Different Regions in Activation Function-1 of the Human Estrogen Receptor Required for Antiestrogen- and Estradiol-dependent Transcription Activation*

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The human estrogen receptor (ER)1 is a ligand-inducible transcription factor that contains two transcriptional activation functions, one located in the NH2-terminal region of the protein (AF-1) and the second in the COOH-terminal region (AF-2). Antiestrogens, such as trans-hydroxytamoxifen (TOT), have partial agonistic activity in certain cell types, and studies have implied that this agonism is AF-1-dependent. We have made progressive NH2-terminal and other segment deletions and ligations in the A/B domain, and studied the transcriptional activity of these mutant ERs in ER-negative MDA-MB-231 human breast cancer and HEC-1 human endometrial cancer cells. Using several estrogens and several partial agonist/antagonist antiestrogens, we find that estrogens and antiestrogens require different regions of AF-1 for transcriptional activation. Deletion of the first 40 amino acids has no effect on receptor activity. Antiestrogen agonism is lost upon deletion to amino acid 87, while estrogen agonism is not lost until deletions progress to amino acid 109. Antiestrogen agonism has been further defined to require amino acids 41–64, as deletion of only these amino acids results in an ER that exhibits 100% activity with E2, but no longer shows an agonist response to TOT. With A/B-modified receptors in which antiestrogens lose their agonistic activity, the antiestrogens then function as pure estrogen antagonists. Our studies show that in these cellular contexts, hormone-dependent transcription utilizes a range of the amino acid sequence within the A/B domain. Furthermore, the agonist/antagonist balance and activity of antiestrogens such as TOT are determined by specific sequences within the A/B domain and thus may be influenced by differences in levels of specific factors that interact with these regions of the ER.

The estrogen receptor (ER)1 is a ligand-inducible transcription factor that regulates gene expression through interaction with cis-acting DNA elements called estrogen response elements (EREs) (for reviews, see Refs. 1–5). Like other steroid hormone receptors, the ER contains specific domains responsible for functions leading to transcription of target genes, such as ligand binding, DNA binding, and transactivation (6–8). The ER contains two distinct, non-acidic activation functions, one activation function at the NH2 terminus (AF-1) and a second, hormone-dependent activation function at the COOH terminus (AF-2), in the hormone binding domain (8–12). AF-2 is highly conserved among species and other nuclear hormone receptors (1, 12, 13), whereas the A/B domain at the amino terminus of the ER, which includes AF-1, is less well conserved among different species and other nuclear receptors (1, 13, 14). The activity of each activation function of ER is cell- and gene promoter-dependent. AF-1 can exhibit transcriptional activity in the absence of AF-2 (8) in some cell contexts but, in most cell and promoter contexts, both AF-1 and AF-2 function in a synergistic manner and are required for full receptor activity (6, 8, 15–22).

Transactivation of estrogen-responsive genes by ER can be antagonized by antiestrogens such as trans-hydroxytamoxifen (TOT) and ICI 164,384 (18, 19). One mechanism by which these antiestrogens inhibit ER action is by competition with estradiol (E2) for binding to the ER. Although antiestrogen-occupied ER binds estrogen response DNA elements in cells (23, 24), it is thought that antiestrogens promote a conformational change which is different from that induced by E2 (24, 25). Some antiestrogens, like TOT, have partial agonistic activity in certain cells, such as chicken embryo fibroblasts (CEF) and MDA-231 human breast cancer cells (18, 26). The cell and promoter dependence of TOT agonism has been attributed to the cell and promoter specificity of AF-1 activity (15–18). Previous studies using chimeric receptors have shown that TOT is unable to induce AF-2 activity, but that TOT can be a strong agonist in cellular and promoter contexts where AF-1 is an efficient transcriptional activator (11, 18, 21).

We have investigated the A/B domain of the ER and its role in the transcriptional activity of ER elicited by estrogens and some antiestrogens, and we find that different regions within this domain are required for transcriptional stimulation by estrogen versus antiestrogen. In the studies presented, we demonstrate that a specific 24-amino acid region of AF-1 of the human ER is necessary for agonism by TOT and other partial agonist/antagonist antiestrogens, but is not required for E2-dependent transactivation. As a consequence, the activity of estradiol and the estrogen agonist/antagonist character of TOT depended markedly, but not always concordantly, on the se-
quences present within the A/B domain in the ER. Our studies show that in the context of the full-length ER, hormone-dependent transcription utilizes a broad range of sequences within the A/B domain and suggest that differences in the agonist/antagonist character of antiestrogens observed in different cells could be due to altered levels of specific factors that interact with these regions.

