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Inhibition of Estrogen-Responsive Gene Activation by the Retinoid X Receptor β: Evidence for Multiple Inhibitory Pathways

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The retinoid X receptor β (RXRβ; H-2RIIBP) forms heterodimers with various nuclear hormone receptors and binds multiple hormone response elements, including the estrogen response element (ERE). In this report, we show that endogenous RXRβ contributes to ERE binding activity in nuclear extracts of the human breast cancer cell line MCF-7. To define a possible regulatory role of RXRβ regarding estrogen-responsive transcription in breast cancer cells, RXRβ and a reporter gene driven by the vitellogenin A2 ERE were transfected into estrogen-treated MCF-7 cells. RXRβ inhibited ERE-driven reporter activity in a dose-dependent and element-specific fashion. This inhibition occurred in the absence of the RXR ligand 9-*cis* retinoic acid. The RXRβ-induced inhibition was specific for estrogen receptor (ER)-mediated ERE activation because inhibition was observed in ER-negative MDA-MB-231 cells only following transfection of the estrogen-activated ER. No inhibition of the basal reporter activity was observed. The inhibition was not caused by simple competition of RXRβ with the ER for ERE binding, since deletion mutants retaining DNA binding activity but lacking the N-terminal or C-terminal domain failed to inhibit reporter activity. In addition, cross-linking studies indicated the presence of an auxiliary nuclear factor present in MCF-7 cells that contributed to RXRβ binding of the ERE. Studies using known heterodimerization partners of RXRβ confirmed that RXRβ/triiodothyronine receptor α heterodimers avidly bind the ERE but revealed the existence of another triiodothyronine-independent pathway of ERE inhibition. These results indicate that estrogen-responsive genes may be negatively regulated by RXRβ through two distinct pathways.

Estrogen-mediated gene regulation plays a fundamental role in development of female sex organs and in reproductive processes in vertebrates. In addition, estrogen has been implicated in control of gene expression unrelated to sexual differentiation and reproduction (12, 25, 33). Activation of estrogen-responsive genes occurs via interaction of the estrogen receptor (ER) with 17-beta estradiol (E_2) and subsequent receptor binding to estrogen-responsive elements (EREs) of target genes. The ER, a member of the nuclear receptor superfamily, is composed of three distinct modular domains with distinct functions (19, 20, 41, 43, 58, 75). Analysis of in *Xenopus* and chicken vitellogenin genes as well as other estrogen-responsive genes have shown that EREs contain a GGTCA motif, often in palindromic repeats (38, 55, 77). Despite the presence of the GGTCA motif in other hormone response elements, DNA sequence requirements for functional ER binding have been found to be rather stringent (42, 54, 59, 61, 65).

Reports suggest that control of estrogen-responsive gene regulation is complex (for a review, see reference 32 and references therein). Several lines of evidence indicate that gene regulation mediated by estrogen and EREs involves not only the ER but also other transcription factors and DNA-binding proteins. Involvement of oncogene products such as c-*jun*/c-*fos* (17, 26), NF-1 (11, 54), and H-*ras* (63) in E_2/ERE-mediated gene regulation has been documented, although these factors do not bind to EREs. In addition, it has been shown that some cells lacking the ER nevertheless contain a nuclear factor(s) that can bind to EREs (21) and that some of these factors appear capable of affecting transcription from ERE-containing promoters (37). Further, other members of the nuclear hormone receptor family such as the thyroid hormone (triiodothyronine [T_3]) receptor (T_3R [28]) and COUP-TF (1) have been shown to bind EREs in addition to their cognate response elements. Despite their ability to bind to EREs, a functional role for these non-ER proteins in estrogen-regulated cells has remained elusive.

We have previously reported isolation of a cDNA for the retinoid X receptor β (mRXRβ; H-2RIIBP), a member of the nuclear hormone receptor superfamily which binds to the region II enhancer element of major histocompatibility complex (MHC) class I genes and other elements containing the GGTCA motif (35). This motif is common to other hormone response elements such as the thyroid response element and retinoic acid (RA) response element (3, 19, 54, 61, 76). We have shown that RXRβ binds a number of these hormone response elements, including EREs (52). Two other members of the RXR subfamily, RXRα and RXRγ, have subsequently been cloned (49). All RXRs bind an isoform of all-*trans* RA, 9-*cis* RA (36, 47), but can control transcription of target genes independently of RA receptors (RARs) (49, 62). Further, we (51) and others (8, 40, 46, 80, 81) have shown that RXRs form heterodimeric complexes with other nuclear hormone receptors, including the T_3R, RAR, and vitamin D receptor (VDR). These studies have suggested that RXR heterodimers are more stable than the RXR homodimer and bind to various hormone response elements

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with much greater affinities than do the homodimers of the cognate receptors. Despite the demonstration of ERE binding by RXRβ, the function of RXRβ with respect to ER-mediated gene activation has not yet been determined.

