Anti-estrogens like hydroxytamoxifen (OHT) have mixed agonist/antagonist activities, leading to tissue-specific stimulation of cellular proliferation. Partial agonist activity of OHT can be observed in vitro in endometrial carcinoma cells like Ishikawa. Here, we have compared several anti-estrogens (including extensively characterized OHT and pure anti-estrogens such as ICI164,384 and RU58,668, which are devoid of uterotrophic activity) for their capacity to stimulate promoters containing estrogen response elements (EREs) or AP1-binding sites (12-O-tetradecanoylphorbol-13-acetate response elements, TREs), the two types of DNA motifs known to mediate transcriptional stimulation by estrogen receptors. Assays were performed in Ishikawa cells either by transient transfection or by using cell lines with stably propagated reporter vectors. In transient transfection experiments, none of the anti-estrogens displayed agonist activity on the promoters tested. In contrast, significant transcriptional stimulation was observed with low concentrations of OHT and RU39,411 in Ishikawa cells stably propagating reporter constructs containing a minimal ERE3-TATA promoter. In addition, micromolar concentrations of OHT, but not of RU39,411, stimulated stably propagated AP1-responsive reporter constructs. No transcriptional stimulation of ERE- or TRE-containing promoters was observed with the pure anti-estrogens ICI164,384 and RU58,668. These results indicate that the presence of estrogen response elements in promoters is sufficient to mediate cell-specific agonism of anti-estrogens at the transcriptional level, and that stimulation of AP1 activity may be restricted to a subset of anti-estrogens possessing agonist activity on EREs. In addition, our results suggest that transient transfections do not fully recapitulate in vivo conditions required to observe agonist activity of anti-estrogens.

The estrogen 17β-estradiol (E2) regulates gene transcription by binding to the estrogen receptor (ER), which interacts with specific target DNA sequences known as estrogen response elements (EREs). When bound to DNA the ER stimulates transcription via two transcription activation domains, AF-1 and AF-2 (1–3). AF-1 is located in the poorly conserved N-terminal A/B domain of the ER (2, 4), whereas AF-2 is found in the C-terminus of region E, the hormone-binding region (5–8). Binding of E2 to the ER is thought to induce a conformational change in the hormone binding domain, stimulating its transactivation properties.

Different types of synthetic compounds have been developed that are capable of antagonizing ER action in reproductive tissues and, in particular, of blocking estradiol stimulation of cellular growth in breast and uterine tissues. These anti-estrogens act by competing with E2 for binding to the ER and block ER-mediated activation of transcription when co-administered with hormone (9). However, tamoxifen, one of the most widely used anti-estrogens in breast cancer treatment (10), can induce uterine cell growth in vivo in animal models (11) and in humans (12, 13). Hydroxytamoxifen also induces cellular proliferation (14, 15) and transcription of endogenous estrogen target genes such as the progesterone receptor (PR) gene in human endometria and cultured human endometrial carcinoma cells (15, 16). Other anti-estrogens, like ICI164,384 (17) or the more recently developed RU58,668 (18), were reported not to stimulate uterine cell growth and may therefore prove more appropriate for breast cancer therapy (17).

To better understand the mechanisms of tissue-specific estrogenic activity of anti-estrogens, we compared transcriptional activation of ERE-containing reporter constructs by full or partial anti-estrogens in the estrogen-dependent breast carcinoma cell line MCF7 and in the endometrial carcinoma cell line Ishikawa. We also assessed whether anti-estrogens may regulate transcription of target genes through AP1-binding sites (TPA response elements, TRES) rather than, or as well as, through EREs. Indeed, estrogenic stimulation of promoters containing TRE sites has been documented (19–22), a possible mechanism being direct interaction between ER and AP1 components (22). Assays used for investigating the contribution of EREs or TRES in transcriptional stimulation by anti-estrogens with partial agonist activity included, in addition to transient transfection assays in MCF7 or Ishikawa cells, direct hormonal stimulation of reporter vectors stably propagated as episomes in these cell lines. The latter assay was selected because of increasing evidence for mechanistic links between transcriptional stimulation and reorganization of chromatin structure.

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Comparison of the two types of assays and implications for the mechanism of cell-specific agonism by tamoxifen are discussed.

EXPERIMENTAL PROCEDURES

Chemicals and Materials—17β-Estradiol (E2) was purchased from Sigma; RUS5,665 (RUP) and RUS9,411 (RUP) were generous gifts from Dr. D. Philibert, Hoechst-Marion-Roussel, Romainville, France. ICI164,384 was kindly provided by Dr. T. Wilson, Glaxo-Wellcome Research Institute, Research Triangle Park, NC. OHT and TAM were purchased from Sigma. Cell culture media, fetal bovine serum, G418, hygromycin B were purchased from Life Technologies, Inc.