MATERIALS AND METHODS

Chemicals and Materials—Cell culture media were purchased from Life Technologies, Inc. Calf serum was from Hyclone Laboratories (Logan, UT) and fetal calf serum was from Sigma. [14C]Chloroamphenicol (50–60 Ci/mmol) was from DuPont NEN. The antiestrogens TOT and ICI 164,384 were kindly provided by Dr. Alan Wakeling, Zeneca Pharmaceuticals, Inc., 100 mM HEPES, 5% calf serum, 100 units of penicillin/ml (Life Technologies, Inc.), and 100 μg of streptomycin/ml (Life Technologies, Inc.). MDA-MB-231 cells or HEC-1 cells were grown in red-free Improved medium, less essential medium and 5% charcoal/dextran-treated calf serum for 2 days prior to transfection.

Cell Culture and Transient Transfections—MDA-MB-231 human breast cancer cells were maintained in Leibovitz’s L-15 Medium with 10 mM HEPES, 5% calf serum, 100 units of penicillin/ml (Life Technologies, Inc.), 100 μg of streptomyacin/ml (Life Technologies, Inc.), 25 μg of gentamycin/ml, 6 ng of bovine insulin/ml, 3.75 μg of hydrocortisone/ml, and 16 μg of glutathione/ml. Human endometrial cancer (HEC-1) cells were maintained in minimum essential medium plus phenol red supplemented with 5% calf serum and 5% fetal calf serum, 100 units of penicillin/ml (Life Technologies, Inc.), and 100 μg of streptomyacin/ml (Life Technologies, Inc.). MDA-MB-231 cells or HEC-1 cells were grown in minimum essential medium plus phenol red supplemented with 5% charcoal/dextran-treated calf serum for 2 days prior to transfection. Cells were plated at a density of 3 × 10^5 cells/100-mm dish in phenol red-free Improved minimal essential medium and 5% charcoal/dextran-treated calf serum and were given fresh medium 24 h before transfection. All cells for transfection were maintained at 37°C in a humidified CO₂ atmosphere. Cells were transiently transfected by the CaPO₄ co-precipitation method (29). One ml of precipitate contained 0.8 μg of pCMV5 as internal control, 6 μg of an ER-containing reporter plasmid (ERE)₅-pS2-CAT reporter plasmid, 100 ng of ER expression vector, and pTZ19R carrier DNA to a total of 15 μg of DNA. Cells remained in contact with the precipitate for 4 h and were then subjected to a 2.5-min glycerol shock (20% transfection medium). Cells were rinsed with Hank’s balanced salt solution and given fresh medium with hormone treatment as indicated.

Promoter Interference Assays—MDA-MB-231 cells were transiently transfected with 2 μg of CMV-(ERE)₅-CAT reporter plasmid, 0.8 μg of pCMVΔ, 12.2 μg of pTZ19R, and 100 ng of ER expression vector/100-mm dish of cells. Cells were treated as described previously for transient transfection, and CAT assays were performed on cell extracts.

RESULTS

Different Regions in the A/B Domain Are Important for Estradiol- and trans-Hydroxytamoxifen-Dependent Transcriptional Activity—Our studies were aimed at identifying regions within the A/B domain that are responsible for E₂-dependent transcription and for TOT agonism. We have generated ER derivatives that contain increasing NH₂-terminal deletions or other deleterional changes in the A/B domain. Fig. 2 shows the structure of the ER derivatives used in this study and the relative expression levels of the receptors observed in cells. Western immunoblot analysis showed that receptors of the predicted sizes were being produced in the cells and that all of the A/B domain altered receptors (Fig. 1B) were expressed at levels very similar to that of the wild type ER.