In this report, we have addressed the role of mRXRβ in transcriptional regulation of an estrogen-responsive gene in the human breast cancer cell line MCF-7. This line of inquiry is supported by the observation that retinoids may function as antiestrogens in breast cancer cells (4, 22). MCF-7 cells were chosen for study since the cells permit a direct analysis of the action of mRXRβ with respect to function of the endogenous ER (13, 15, 38, 68, 72). We show here that the endogenous RXRβ expressed in MCF-7 cells contributes to ERE binding activity, independently of the ER. Moreover, we show that transient overexpression of RXRβ results in specific inhibition of ERE-driven reporter activity. Deletion analysis of RXRβ indicates that the mechanism of inhibition is more complex than a direct interference of ER binding to the target ERE by RXRβ. Furthermore, inhibition of ERE-driven promoter activity by RXRβ was observed to occur via two pathways: (i) a ligand-independent pathway involving RXRβ and an unidentified auxiliary factor present in MCF-7 cells and (ii) a pathway involving RXRβ in combination with thyroid hormone and the T_3R. These data therefore show that estrogen-responsive genes may be negatively regulated by RXRβ through two distinct inhibitory pathways.

MATERIALS AND METHODS

Cell culture. MCF-7 cells (passage 148) were obtained from American Type Culture Collection (Rockville, Md.) and maintained at 37°C in improved minimal essential medium (IMEM; Biofluids, Rockville, Md.) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) in an atmosphere of 5% CO_2. Experiments were routinely conducted on cells (passages 149 to 170) maintained for a minimum of 24 h in phenol red-free IMEM plus 5% charcoal-stripped fetal bovine serum (13). MDA-MB-231 cells (9) were a gift of Kim Leslie (University of Colorado, Denver) and were cultured in conditions identical to those for MCF-7 cells. Hormones were added to the media as indicated at the following concentrations: E_2, 10^{-7} M; T_3, 10^{-7} M; all-*trans* RA, 10^{-6} M; 9-*cis* RA, 10^{-6} M; and dexamethasone, 10^{-7} M. Controls for hormone-treated cells were treated with identical concentrations of vehicle alone which did not exceed 0.02% (vol/vol).

Gel retardation assays. Nuclear extracts were prepared by the method of Dignam et al. (16), with the minor modification of omitting the final dialysis step and freezing extracts in buffer D. Five micrograms of nuclear proteins was mixed with approximately 2 fmol of ^{32}P-radiolabeled oligonucleotide (A2 ERE sequence [5′-GGCTGATCAGGTCACTGT GACCTGACTT-3′]) in a 20-μl volume of binding buffer consisting of 25 mM Tris-HCl (pH 7.6), 75 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 1 mM $MgCl_2$, and 2 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia Fine Chemicals) with or without 200-fold excess competitor DNA (*H-2L*^{d}, region I sequence [5′-GCTGGGGATTCCCCAT-3′] or AP-1 [5′-AATTAGTCAGCCATGGGG-3′]). Unless otherwise stated, after a 30-min binding reaction at 4°C, the mixture was electrophoresed at 140 V in a 4% polyacrylamide gel in low-ionic-strength conditions of 0.4× TBE (52) buffer. Recombinant ER produced in a baculovirus expression system was a generous gift of Myles Brown (Dana Farber Cancer Institute, Boston, Mass. [7]), COUP-TF was kindly donated by Ming-Jer Tsai (Baylor College of Medicine, Houston, Tex.), and $T_3R\alpha$ was donated by Vera Nikodem (National Institute of Diabetes and Digestive and Kidney Diseases [34]). The preparation of mRXRβ is described elsewhere (52). Baculovirus-infected Sf9 cell extracts were added at a concentration of 2 μg of total proteins per lane unless otherwise stated. Anti-ER monoclonal antibody H222 was a gift of Geoffrey Greene (Ben May Institute, University of Chicago [31]). Anti-RXRβ monoclonal antibody MOK 13.17 has been described elsewhere (52). Monoclonal antibody MOK 15.55, specific for RXRβ, will be described elsewhere (53).