Plasmid Recombinants—Expression vectors pSG5, pSG5-HEG0, and reporter recombinants Vit-tk-CAT (23) and STR-CAT (equivalent to construct STR-CAT in Ref. 24) were kindly provided by Dr. P. Chambon (Ilkirch, France). ERE3-tk-CAT was constructed by insertion of three copies of double-stranded oligonucleotides containing the 15-bp Xenopus vitellogenin A2 ERE sequence (25) flanked by HindIII and XbaI sites between the HindIII and Xbal sites of pBLCAT8+. ERE3-TATA-CAT was constructed in several steps from GRE5-CAT (26). First, the BgIII site upstream of the CAT gene in GRE5-CAT was deleted by filling-in with Klenow, creating GRE5-CAT-BgIII. A fragment containing the ERE3-TATA promoter was then excised from the vector ERE3-pAL10 (27) by digestion with BamHI, filling-in with Klenow, creating GRE5-CAT[−10] and digestion with XhoI; this fragment was inserted into GRE5-CAT[−10] by digestion with HindIII and XhoI to remove the GRE5-BgIII fragment. The resulting GRE5-TATA-CAT plasmid is identical to GRE5/CAT (26). AP1-responsive promoters were inserted either in non-episomal vectors derived from GRE5/CAT (TRE6-TATA-CAT) or in EBV vectors (TRE2-TATA-CAT/EBV and TRE6-TATA-CAT/EBV).

Cell Culture and Transfections—Ishikawa and MCF7 Cells—Ishikawa or MCF7 cells were transfected with 15 μg of pSG5 or pSG5-HEG0 expression vectors. Cells were harvested 36 h later, and extracts were prepared in gel retardation buffer 4 × (20 mM Tris-HCl, pH 7.5, 20% glycerol, 400 mM KCl, 0.1 mM EDTA, pH 8.0, 2 mM dithiothreitol, supplemented with protease inhibitor mixture) by three cycles of freeze-thawing on ice. For gel retardation assays, extracts were incubated with 2 μg of GRE5-TATA-CAT plasmid (26), 1 μg of internal control RSV-LacZ, and Bluescribe M13+ as carrier DNA (total 15 μg). Progesterone was added 1 day later, after removal of calcium-phosphate precipitates by two consecutive washes. Cells were harvested 24 h later, and CAT activity was assayed as described above. All CAT assays were reproduced a minimum of three times.

COS-1 cells were grown in DMEM supplemented with 5% FBS. Cells were plated at a confluency of 1.5 × 10^6 cells/10-cm plate and transfected with 15 μg of pSG5 or pSG5-HEG0 expression vectors. Cells were harvested 36 h later, and extracts were prepared in gel retardation buffer 4 × (20 mM Tris-HCl, pH 7.5, 20% glycerol, 400 mM KCl, 0.1 mM EDTA, pH 8.0, 2 mM dithiothreitol, supplemented with protease inhibitor mixture) by three cycles of freeze-thawing on ice. For gel retardation assays, extracts were incubated with 2 μg of GRE5-TATA-CAT plasmid (26), 1 μg of internal control RSV-LacZ, and Bluescribe M13+ as carrier DNA (total 15 μg). Progesterone was added 1 day later, after removal of calcium-phosphate precipitates by two consecutive washes. Cells were harvested 24 h later, and CAT activity was assayed as described above. All CAT assays were reproduced a minimum of three times.

Generation and Hormonal Treatment of Stably-transfected Cell Lines Derived from Ishikawa and MCF7 Cells—Ishikawa or MCF7 cells were transfected with 15 μg of ERE3-TATA-CAT/EBV, TRE2-TATA-CAT/EBV, or TRE6-TATA-CAT/EBV (10-cm plates, 1.5 million cells). Forty-eight hours after transfection, cells were passaged into 15-cm plates using medium containing 150 μg/ml hygromycin B and maintained in this medium for about 2 weeks until disappearance of all cells in control non-transfected plates (28). Surviving cells in each 15-cm plate were then pooled, propagated, and tested for estrogen or TPA induction of CAT activity. Different pools of cells carrying the same reporter plasmid were found to behave similarly. For generation of stable cell lines containing non-episomal TRE-based reporter vectors, Ishikawa cells were cotransfected with 15 μg of TRE6-TATA-CAT vectors and 1.5 μg of neomycin resistance gene expression vector Ro/RSV (Inigro). 48 h after transfection, surviving cells were selected in medium (α-minimum Eagle’s medium containing 5% FBS and 1 mg/ml G418). Two weeks later, individual clones were selected, expanded, and tested for stimulation of CAT activity by incubation with TPA (100 ng/ml) for 24 h. Established cell lines were subsequently maintained in medium containing half the concentration of antibiotic used for selection. For hormonal treatment, cells were preincubated for 72 h in medium without phenol red before being supplemented with charcoal-treated serum. Incubation with estrogen or anti-estrogens was then carried out for 24 h except when indicated otherwise.