These ER mutants were then analyzed for their ability to transactivate an ERE-containing pS2 promoter-reporter gene in ER-negative MDA-231 human breast cancer cells. Wild type ER or receptors with deletions of amino acids 1–20 (N21), 1–40 (E41), 1–86 (A87), 1–108 (M109), or 1–179 (AAB) were transiently transfected into MDA-231 cells, and transcriptional activity was measured in response to increasing concentrations of E₂. ER mutants N21, E41, and A87 showed dose-response curves for transcriptional activity virtually identical to that observed with wild type ER (Fig. 2A). In contrast, deletion of the first 108 amino acids resulted in receptors that showed a great loss of activity; M109 receptors showed only about 20% of wild type ER transcriptional activity at 10⁻⁸ M E₂, suggesting that residues between amino acids 87 and 108 are important for estradiol-stimulated activity. Deletion of the complete A/B domain (amino acids 1–179) gave a receptor that showed no activity in this cell system.

Similar studies were conducted using the NH₂-terminal deletion mutants to examine transcriptional response to the triphenylethylen compound trans-hydroxytamoxifen, TOT (Fig. 2B). MDA-231 cells were again used in these studies, since with wild type ER, TOT behaves as a relatively strong agonist. TOT (10⁻⁷ M) stimulates transcriptional activity to approximately 30% the level evoked by maximal (10⁻⁵ M) E₂ stimulation. Compared with the wild type ER, deletion of amino acids 1–20 or 1–40 had no effect on either the E₂ response or TOT agonism. However, deletion of amino acids 1–86, which had no effect on E₂-induced activity, abolished TOT agonism completely (Fig. 2B). The further deleted mutant, M109, which was transcriptionally impaired in response to E₂, treatment, did not exhibit any measurable response to TOT. The loss of TOT agonism observed selectively with the A87 mutant suggested that sequences between 41 and 87 may be important contributors to TOT agonism, but are not essential for the response to E₂.

Deletion mutant Δ41–64, which lacks only amino acids 41–64, was constructed and tested for its transactivational ability in response to E₂ and TOT. Δ41–64 retained 100% of wild type E₂-dependent activity (Fig. 2C) yet displayed no measurable response to TOT (Fig. 2D). These results are consistent with the loss of TOT response with the A87 mutant as they implicate residues 41–64 as a major contributor to TOT agonism but not to E₂ response.

A/B Deletion Mutants Exhibit Differential Response to Other Estrogens and Antiestrogens—Further examination of the ligand-dependent transcriptional activity of these mutants revealed...
FIG. 1. Structure and expression of ER derivatives. A, the functional domains (A/B, C, D, E, F) and activation functions (AF-1 and AF-2) of ER are shown at the top along with schematics for the A/B domain mutants studied in this report. The values to the right of the receptor...
that another full estrogen, the resorcylic lactone P1496 (31), showed a pattern of activity identical to that observed with E2. Like E2, transcriptional response to P1496 was fully retained in N21, E41, and A87 receptors, but was impaired with the deletion of the first 108 residues (Fig. 3A). Similar results to those seen with TOT were observed with the antiestrogen compounds BF and BT (32). Like TOT, these heterocycle-based antiestrogens were significant agonists, evoking transcriptional activity that was similar in magnitude to that obtained with TOT (~30% of E2 stimulation). As seen in Fig. 3A, antiestrogen stimulation of CAT activity was lost with the mutants A87 and Δ41–64 for the three antiestrogen compounds (TOT, BF, and BT), while estrogen (E2 and P1496) stimulation of transcriptional activity was still maintained maximally in these two constructs. No stimulation of wild type ER or any ER mutants was seen with the pure antiestrogen ICI 164,384 (data not shown).

These A/B domain mutants were also tested in a different cell background utilizing an ER-negative human endometrial cancer cell line (HEC-1 cells). In these cells, wild type ER also responds to TOT as an agonist, showing about 30–40% of wild type E2 response (Fig. 3B). Similar results to those seen previously in MDA-231 breast cancer cells were observed with the A/B domain deletion mutants in these endometrial cancer cells; both A87 and Δ41–64 receptors retained full wild type transcriptional activity in response to E2, but did not exhibit any response to TOT. These results demonstrate again that a region between amino acids 40 and 65 is critical for TOT agonism yet is not required for E2-dependent transcription.