Methylation interference. An antibody-mediated methylation interference assay using dimethyl sulfate-treated oligonucleotides was performed as described previously (52). Briefly, to determine G residues involved in binding of homodimeric RXRβ, baculovirus-produced RXRβ was first complexed to anti-mouse immunoglobulin beads via a monoclonal antibody (MOK 13.17) to RXRβ. Methylated labeled probes were then incubated with the bound RXRβ, the beads were washed, and probe remaining in the precipitated complex was then extracted with phenol-chloroform and processed as described previously (52). To determine G residues involved in $T_3R\alpha$/RXRβ heterodimer binding, 3.5 μg of Sf9 cell extracts containing each receptor was subjected to gel shift, using methylated probe to visualize the heterodimeric complex. The shifted probe was then eluted and processed as described previously (52).

CAT assay. Twenty-four hours prior to transfection, 1×10^6 to 3×10^6 cells were plated in 100-mm^2 plates containing phenol red-free IMEM and 5% charcoal-stripped serum. Medium was routinely changed 3 to 4 h prior to addition of DNA. Unless otherwise stated, a total of 15 to 20 μg of DNA was added to plates by using the BES (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid) buffer-based calcium phosphate precipitation method of transfection (62). Cells were washed with phosphate-buffered saline approximately 20 h later, and fresh medium containing hormones was added as indicated. Following an additional 36- to 48-h incubation, cells were harvested and lysed by freeze-thaw in 100 mM $KHPO_4$ (pH 7.8) according to the method of deWet et al. (14). As indicated in figure legends, cell extracts were normalized for protein concentration by using the Bio-Rad assay system and/or for luciferase activity produced by cotransfected Rous sarcoma virus (RSV)-luciferase by using a Monolight 2010 luminometer (Analytical Luminescence Laboratory) and assayed as described previously (14). Chloramphenicol acetyltransferase (CAT) activity was assayed as described elsewhere (29). Quantitation of the percentage of conversion to acetylated chloramphenicol was performed by using an AMBIS Radioanalytic Imaging system. Unless otherwise stated, plotted values represent the mean of three experiments ± standard error of the mean.

Plasmid construction and DNA preparation. Construction of the expression vector pRSV-H-2RIIBP and the RXRβ domain deletion mutants has been described elsewhere (62). *Nco*I digests of the respective deletion mutants were subcloned into pEXPRESS-O (24) in order to transcribe and translate the deletion mutants in vitro. Correct insertion of the mutants was confirmed by dideoxy sequencing. The thymidine kinase (*tk*)-CAT reporter constructs A2 ERE tkCAT, GRE (glucocorticord response element) tkCAT, Δ2 ERE tkCAT, and basal tkCAT are described elsewhere (56). The $T_3R\alpha$ expression vector was a generous gift of V. Nikodem and is described elsewhere (64). The expression vector for the estrogen receptor, HEO, and control vector,