Reverse-transcription-PCR Amplification of Actin and CAT mRNAs—Ishikawa/ERE3-E2/BV cells maintained in phenol red-free DMEM, 5% charcoal-treated FBS were incubated with E2 (25 nM), OHT (100 nM), or ethanol for 8 h before harvesting and isolation of total RNA by CsCl gradient centrifugation. 3 μg of total RNA were precipitated, resuspended in 6 μl of 1x DNase I digestion buffer (Promega) containing 0.5 units of DNase I, incubated for 15 min at 37 °C and for 10 min at 75 °C, and then transferred on ice. DNase I-treated RNAs (2 μl) were reverse-transcribed using Superscript II RNAse H reverse transcriptase (Life Technologies, Inc.) and 0.5 μM random hexamer in a 20-μl final volume of RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 5 mM dithiothreitol supplemented with 0.5 mM dNTPs) at 37 °C for 1 h, followed by 75 °C for 10 min. Aliquots of resulting cDNAs (2 μl) were amplified by PCR using Taq DNA polymerase (Amersham Pharmacia Biotech) in a 50-μl final volume of 1x Taq buffer supplemented with 0.2 μM dNTPs and 0.5 μM forward and reverse CAT or Actin primers as follows: CAT forward, 5’-CCACGCATCCTGCACTCC-3’ and CAT reverse, 5’-GCAGTTGCGAGATGAAAGC-3’; β-actin forward, 5’-GCGTGGCTATCCGTGACTCC-3’ and β-actin reverse, 5’-GCCATGTTGATGACGGCG-3’. 24 cycles of PCR (95 °C for 30 s, 56 °C for 1 min, and 72 °C for 25 s) were performed for amplification of CAT CNAs and 25 cycles were performed (95 °C for 30 s, 58 °C for 1 min, and 72 °C for 30 s) for amplification of CAT sequences, followed by a final elongation step.
when using extracts from cells transfected with the parental expression vector pSG5 (Fig. 2, 1st lane) or when HEG0-containing extracts were incubated with a glucocorticoid-response element instead of an ERE (data not shown). Estrogen treatment of the cellular extracts increased the electrophoretic mobility of the ER-ERE complex (Fig. 2, E2, arrow 3). OHT treatment, on the other hand, resulted in a slower migrating complex (Fig. 2, OHT, arrow 1), and RUf treatment generated a complex migrating at a position similar to that of OHT (Fig. 2, RUf, arrow 1). Complexes between ER and the anti-estrogen RUf migrated at a position intermediary between those of ERE2 and ER-OHT (Fig. 2, RUf, arrow 2), and indistinguishable from that of the ER-ICI164,384 complexes (Fig. 2, ICI, arrow 2). Similar results were obtained using the ER mutant HE0, except that complexes were only observed in the presence of estrogen or of the various anti-estrogens and not in the absence of ligand (data not shown), consistent with the destabilizing effect of the G400V mutation on unbound ER (34, 35).

In conclusion, the electrophoretic mobilities of ER liganded with RUf or RUf are consistent with their identification as partial and full anti-estrogens, respectively.

**Differential Induction of Progesterone Receptor Gene Expression by Anti-estrogens in Ishikawa Cells**—Hydroxytamoxifen was previously reported to induce expression of the estrogen target gene human progesterone receptor in Ishikawa cells (15, 16). We decided to investigate whether RUf, which similarly affects ER migration in gel shift assays, can also induce expression of PR in Ishikawa and MCF7 cells. Cells were pretreated with estrogen or anti-estrogens prior to transient transfection with GRE5-TATA-CAT/EBV (Fig. 1), followed by addition of progesterone for 24 h (see “Experimental Procedures”). No stimulation of CAT activity by progesterone could be observed in extracts of cells in the absence of treatment with estrogen or anti-estrogens (Fig. 3A, lane 2). In cells treated with estrogen or with the ER agonist moxestrol, the 11β-methoxy derivative of ethynyl estradiol (36, 37), CAT activity was induced over 20-fold in the presence of progesterone (Fig. 3A, lanes 4 and 12). In cells pretreated with OHT, progesterone-induced CAT expression levels were 12% of those obtained with estrogen pretreatment (Fig. 3A, lane 6). The anti-estrogen RUf induced PR expression to comparable levels (Fig. 3A, lane 10), whereas no stimulation could be detected following incubation with RUf (Fig. 3A, lane 8). Contrary to what was observed with Ishikawa cells, none of the anti-estrogens assayed detectably stimulated PR transcriptional activity in MCF7 cells using this assay (Fig. 3B, compare lanes 5–10 to lanes 1 and 2). These observations confirm that partial agonist activity of anti-estrogens on expression levels of endogenous estrogen target genes can be observed in Ishikawa cells (15, 16).

