *et al.*, 1994). In extensive studies carried out by Chambon and co-workers, TAF1 and TAF2 of human ER were found to act synergistically on a simple ERE-TATA-CAT promoter in HeLa cells. However, in chicken embryo fibroblasts, TAF1 exhibited 56% of the activity of wild-type hER, while TAF2 showed less than 5% of wild-type hER activity (Berry *et al.*, 1990). On a complex VIT-TK-CAT promoter, hER mutants containing only TAF2 exhibited 100% of wild-type ER activity, while hER mutants containing only TAF1 exhibited only the basal activity contributed by the promoter itself. In a recent study, reported after the completion of this work, Tzukerman *et al.* examined the effect of mutations which abolish TAF1 and TAF2 activity and concluded that the ligand-independent TAF1

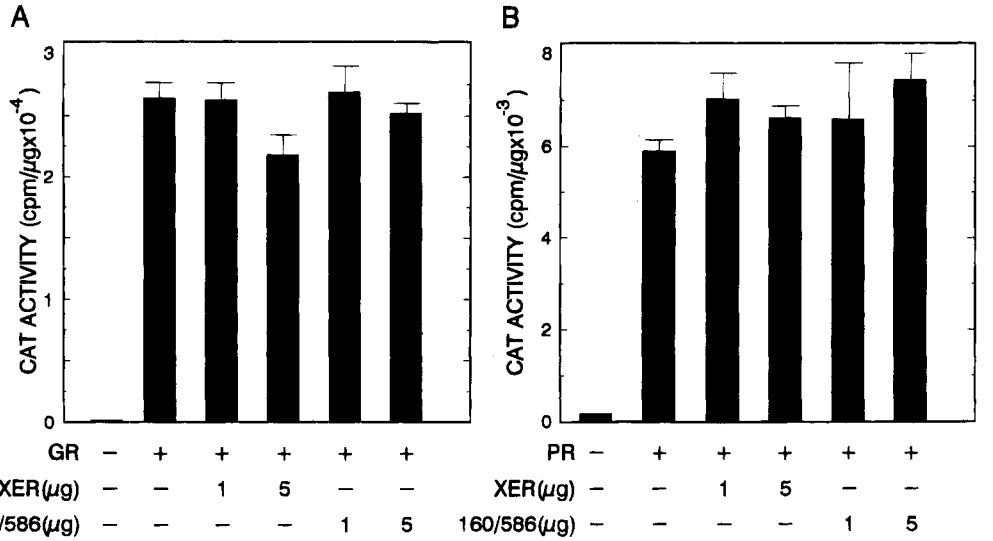

FIGURE 5: Neither XER not the 160/586 mutant blocks the activity of glucocorticoid receptor or progesterone receptor. In panel A XL110 cells were cotransfected with 5 μg of the mouse mammary tumor virus (MMTV)-CAT reporter gene, 1 μg of the glucocorticoid receptor expression vector ($RSVGR_a$), and either the wild-type XER or the XER160/586 expression vector. Cells were treated with 1 μM hydrocortisone and 100 nM E_2 for 60 h after glycerol shock. In panel B XL110 cells were cotransfected with 5 μg of the mouse mammary tumor virus-CAT reporter gene and 200 ng of progesterone receptor expression vector ($CMV\text{-}PR_B$) along with either the wild-type XER or the XER160/586 expression vector. Cells were treated with 10 nM R5020 and 100 nM E_2 for 60 h after glycerol shock. The data for the activity of GR or PR alone was not significantly different (at $P < 0.05$) from the corresponding data in the presence of XER or XER160/586. The data represent the mean ± SEM for three separate transfections.

"...is the dominant transcriptional activator, and TAF2 functions as a transcriptional facilitator...but cannot transactivate on its own" (Tzukerman *et al.*, 1994).

In this work we used a nearly homologous system in which the XER mutants were assayed in a *Xenopus* liver cell line. Using a simple 2ERE promoter, both transactivation domains were required for activity, and XER160/586 exhibited <10% of the activity of wild-type XER (Figures 1 and 2). Using the complex vitellogenin-derived promoter ERE-VIT-CAT, XER160/586 exhibited about 30% of wild-type activity (Figure 1). While these data indicate that an AF1 deletion can exhibit significant activity on some promoters, they are consistent with the widely held view that on simple promoters the AF1 and AF2 transactivation domains synergize with each other to produce fully active ER.