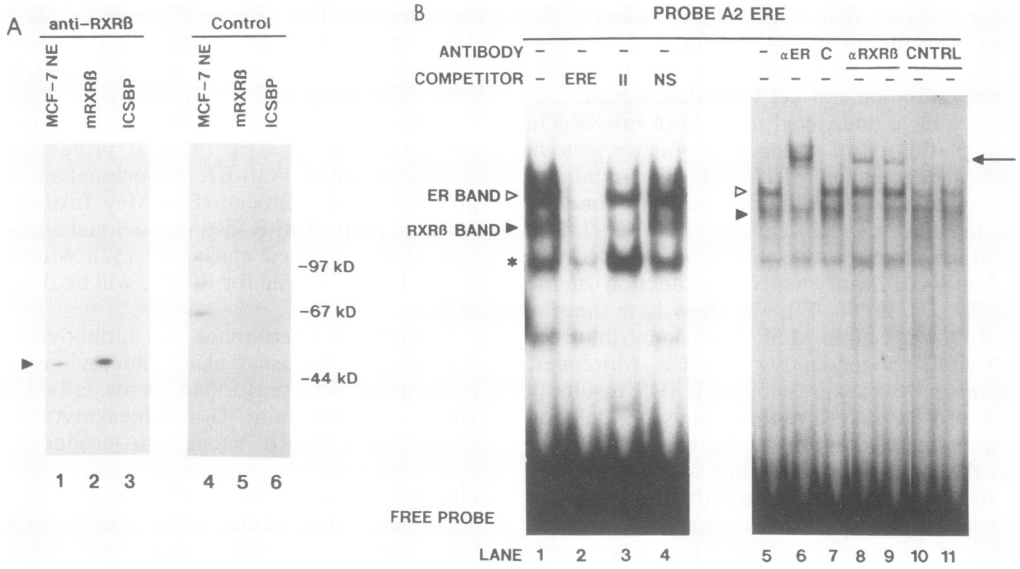

FIG. 1. Evidence that MCF-7 cells contain an endogenous ERE binding activity attributable to RXRβ. (A) Western analysis. Nuclear extracts prepared from estrogen-treated MCF-7 cells or in vitro-translated mRXRβ were probed with a monoclonal antibody directed against RXRβ (MOK 13.17; lanes 1 to 3). In vitro-translated murine ISCBP protein served as a negative control. The arrowhead indicates the RXRβ band. Lanes 4 to 6, control antibody (MOK 15.42) directed against an unrelated antigen. (B) Gel retardation assay of ERE binding activity in MCF-7 nuclear extracts. Nuclear extracts prepared from estrogen-treated MCF-7 cells were incubated with an oligonucleotide corresponding to the vitellogenin A2 ERE. Three reproducible bands are marked: open arrowhead, black arrowhead, and asterisk. Competition was performed with a 200-fold molar excess of A2 ERE, MHC class I region II (lane 2), or an unrelated oligonucleotide, MHC class I region I (NS, lane 4). For supershift experiments, a monoclonal antibody reacting with the ER (αER; 1 μl; lane 6) or RXRβ (MOK 15.55; αRXRβ; lanes 8 and 9, 4 and 2 μl, respectively) or a control antibody (MOK 15.42; CNTRL; lanes 10 and 11, 4 and 2 μl, respectively) was added to the reaction mixture as culture supernatant for 15 min at 30°C prior to electrophoresis.

pKCR2 (6), were gifts of P. Chambon (30). pRSV-luciferase was given by D. R. Helinski (14). Plasmids pRSV-Jm66 (62) and RSV-β-galactosidase (β-gal) (a generous gift of Chris Sax, National Eye Institute [4]) were used as controls.

Western immunoblot analysis. Western blots were performed as described previously, using the anti-RXRβ antibody MOK 13.17 and a nonspecific isotype-matched antibody, MOK 15.42 (52). Fifty micrograms of MCF-7 nuclear and cytoplasmic extracts was loaded per lane on 10.5% polyacrylamide gels for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and subsequent transfer.

Streptavidin-biotin-coupled DNA/cross-linking assay. Unless otherwise indicated, the procedure was performed as described elsewhere (51), using a 27-bp oligonucleotide identical to the A2 ERE sequence used in mobility shift assays but synthesized with a biotin group on the 5′ nucleotide of one strand (10). One to two microliters of radiolabeled, in vitro-translated protein produced from transcription of the respective pEXPRESS-RXRβ deletion constructs was incubated in 50 μl of buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 50 mM NaCl, 1 mM EDTA, 5% [wt/vol] glycerol, 0.05% [vol/vol] Triton X-100) containing 1.4 μM nonspecific oligonucleotide (5′-CGAATCTAACGCTATTTTTGGAATGCAA AT-3′) with either (i) no added extracts, (ii) 15 μg of nuclear proteins from MCF-7 cells treated for 48 h with 10^{-7} M E_2, or (iii) 15 μg of NTera-2 cytoplasmic extracts. After a 10-min preincubation on ice, biotinylated oligonucleotide was added to 0.1 μM and samples were placed at 4°C for 1 h. Disuccinimidyl suberate (Pierce) was then added to reaction mixtures at 0.25 mM for 30 min.; disuccinimidyl suberate was then quenched with 10 mM NH_4Cl. Ten microliters of streptavidin-agarose beads (Sigma) was added for a final 30- to 60-min incubation. Beads were then washed, and proteins were resolved on an SDS–8% polyacrylamide gel as described. Parallel control experiments (not shown) were performed with a biotinylated oligonucleotide corresponding to the murine *H-2L*^{d} region I element (5′-GCCCAGGGCTGGG GATTCCCCATCTCCT-3′).

RESULTS

RXRβ is a nuclear protein expressed in MCF-7 cells. The expression of RXRβ was examined by Western blot analysis of nuclear and cytoplasmic extracts prepared from estrogen-treated MCF-7 cells. As seen in Fig. 1A, a monoclonal antibody (MOK 13.17) directed against RXRβ detected a single 44-kDa band in nuclear extracts (lane 1, arrowhead). This band corresponded with the size of RXRβ detected in extracts of a number of mammalian cells (53). This band comigrated with the band produced by in vitro translation of the mRXRβ cDNA (lane 2). The same antibody exhibited no reactivity to an unrelated in vitro-translated protein, ICSBP (18). Analysis of MCF-7 cytoplasmic extracts confirmed nuclear localization of RXRβ in these cells (not shown). As expected, the same proteins probed with an isotype-matched control antibody of unrelated specificity (MOK 15.42; lanes 4 to 6) did not produce this band. These results indicate that RXRβ is expressed in MCF-7 cells as a 44-kDa nuclear protein.