**Lack of Agonist Activity of Anti-estrogens in Transient Transfection Assays of ERE-containing Reporter Vectors**—In order to analyze the mechanisms of transcriptional regulation by anti-estrogens with partial agonist activity in Ishikawa cells, we examined whether synthetic estrogen-responsive promoters can be stimulated by these anti-estrogens in transient transfection assays (Fig. 4). Three estrogen-sensitive reporter recombinants, ERE3-TATA-CAT/EBV, ERE3-tk-CAT, or Vit-tk-CAT (Fig. 1), were transiently transfected into Ishikawa cells in the absence of cotransfected estrogen receptor expression vector (Fig. 4A). Anti-estrogens did not detectably stimulate CAT expression from ERE3-TATA-CAT/EBV (Fig. 4A, lanes 3–6), ERE3-tk-CAT (Fig. 4A, lanes 10–13), or Vit-tk-CAT (Fig. 4A, lanes 17–20) under conditions where estradiol stimulated these reporter constructs 35-, 10-, and 25-fold, respectively (Fig. 4A, lanes 2, 9, and 16). Similar results were observed when CAT assays were repeated with 10 times more extract.
than in Fig. 4A to confirm that none of the anti-estrogens tested stimulated CAT activity higher than vehicle (data not shown).

Cotransfection of an expression vector for wild-type ER (pSG5-HEG0) along with ERE3-TATA-CAT/EBV, ERE3-tk-CAT, or Vit-tk-CAT resulted in increased background levels in the absence of hormonal treatment (Fig. 4B, lanes 1, 8, and 15). Treatment with anti-estrogens did not increase CAT expression levels compared with vehicle alone (Fig. 4B, lanes 3–6, 10–13, and 17–20), although CAT levels in the presence of OHT or RUp were slightly higher than with other anti-estrogens. Background CAT expression in the absence of hormonal treatment could result from binding of residual estrogens in media, which would be competed out by incubation with anti-estrogens. Alternatively, unliganded ER may be a weak transcriptional activator under the conditions of this assay.

Results similar to those observed in Ishikawa cells were obtained by transient transfection of MCF7 cells (data not shown). In both cell lines, the concentrations of anti-estrogens used (100 nM) were sufficient to totally repress stimulation by 1 nM estrogen, demonstrating that estrogen receptors are fully saturated by these anti-estrogens under the conditions used to assay for agonist activity (data not shown). In conclusion, agonist activity of anti-estrogens could not be detected in transient transfection assays of ERE-containing reporter vectors either in Ishikawa or in MCF7 cells.

Detection of Cell-specific Agonist Activity of Anti-estrogens Using a Stably Propagated ERE3-TATA-CAT Episomal Reporter Vector—The failure to detect significant transcriptional activation by OHT or other anti-estrogens in transient transfections raised the possibility that in vivo conditions required for agonist activity of OHT are not fully reconstituted in this assay. Therefore, we established Ishikawa and MCF7 cell lines stably propagating the ERE3-TATA-CAT/EBV episomal plasmids, selecting pools of transfected cells by addition of hygromycin B in cell culture medium (see “Experimental Procedures”); note that for brevity cell lines stably propagating
ERE3-TATA-CAT/EBV episomal plasmids will be indicated by the suffix -ERE3/EBV. Stimulation with estrogen (25 nM, 24 h) of Ishikawa-ERE3/EBV cells led to a marked stimulation of CAT expression levels (~17-fold, Fig. 5A, lane 2). No stimulation was observed with the pure anti-estrogens RUf or ICI (100 nM, Fig. 5A, lanes 5 and 6). In contrast, OHT and RUf stimulated CAT activity to ~22 and 30% of the levels obtained with estrogen, respectively (Fig. 5A, lane 3 and 4). These results were obtained with different pools of Ishikawa-ERE3/EBV cells generated by two independent rounds of selection. On the other hand, neither the partial anti-estrogens OHT and RUf nor the full anti-estrogens ICI or RUf stimulated CAT expression more than background in MCF7-ERE3/EBV cells; note, however, that the fold stimulation by estrogen was lower in stably transfected MCF7 cells than in Ishikawa-derived cell lines (Fig. 5B, lanes 1–7).

Agonist activity of OHT was dose-dependent, and stimulation of CAT activity could be detected with concentrations as low as 1 nM, whereas ICI or RUf did not induce levels of CAT activity above basal levels at any of the concentrations tested (Fig. 5C). Note that none of the anti-estrogens were capable of inducing CAT expression in a cell line derived from Ishikawa cells by stable transfection of an episomal vector containing five glucocorticoid response elements (GRE5) instead of three EREs (GRE5-TATA-CAT/EBV, Ref. 28), demonstrating that the presence of EREs is required for transcriptional activation by anti-estrogens (data not shown).