Upstream Activator Proteins Can Partially Replace the AF1 Transactivation Domain. A number of studies have demonstrated synergistic activation of transcription in promoters containing two steroid receptor binding sites located in close proximity (Ankenbauer *et al.*, 1988; Cato *et al.*, 1988; Schule *et al.*, 1988a; Wright & Gustaffson, 1991) and in promoters containing a receptor binding site and an adjacent transcription factor binding site (Schule *et al.*, 1988b; Strahle *et al.*, 1988; Day *et al.*, 1990; Bruggemeier *et al.*, 1991; Chang *et al.*, 1992; Voz *et al.*, 1992). The failure of an N-terminal ER deletion mutant lacking AF1 to show strong synergism with test promoters, such as the complement factor 3 and adenovirus major late promoters, led Tzukerman *et al.* to conclude that AF1 was the major transactivation domain in the ER. Similarly, The N-terminal TAU region of the glucocorticoid receptor is solely responsible for synergistic interactions between two copies of adjacent glucocorticoid response elements (Wright & Gustaffson, 1991). Our data clearly demonstrated that the N-terminal XER160/586 mutant is capable of strong synergistic interaction with three different upstream activator proteins (NF1, AP1, and vitellogenin activator). These studies extend earlier work (Martinez *et al.*, 1990) which concluded that both AF1 and AF2 of ER are capable of synergistic interactions with the NF1 transcription factor. Our data (Figure 2) demonstrates that the XER160/586 mutant exhibits a 2-fold greater degree of synergism than wild-type XER with all three upstream activators. The strong approximately 6-fold synergism observed between XER160/586 and either the NF1, AP1, or VA upstream activator protein provides clear evidence that AF2 can synergise with other transcription activators. It therefore appears that in mutants, such as XER160/586, in which AF1 is deleted, the transactivation domains in other upstream activator proteins can partially substitute for the deleted AF1 domain. Our observation that the activity of XER160/586 rises from <10% of wild-type activity on the simple 2ERE promoter to approximately 30% of wild-type XER on the complex ERE-VIT promoter (which contains NF1 and VA binding sites) also supports this conclusion. Our data therefore suggest a more significant role in transactivation for the ligand-dependent AF2 transactivation domain than proposed by Tzukerman *et al.* (1994). The apparent differences between our finding of strong synergism between an XER containing an AF1 deletion and upstream activators and their failure to observe such synergism is most likely to be due to the use of different promoters and cell lines, but it may also result from our use of the XER.

Deletion of the N-terminal Region of the XER Increases Its Affinity for the ERE. We used the promoter interference assay to investigate the interaction of the ER and the ER mutants with the ERE. The promoter interference assay was originally described by Hu and Davidson (1987), who used it to show that *lac* repressor bound to its operator near a transcription initiation site strongly repressed the activity of a CAT reporter gene. Their data supported the conclusion that reduced CAT activity was the result of inhibition of transcription due to the binding of lac repressor to its operator. In contrast to gel mobility shift assays, the

promoter interference assay has the advantage of actually measuring the interaction of the ER with the ERE within the cell.

When we analyzed the ability of XER160/586 to compete for binding in the promoter interference assay, we obtained the surprising result that it showed a clear increase in affinity for the ERE. This was not due to overexpression of XER160/586, as it was actually expressed at a somewhat lower level than wild-type XER. Because the observation that deletion of the N-terminal A and B domains from the XER actually increased affinity for the ERE was unexpected, we determined whether XER160/586 exhibited increased affinity for the ERE across a broad range of concentrations of transfected DNA. The increased affinity of XER160/586 for the ERE was apparent at both low and high levels of transfected DNA. At high levels of transfected DNA (5 and 10 μg) interference with promoter activity by wild-type XER reaches a plateau at 45−50%, while interference with the promoter by the XER160/586 mutant plateaus at approximately 80%. The increased ability of XER160/586 to interfere with the promoter is unlikely to be due to simple steric interference because >25% of the XER has been deleted in the XER160/586 mutant. One potential explanation for the increased ability of the XER160/586 mutant to block transcription of the CMV promoter is that when inhibition of the promoter reaches a plateau, binding of the XER to the EREs reaches saturation. The extent to which transcription of the promoter is inhibited is a function of the ability of cellular transcription factors to displace the XER from the EREs. In this model the increased ability of the XER160/586 mutant to interfere with transcription would reflect tighter binding to the EREs, making displacement by cellular transcription factors more difficult. The existence of a plateau also suggests that once transcription of the promoter is initiated, a stable transcription complex forms and cannot be displaced by XER. Although this model provides a plausible explanation of our observations, other explanations remain possible. While we would have preferred to confirm the enhanced binding of XER160/586 to the ERE with gel mobility shift assays, the low levels of expression of XER in transfected *Xenopus* and mammalian cell lines have so far made gel mobility shift assays impossible (S. Mattick and D. Shapiro, unpublished observations).