Specific Regions in the A/B Domain Are Required to Support TOT Agonism—Since TOT was not a full estrogen agonist in these assays, and is known to show mixed estrogen agonist and antagonist activity in many cells (15–18), we also examined the antagonist activity of TOT and how this was impacted by changes in the A/B domain of ER (Fig. 4). TOT agonism was apparent in wild type ER, N21, and E41 receptors and, in these three receptors, TOT (at a 10-fold excess concentration relative to that of E2) was also able to suppress E2-stimulated activity to that of its own inherent level of agonism (i.e., approximately 30% of the E2-stimulated level). Thus, with these receptors, this compound showed partial agonist and partial antagonist activity. Of interest, in the A87, M109, and Δ41–64 receptors where TOT showed no agonistic activity, TOT behaved as a pure antiestrogen and was now a complete antagonist of the E2 stimulation. Thus, the agonist/antagonist character of the antiestrogen TOT differed with the nature of the ER A/B domain.

Deletions in the A/B Domain Do Not Affect Receptor Level or DNA Binding—Since certain A/B deletion mutants exhibited a differential response to estrogens and antiestrogens, the levels of these receptors and the DNA binding abilities of these mutant ERs were determined following exposure to E2 or TOT in order to determine whether differences in response to these two ligands might be attributable to ligand-induced alteration in receptor stability or DNA binding ability. As seen in Fig. 5A, levels of wild type ER, Δ41–64 ER and A87 ER were similar following cell treatment with E2 or TOT. Thus, differential turnover of these receptor proteins in response to TOT versus E2 is not likely to explain the very different transcriptional response of these receptors to these two ligands.

DNA binding studies were conducted with several of the mutants by use of a promoter interference assay, in order to assess whether differences in DNA binding of the TOT-ER

schematics indicate the transcriptional activity of the receptors in response to $10^{-8} M$ E2 or $10^{-7} M$ TOT and summarize data that are derived from dose response experiments detailed later in this paper. B, the expression of wild type and mutant estrogen receptors from cytomegalovirus promoter-containing expression vectors was determined following transfection into ER-negative COS-1 cells. Equal amounts of protein were used and immunoblotting was done with the anti-ER monoclonal antibody H222.
versus E$_2$-ER complexes might explain their different transcriptional efficacy (Fig. 5B). This promoter interference assay measures the ability of ER to bind to ERE DNA in intact cells (23). Binding of ER to the ERE is assayed by assessing the ability of ERE-bound ER to block transcription from the constitutively active cytomegalovirus (CMV) promoter, with the repression of CAT activity being a measure of the binding of ER to the ERE-containing promoter. A87, which responds to E$_2$ but not to TOT, and M109, which is impaired in both E$_2$- and TOT-dependent activity, were both able to bind to the EREs and to interfere with promoter activity to the same extent as the wild type ER (Fig. 5B). Therefore, differences in E$_2$- and TOT-dependent transactivation exhibited by these ER derivatives do not appear to be caused by differences in receptor protein level or by differential DNA binding.

**Residues 41–109 Encompass Sequences Important for Both Estradiol- and TOT-dependent Transcription—** Additional analysis of the A/B region was made to further characterize sequences important for E$_2$- and TOT-dependent transcription. Since transcriptional response to E$_2$ was almost completely lost in going from the A87 to the M109 ER, we wished to directly assess the importance of amino acids 87–108 in E$_2$-dependent activity. To do so, we tested an ER mutant lacking only amino acids 87–108 (D$_{87-108}$). Full dose-response studies employing 10$^{-12}$ to 10$^{-7}$ M E$_2$ and 10$^{-12}$ to 10$^{-7}$ M TOT were conducted for this mutant and all other mutants described below, as done for the mutant ERs shown in Fig. 2. Values are the mean ± S.E. for three or more determinations from separate experiments. Some error bars are too small to be visible. Further analysis of the A/B region was made using segment ligated mutants (Fig. 1A, entries 8–10). To examine the region between residues 40 and 65, which were required for TOT agonism, we constructed a segment ligated ER derivative, 41–66-CDEF, containing only amino acids 41–66 of the A/B domain linked directly to the intact ER domains C through F and assayed this receptor for its ability to transactivate an ERE-containing reporter gene in the presence of E$_2$ or TOT. This mutant was surprising in its ability to activate the reporter gene in response to E$_2$ (Fig. 1A, entry 8), even though deletion of amino acids 41–64 resulted in no change in E$_2$-stimulated activity. The ER mutant...
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![Diagram](http://www.jbc.org/)  