The levels of CAT mRNAs after stimulation by estrogen and OHT for 8 h were measured by semi-quantitative RT-PCR in order to confirm that the anti-estrogen increased CAT mRNA levels. CAT mRNA levels obtained after OHT stimulation were ~40% of those obtained after incubation with estrogen, whereas actin mRNA levels did not vary between the different samples (Fig. 5D). These results are in good agreement with those obtained by measuring levels of CAT enzyme activity. The agonist effect of OHT and RUf persisted when incubations were performed for longer periods than 24 h. Treatment for 2–5 days with OHT or RUf consistently generated increased CAT expression compared with treatment with full anti-estrogens or vehicle (Fig. 5E).

In order to rule out the possibility that differences in the protocols used for transient transfection assays and experiments with stable cell lines might be the source of the discrepancy in the results observed with partial anti-estrogens (i.e. no stimulation of CAT activity in transient transfection versus stimulation in stable cell lines), we repeated these experiments in parallel using Ishikawa and Ishikawa-ERE3/EBV cells, with or without mock transfection of Ishikawa-ERE3/EBV cells (i.e.
transfection with carrier DNA only). Estrogen stimulation of CAT activity was much higher in the transient transfection assay than with the stable cell line (55- and 10-fold, respectively), whereas background levels of CAT activity were similar (Fig. 5F, compare lanes 1 and 2, and 13 and 14). Despite the high levels of stimulation seen with E2, CAT activities observed with transient transfections performed in the presence of OHT or RuP were lower than those obtained with vehicle (compare lanes 15–17 with lane 13) and lower than those observed with OHT or RuP in Ishikawa-ERE3/EBV cells (compare lanes 15–17 with lanes 3–5; see also inset). Mock transfection of Ishikawa-ERE3/EBV cells slightly reduced stimulation with both estrogen and RuP while increasing background, thereby blunting the stimulation by anti-estrogens but not suppressing it. Therefore, differences in the results observed using the two assays seem to be due at least in part to the status of reporter vectors within cells, i.e. number of copies per cell and/or integration into chromatin.

Taken together, these results suggest that anti-estrogens with partial agonist activity, but not full anti-estrogens, can stimulate transcription directed by endogenous levels of estrogen receptors bound to minimal ERE-containing promoters in Ishikawa cells, even though this activity is undetectable in transient transfection assays using the same reporter vectors.

Minimal Promoters Containing AP1-binding Sites Are Not Activated by Estrogen or Anti-estrogens in Transient Transfection Assays of Ishikawa Cells—Previous reports have described induction of AP1 activity by estrogen treatment in several cell lines (19–22) and by anti-estrogens in transient transfection assays of Ishikawa cells (22). To test whether TRE motifs are sufficient to mediate stimulation by estrogen and anti-estrogens in Ishikawa cells, we transiently transfected reporter vectors containing a minimal promoter composed of six TRE motifs upstream of a TATA box (Fig. 1) or a reporter vector containing the AP1-responsive rat stromelysin promoter, STRCAT (Fig. 1, see also Ref. 24). Stimulation of expression from the STR-CAT reporter vector could be observed in the presence of estrogen (3-fold, compare lane 2 to lane 1 in Fig. 6A) but not in the presence of anti-estrogens. TPA-stimulated CAT expression levels 5–10-fold, and no additional increase was obtained in the presence of estrogen or anti-estrogens (Fig. 6A, lanes 5–8). No stimulation of the minimal TRE6-TATA-CAT reporter constructs could be observed after incubation of the transiently transfected cells with either estrogen or anti-estrogens (Fig. 6B). When the same promoter was incorporated into an episomal vector, a lower basal activity was observed (10-fold lower, data not shown) and a weak stimulation by estrogen (2.5-fold) could be detected in the absence but not in the presence of TPA (Fig. 6C, compare lanes 2 and 6 to lanes 1 and 5). Anti-estrogens had no effect on CAT expression levels directed from this reporter construct (Fig. 6C, lanes 3 and 4, and 7 and 8).

From these experiments, we conclude that stimulation of expression from promoters containing TRE motifs by estrogen is dependent on the promoter context. Although we did not observe transcriptional activation by anti-estrogens using these promoters, we cannot rule out the possible existence of promoter-specific effects.

Micromolar Concentrations of OHT, but Not Of Other Anti-estrogens, Can Stimulate Transcription from Minimal Promoters Containing TRE Motifs in Stably Transfected Ishikawa Cells—To investigate further the potential role of anti-estrogens in stimulation of the AP1 signaling pathway, cell lines were derived from Ishikawa cells by stable transfection of TRE6-TATA-CAT vectors (clonal selection by integration into the cellular genome) or of the episomal vectors TRE6-TATA-CAT/EBV or TRE2-TATA-CAT/EBV.