In a recent study in yeast, a repressor protein which binds to the N-terminal region of hER was reported (McDonnell *et al.*, 1992). Whether the absence of this protein bound to the ER plays a role in the enhanced binding of XER160/586 to the ERE is not known. It will be interesting to determine whether deletions of the A and B domains of other members of the steroid/nuclear receptor gene superfamily also display enhanced binding to their hormone response elements.

Deletion of the AF1 Transactivation Domain Generates an Effective Dominant Negative XER. Several studies have described dominant negative mutants of nuclear receptors (Damm *et al.*, 1989; Sap *et al.*, 1989; Ince *et al.*, 1993; Nagaya & Jameson, 1993; Ways *et al.*, 1993). In one well-studied system, expression of the *v-erbA* oncogene, which lacks a functional thyroid hormone binding domain, can suppress the intracellular activity of wild-type thyroid receptor by up to 90% (Damm *et al.*, 1989). In a recent study of dominant negative estrogen receptors, three ER mutants with disruptions or deletions in the AF2 transactivation domain were shown to be potent dominant negative mutants. When equal amounts of DNA encoding the ER mutants and wild-type ER were cotransfected into Chinese hamster ovary (CHO) cells, the mutants suppressed the activity of wild-type ER by 55−80% (Ince *et al.*, 1993). Here we report that deletion of the hormone-independent AF1 domain in the N-terminus of ER also generates an effective dominant negative mutant. Using the same 2ERE reporter gene (Chang *et al.*, 1992) used in the study of Ince *et al.* (1993), we found that when equal amounts of DNA encoding the XER160/586 mutant and wild-type XER were transfected into cells, the activity of wild-type XER was suppressed by 57%. Since the level of expression of wild-type XER is higher than the level of expression of XER160/586, overexpression of XER160/586 is not responsible for its effectiveness in suppressing the activity of wild-type XER. Even without correction for the lower level of expression of XER160/586, it suppresses wild-type ER activity about as well as the recently reported C-terminal deletion hER1−530 (Ince *et al.*, 1993). Taken together, the data demonstrates that, on promoters which require both transactivation domains for efficient activation of transcription, disruption or deletion of either of the two transactivation domains in ER is sufficient to generate a powerful dominant negative mutant. The data also suggests that transactivation domain disruptions or deletions, which do not interfere with DNA binding, ligand binding, or dimerization, may be the preferred method for generating dominant negative mutants of other steroid/nuclear receptors and transcription factors.

There are several potential mechanisms by which XER160/586 might act as an effective dominant negative mutant. Since XER160/586 does not suppress transcription by either glucocorticoid receptor or progesterone receptor, it is unlikely to act by poisoning or squelching the basal transcription apparatus or a protein required for all steroid receptors to function. Because XER160/586 exhibits enhanced binding to the ERE, a considerable part of its activity as a dominant negative mutant is likely to be due to its ability to outcompete wild-type XER for binding to the ERE. This view is supported by a recent study of dominant negative mutants of the thyroid receptor which concluded that competition for binding to the thyroid response element was the most important factor contributing to their activity as dominant negative mutants (Katz & Lazar, 1993). XER160/586 retains the region of the hormone binding domain essential for dimerization (Fawell *et al.*, 1990). It is therefore possible that heterodimers form between XER160/586 and wild-type XER and are unable to activate transcription when bound to the ERE. In addition, it is possible that, although it is unable to activate transcription, XER160/586 still interacts with an unidentified ER-specific component of the transcription apparatus. The existence of such a class of bridge proteins has been proposed (Dynalecht *et al.*, 1991). If this component were present in limiting amounts, unproductive complexes with XER160/586 could reduce the ability of wild-type XER to activate transcription. Precise determination of the relative contributions of each of these mechanisms to the dominant negative phenotype of XER160/586 requires further investigation.

In this work we show that an XER mutant (XER160/586), in which the AF1 transactivation function located in the N-terminal region of the XER is deleted, exhibits dramati-

cally reduced basal and hormone-dependent activity. We find that the deletion of AF1 can be partially compensated by enhanced synergism of the XER160/586 mutant with upstream activator proteins. The XER160/586 mutant is unusual in that it exhibits an enhanced *in vivo* binding to the ERE in the presence of estrogen. Our data provides the first indication that the N-terminal A/B region of ER may contain sequences which modulate binding to the ERE. The ability of XER160/586 to act as a powerful dominant negative mutant suggests that disruption of the transactivation domains of gene regulatory proteins provides a general strategy for the production of dominant negative mutants.

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