**Fig. 5.** Protein levels and DNA-binding abilities of wild type ER and ER mutants treated with estrogen or antiestrogen. A, levels of wild type ER and ER mutants were examined following transfection and treatment of COS-1 cells with either 10⁻⁸ M E₂ or 10⁻⁷ M TOT for 24 h. Immunoblotting was done with the anti-ER monoclonal antibody H222. B, MDA-231 cells were transfected with the constitutively active CMV-(ERE)₂-CAT promoter interference plasmid and wild type ER or mutant ERs. Cells were treated with control vehicle, 10⁻⁸ M E₂, or 10⁻⁷ M TOT, and CAT activity was analyzed as described in the legend to Fig. 2. Values are the mean ± S.E. for three or more determinations from separate experiments. For some values, error bars are too small to be visible.

41–66-CDEF, however, exhibited no measurable response to TOT. This suggests that residues 41–64 are necessary for TOT agonism, but that they alone are not sufficient for TOT-directed transcription. Extension of the A/B domain toward the COOH terminus (Fig. 1A, entry 9) to include amino acids 41–87 (41–87-CDEF) did not result in any increase in E₂- or TOT-dependent transcription compared with 41–66-CDEF. However, extension to amino acid 109 (41–109-CDEF) did result in a 2-fold increase in E₂-dependent transcriptional activity compared with 41–66-CDEF and a dramatic increase in TOT agonism such that the activity measured was approximately 80% of wild type ER activity for both E₂ and TOT (Fig. 1A, entry 10). This indicates that the region encompassing residues 41–109 contains almost all of the A/B domain sequence needed both for E₂ and TOT stimulatory activity.

Interestingly, the transcriptional activity of 80% observed with 41–109-CDEF is in agreement with the observation that only 20% of wild type E₂-stimulated activity is retained upon deletion of the first 108 residues. These results suggest that residues 87–108 play a significant role in E₂-stimulated transcriptional activity but are supported by other sequences in the A/B domain. This is highlighted by the Δ87–108 mutant (Fig. 1A, entry 7), which lacks residues 87–108 in the A/B domain. This mutant is only weakly impaired in response to E₂ and TOT compared with wild type ER, consistent with residues 41–109 being important for full AF-1 function. Together, these results demonstrate that E₂- and TOT-dependent transcription utilizes other flanking sequences beyond amino acids 87–108 within the A/B domain to achieve full receptor activity. These required regions could serve as a portion of the activation function or could serve a structural purpose, perhaps maintaining proper three-dimensional structure of the receptor protein.

**DISCUSSION**

The human estrogen receptor contains two transcriptional activation functions, AF-1 located in the A/B domain and AF-2 in the hormone-binding domain. Both transcriptional activation functions act in a promoter- and cell type-dependent manner. The amino acid sequences of these activation functions are not similar to other known activation sequences, so elucidation of their precise mechanism of action is of interest. Our studies have defined AF-1 regions within the A/B domain of ER that support the transcriptional response to estrogens (E₂, P1496) and those that support the transcriptional response to several antiestrogens. While considerable overlap in the transcription-supporting regions is observed for both categories of ligands, we found that there are some distinct sequence requirements.

There are limitations in the applications of mutational methods to precisely define regions of the A/B domain that support the transcriptional agonism of these different ligands, as these activities appear to be distributed over more than one discrete segment. To address these issues we have, in fact, made three different types of alterations in the A/B domain, namely progressive NH₂-terminal deletions, segmental deletions, and segmental ligations. In many cases, we obtained consistent results regarding the transcription-supporting role of a particular region of the A/B domain by making the different types of mutations; however, we did not always get identical results using all three approaches.