Two clones obtained by selection for integration of the TRE6-TATA-CAT reporter vector responded to TPA stimulation (100 ng/ml) by an increase in CAT expression (3–7-fold; Fig. 7A, lanes 2–4 and 6–8). Similar results were obtained with the other clone (data not shown). Note that estrogen stimulated expression from an ERE3-hsp68-LacZ reporter vector transiently transfected in Ishikawa-TRE6 cells, demonstrating that lack of induction of the API pathway in these cell lines was not due to loss of ER function (data not shown).

Pools of cells were selected for propagation of the TRE6-TATA-CAT/EBV vector, generating the Ishikawa-TRE6/EBV cell line (CAT activity was stimulated ~5-fold by TPA in these cells, data not shown). Treatment of Ishikawa-TRE6/EBV cells as well as of Ishikawa-TRE6 clone 29 cells with estrogen or anti-estrogen did not lead to detectable stimulation of CAT activity (Fig. 7B, lanes 1–10), whereas in the same assay strong agonist activity could be observed using the Ishikawa-ERE3/EBV cells (Fig. 7B, lanes 11–15). Similar results were obtained.
in the presence of TPA, except that levels of CAT activity were higher in the presence of TPA in the cells containing AP1-responsive promoters but not in Ishikawa-ERE3/EBV cell lines (data not shown).

Because the conditions used for tissue culture in the above-described experiments (phenol red-free DMEM supplemented with 5% charcoal-treated FBS) may mask stimulation of AP1 activity by estrogen or anti-estrogens, we performed these assays again in the absence of serum. CAT activity was induced by estrogen (10-fold) and anti-estrogens (3–5-fold) in the Ishikawa-ERE3/EBV cells (Fig. 7C, lanes 1–10) but not in Ishikawa-TRE6/EBV cells (Fig. 7C, lanes 11–15) or Ishikawa-TRE2/EBV cells (Fig. 7C, lanes 16–20), which propagate EBV episomal vectors containing only two TPA response elements. However, when higher concentrations of anti-estrogens were used (5 μM instead of 0.1 μM), small but reproducible stimulations of CAT activity were observed with OHT (3-fold) or with tamoxifen (2-fold) (Fig. 7D, compare lanes 3 and 4 to lane 1) but not with estrogen or with other anti-estrogens (Fig. 7D, lanes 2, 5 and 6).

These results indicate that TRE elements together with a TATA box can only mediate transcriptional stimulation by high concentrations of OHT (or TAM) in Ishikawa cells, whereas estrogen response elements can be activated at lower concentrations by both OHT and RU38477 under the same conditions.

**DISCUSSION**

Mechanisms underlying the partial agonist activity of anti-estrogens are still poorly understood (8). In this report, we have investigated whether the partial agonist activity of anti-estrogens in Ishikawa cells can be mediated at the level of regulation of gene expression by typical estrogen response elements and/or by TPA response elements, which can mediate estrogen stimulation in some promoters (19–22). Although transcriptional activation of ERE-containing promoters by OHT has been documented using transient transfection assays in a number of cell lines, including HeLa cells and chicken embryo fibroblasts (3), agonist activity of OHT and RU38477 was not observed in transiently transfected Ishikawa cells using minimal promoters containing EREs (ERE3-TATA) or more complex promoters (ERE3-tk, Vit-tk). Failure to detect transcriptional stimulation by these receptors may be due to the absence of a TATA box in these promoters.
tional activation of ERE-containing promoters by OHT in transiently transfected Ishikawa cells is in agreement with previous observations (22). Contrary to results obtained with transient transfections, we observed significant levels of transcriptional activation by OHT and RuP in Ishikawa, but not in MCF7 cells, when the ERE3-TATA-CAT/EBV reporter vector was stably propagated as an episome. These results correlate well with the observed agonist effect of these two anti-estrogens on expression levels of the endogenous progesterone receptor (note that the human progesterone receptor upstream sequences contain a half-palindromic TGACC motif but no consensus EREs, Ref. 38). In addition, OHT and RuP, although structurally unrelated, induced similar shifts in mobility of ER:ERE complexes in gel retardation assays. The migration of these complexes was found to be distinct from those formed in the presence of the full antagonists RuF and ICI.

Differences observed in transcriptional activity in the presence of anti-estrogens in transient transfection assays and using “reporter cell lines” may reflect the different status of the reporter vectors, which are present at a lower copy number when maintained as episomes (generally less than 50 copies per cell, Ref. 39) and are incorporated into chromatin to a higher degree (40) compared with transiently transfected reporter plasmids. These results suggest that stimulation of ER:mediated transcription by anti-estrogens requires cofactor(s) limiting in amounts or availability in transient transfection assays in Ishikawa cells, whereas estrogen-ligated ER may recruit other, non-limiting cofactors (41, 42). Of interest is the fact that capacity to remodel chromatin structure via histone acetyltransferase or deacetylase activities has been attributed to an increasing number of nuclear receptor co-activators and co-repressors (42–49), demonstrating that incorporation of target promoters into chromatin is an integral part of the mechanism of transcriptional activation by nuclear receptors. It is not clear at present whether ER can interact with these cofactors in vivo when bound by anti-estrogens with partial agonist activity. It is possible that cofactors specific to OHT-bound ER, such as the newly described co-activator L7/SPA (50) may mediate the agonist activity of this anti-estrogen. Future functional characterization of cofactors interacting with ER in the presence of OHT and RuP should provide insights into the molecular mechanisms of action of anti-estrogens with partial agonist activity.

Low concentrations of OHT or RuP, which were sufficient for activation of ERE3-TATA promoters in stably propagated vectors, did not yield detectable transcriptional stimulation of promoters containing TRE sites inserted upstream of a TATA box either in transient transfection assays or using stably propagated vectors. Others have previously documented transcriptional activation of the TRE-containing collagenase promoter by anti-estrogens in transiently transfected Ishikawa cells (22). Discrepancy between these and our results could be due to the promoter context of TRE elements. Along the same line, our results indicate that promoter context influences stimulation of AP1-responsive promoters by estrogen in transient transfection assays. Alternatively, differences in cell lines or transfection methods could be the source of this discrepancy. Side-by-side comparison of cell lines carrying episomal reporter vectors whose promoters differed only by the response elements present in the minimal synthetic promoters confirmed that transcriptional stimulation by anti-estrogens like RuP or OHT could be mediated by estrogen response elements but not TPA response elements at low concentrations of anti-estrogens. Stimulation of AP1-responsive promoters could only be observed in serum-free medium using 5 μM OHT or TAM but not estrogen or other anti-estrogens. Whether this effect is mediated by estrogen receptors or is initiated at the cellular membrane remains to be investigated.

In conclusion, our results suggest that transcriptional stimulation by anti-estrogens can be mediated by consensus EREs in Ishikawa cells, this observation being consistent with the absence of TRE sites in the promoters of genes whose expression can be induced by hydroxytamoxifen in the uterus (38, 51). In addition, TREs are also capable of mediating transcriptional stimulation by anti-estrogens, although in a manner that is restricted by both the nature and the concentration of the anti-estrogen. Finally, while transient transfection has proven to be a powerful tool for analyzing intracellular signaling pathways, our study emphasizes that this assay only partially recapitulates the conditions required for initiation of transcription in vivo.