When making progressive NH₂-terminal deletions, TOT agonism is lost when the A/B domain is truncated from E₄₁ to A₈₇, whereas the effect of E₂ is reduced only upon further deletion to M₁₀₉. Therefore, TOT agonism appears to require a region between residues 41–86, whereas E₂ induction requires the 87–108 sequence. Segmental deletion of residues 41–64 does, in fact, eliminate TOT agonism without affecting E₂ induction. However, the 87–108 segmental deletion, which has a limited effect on TOT agonism, causes only a modest reduction in E₂ induced transcription. Thus, whereas the region 87–108 appears to be critical to the E₂ effect in the absence of residues 1–86 (i.e. by progressive NH₂-terminal deletion), it appears that much of the E₂ effect can be supported by the 1–86 segment (perhaps together with the 109–180 segment) that is still present in the Δ87–108 segment-deleted mutant. The segment ligation approach confirms the importance of the 41–109 region, as this segment alone restores most of the agonistic effect of TOT and gives nearly full induction with E₂. It is clear from our findings that distinctly different regions of the A/B domain are responsible for supporting the transcriptional activation induced by E₂ and the agonism effected by TOT and that in certain situations these regions may act in concert with other A/B segments.

Metzger et al. (21) analyzed the role of A/B segments in chicken embryo fibroblast (CEF) and yeast cells in which AF-1 is able on its own to stimulate transactivation. They observed in CEF cells that deletion of the first ~60 or 80 residues resulted in a decrease in E₂-stimulated transcription of 40 and
70%, respectively. In our studies in 231 human breast cancer and HEC-1 human endometrial cancer cells, deletion of the first 40 amino acids, had no effect on transcriptional activity, while deletion of the first 108 amino acids nearly completely eliminated transcriptional response to E2. Response to E2 was fully retained in our A87' mutant, yet this mutant lost its ability to respond to TOT. In this and some other A/B domain mutants, we observed considerable differences in the ability of TOT versus E2 to stimulate transcription, whereas in the several mutants analyzed for response to E2 and TOT in CEF cells, which contained deletions of only certain NH2- or COOH-terminal portions of the A/B domain, differences between E2 and TOT were not seen. The differences in our findings and those of Metzer et al. (21) may reflect differences in the cell types and promoters studied, but may also reflect the fact that deletions in only the central portion of the A/B domain were not studied by Metzer et al. (21).

Tamoxifen is well known to show cell- and gene-specific agonism, being a relatively pure estrogen antagonist in some cells, and a partial agonist/antagonist or a relatively strong agonist in others (5, 22). Our current findings suggest that cellular processes that impinge on the specific A/B domain sequences we have identified should be key determinants of whether ligands such as tamoxifen will function as agonists, antagonists, or partial agonists/antagonists in any specific cell system. In a recent study, we have shown that the binding of both estrogens and antiestrogens to ER promotes an interaction between AF-1 in the A/B domain and AF-2 in domain E (27). This AF-1/AF-2 interaction appears to be an essential prerequisite for the competence of ER-ligand complexes to induce transcription. It is known that there are conformational differences in ER-estrogen and ER-antiestrogen complexes (24, 25, 33), which are presumed to occur in the ligand binding AF-2 region. Since the interaction of AF-2 with AF-1 is required for optimal transcriptional activity in the cell contexts we have examined, it is not surprising that distinctly different sequences within AF-1 are involved in supporting the transcription activation induced by these different ligand classes.

The mechanisms by which ligand-induced AF-1/AF-2 interaction occurs or by which ER-ligand complexes are able to elicit gene transcription are not well understood. These activation functions have been shown to have squelching effects on their own activity and on acidic activators (9). This transcriptional interference provides evidence that AF-1 and AF-2 interact with a titratable cellular factor(s) indispensable for different classes of activation functions (8, 9). A number of activation function-interacting proteins may be involved in these processes (Ref. 22 and references therein) and may account, as well, for the varying levels of agonism that TOT displays in different cells and on different promoters. For example, in systems in which TOT has agonist activity, a co-regulator or transcription factor that interacts specifically with the 41–64 region of AF-1 in the ER-TOT complex may support transcription, whereas systems in which TOT is a pure antagonist may lack this factor. E2-induced transcription, which operates via somewhat different AF-1 sequences, may not utilize this factor or may utilize other factors. Our identification of differences in the sequences within ER that are required for TOT versus estradiol agonism should aid in elucidating the underlying mechanisms regulating the cell-specific pharmacology and biocharacter of antiestrogens.

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