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REFERENCES

1. Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. (1989) Cell 59, 477–487
2. Metzger, D., Ali, S., Bornert, J.-M., and Chambon, P. (1995) J. Biol. Chem. 270, 3535–3542
3. Berry, M., Metzger, D., and Chambon, P. (1999) EMBO J. 18, 2811–2818
4. McInerney, E. M., and Katznenellenbogen, B. S. (1996) J. Biol. Chem. 271, 24172–24178
5. Heilberg, A. M., and Evans, R. M. (1988) Cell 55, 899–906
6. Webster, N. J., Green, S., Jin, J. R., and Chambon, P. (1998) Cell 94, 199–207
7. Henttu, P. M., Kalkhoen, E., and Parker, M. G. (1997) Mol. Cell. Biol. 17, 1832–1839
8. Danielian, P. S., White, R., Lees, J. A., and Parker, M. G. (1992) EMBO J. 11, 1025–1033
9. Katznenellenbogen, B. S., Montano, M. M., Le Goff, P., Schodin, D. J., Kraus, W. L., Bharatwaj, B., and Fujimoto, N. (1995) J. Steroid Biochem. Mol. Biol. 53, 387–393
10. Jordan, V. C. (1990) Breast Cancer Res. Treat. 15, 125–136
11. Goettl, M. M., Robinson, S. P., Satyaswaroop, P. G., and, Jordan, V. C. (1988) Cancer Res. 48, 812–815
12. Van Leeuwen, F. E., Benraadt, J., Coebergh, J. W., Kiemeney, L. A., Gimbrere, C. H., Otter, R., Schouten, L. J., Danahous, R. A., Bontenbal, M., Dep-horst, F. W., van den Belt-Dusebout, A. W., and van Tinteren, H. (1994) Lancet 343, 448–452
13. Fisher, B., Costantini, J. P., Bedon, C. K., Fisher, E. R., Wicherker, D. L., and Creinin, W. M. (1994) J. Natl. Cancer Inst. 86, 527–537
14. Anzai, Y., Holinka, C. P., Kuramoto, Y., and Kamada, T. (1994) EMBO J. 13, 2362–2365
15. Jamal, A., Creastall, J. D., and White, J. O. (1991) J. Mol. Endocrinol. 6, 1–211
16. Schwartz, L. B., Krey, L., Demopoulos, R., Goldstein, S. R., Nachigal, L. E., and Merttul, K. (1997) Am. J. Obstet. Gynecol. 176, 129–137
17. Weisinger, A. E. (1990) J. Steroid Biochem. Mol. Biol. 38, 177–179
18. Van de Velde, P., Nique, F., Bouchoux, F., Bremaud, J., Hameau, M.-C., Lucas, T., Yamasaki, Y., Kajimoto, Y., and Kamada, T. (1994) J. Biol. Chem. 269, 176, 187–196
19. Gaub, M. P., Bellard, M., Scheuer, I., Chambon, P., and Sassone-Corsi, P. (1990) Cell 63, 1267–1276
20. Philips, A., Chabos, D., and Rochevert, H. (1993) J. Biol. Chem. 268, 14103–14108
21. Umayaehara, Y., Kawamori, R., Watada, H., Imano, E., Iwama, N., Morishima, T., Yamasaki, Y., Kajimoto, Y., and Kamada, T. (1994) J. Biol. Chem. 269, 16435–16442
22. Webb, P., Lopez, G. N., Uht, R. M., and Kushner, P. J. (1995) Mol. Endocrinol. 9, 443–456
23. Klein-Hitpass, L., Schorpp, M., Wagner, U., and Ryffel, G. U. (1986) Cell 46, 1053–1061
24. Nicholson, R. C., Mader, S., Nagai, S., Leid, M., Rochette-Egly, C., and Chambon, P. (1990) EMBO J. 9, 4443–4454
25. Klein-Hitpass, L., Ryffel, G. U., Hetingter, E., and Cato, A. (1988) Nucleic Acids Res. 16, 647–663
26. Mader, S., and White, J. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5603–5607
27. Ponglikitmongkol, M., White, J. H., and Chambon, P. (1990) EMBO J. 9, 2221–2231
28. White, H. J., McCuaig, K. A., and Mader, S. (1994) Bio/Technology 12, 1003–1007
29. Berthois, Y., Katznenellenbogen, J. A., and Katznenellenbogen, B. S. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 2456–2500
30. Banerji, J., Rusconi, S., and Schaffner, W. (1981) Cell 27, 299–308
31. Seed, B., and Shen, J. Y. (1988) Gene (Amst.) 67, 271–277
32. Metzger, D., Berry, M., Ali, S., and Chambon, P. (1995) Mol. Endocrinol. 9, 579–591
33. Jin, L., Berras, M., Lacroix, M., Legros, N., and Leclercq, G. (1995) Steroids 60, 512–518
34. Tora, L., Mulliek, A., Metager, D., Ponglikitmongkol, M., Park, I., and Cham- 
bon, P. (1989) EMBO J. 8, 1981–1986
35. Aumais, J. P., Lee, H. S., Lin, R., and White, J. H. (1997) J. Biol. Chem. 272, 12229–12235
36. Salmon, J., Coussediere, D., Cousty, C., and Raynaud, J. P. (1983) J. Steroid 
Biochem. 18, 565–573
37. Raynaud, J. P., Martin, P. M., Bouton, M. M., and Ojasoo, T. (1978) Cancer 
Res. 38, 3044–3050
38. Kastner, P., Krust, A., Turcotte, B., STropp, U., Tora, L., Gronemeyer, H., and 
Chambon, P. (1990) EMBO J. 9, 1603–1614
39. Yates, J. L., Warren, N., and Sugden, B. (1985) Nature 313, 812–815
40. Pazin, M. P., and Kadonaga, J. T. (1997) Cell 89, 325–328
41. Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S., and 
Tung, L. (1996) Mol. Endocrinol. 10, 1167–1177
42. Glase, C. K., Rose, D. W., and Rosenfeld, M. G. (1997) Curr. Opin. Cell Biol. 9, 222–232
43. Bannister, A. J., and Kouzarides, T. (1996) Nature 384, 641–643
44. Ogryzko, V. V., Schiltz, R. L., Russanov, V., Howard, B. H., and Nakatani, Y. 
(1996) Cell 87, 953–959
45. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., 
Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580
46. Jenster, G., Spencer, T. E., Burcin, M. M., Tsai, S. Y., Tsai, M. J., and O'Malley, 
B. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7879–7884
47. Nagy, L., Kao, H. Y., Chakravarti, D., Lin, R. J., Hassig, C. A., Ayer, D. E., 
Schreiber, S. L., and Evans, R. M. (1997) Cell 89, 373–380
48. Alland, L., Muhle, R., Hou, H., Jr., Potes, J., Chin, L., Schreiber-Agus, N., and 
DePinho, R. A. (1997) Nature 387, 49–55
49. Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., 
Torshia, J., Yang, W. M., Brard, G., Nyo, S. D., Davie, J. R., Sets, E., 
Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997) 
Nature 387, 43–48
50. Jackson, T. A., Richer, J. K., Bain, D. L., Takimoto, G. S., Tung, L., and 
Horwitz, K. B. (1997) Mol. Endocrinol. 11, 683–705
51. Norris Daju Fan, J. D., Wagner, B. L., and McDonnell, D. P. (1996) Mol. 
Endocrinol. 10, 1605–1616
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