Endogenous RXRβ constitutes an ERE binding activity in MCF-7 cells. To determine whether the endogenous RXRβ contributes to an ERE binding activity in MCF-7 cells, we performed gel mobility shift experiments using oligonucleotides corresponding to the ERE of the *Xenopus* vitellogenin

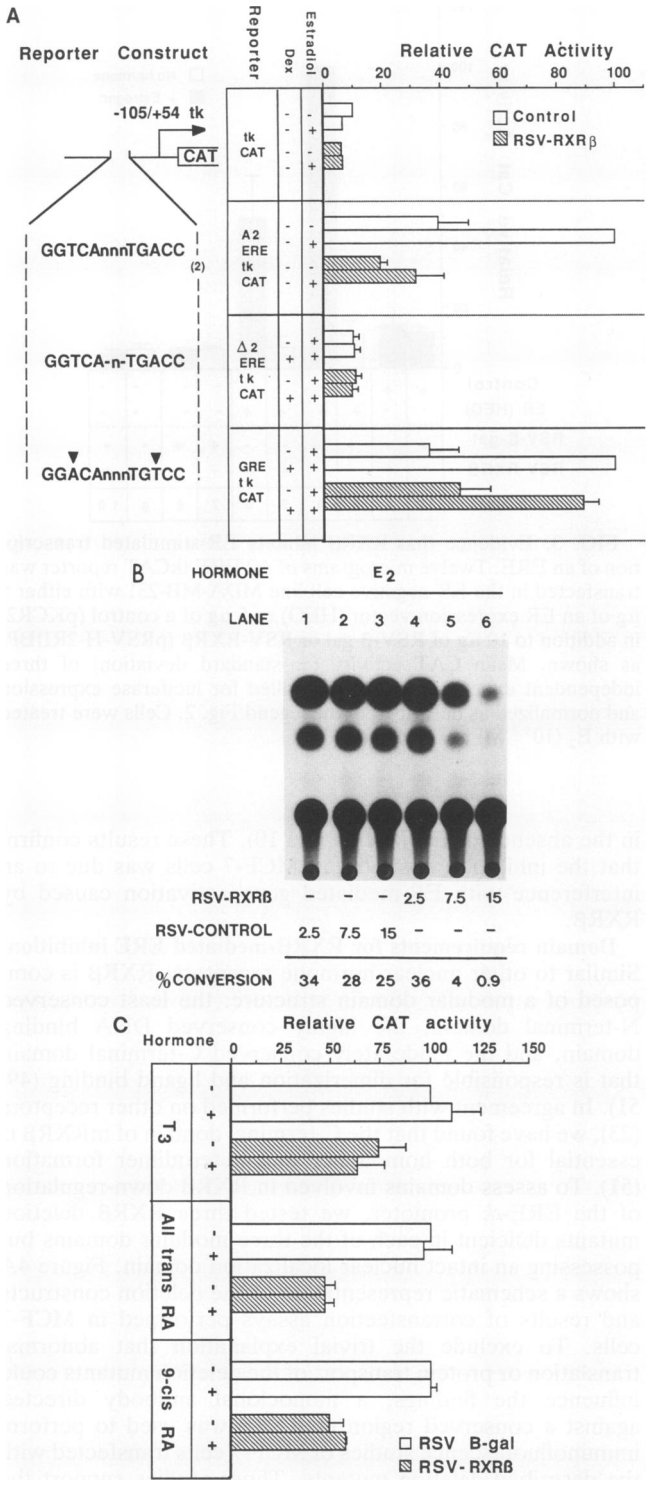

FIG. 2. Inhibition of ERE tkCAT activity by RXRβ in MCF-7 cells. (A) Design of *tk*-CAT reporter constructs. MCF-7 cells were transfected with the indicated reporter in addition to 10 μg of expression vector for either RXRβ (RSV-RXRβ) or control (RSV-β-gal). Mean relative CAT activity (± standard error of the mean) from three independent experiments controlled for luciferase activity is shown. Data were normalized such that estrogen-stimulated (i.e., maximal) CAT activity of the A2 ERE *tk*CAT reporter construct was 100%. Cells were treated with E_2 (10^{-7} M) or dexamethasone (Dex; 10^{-7} M) 24 h after transfection as indicated. Results using the GRE tkCAT and Δ2 ERE tkCAT reporters were obtained in the presence of estradiol (10^{-7} M) and were normalized such that dexamethasone-stimulated reporter activity was 100%. (B) Dose dependence of RXRβ inhibition. Microgram amounts of control plasmid pRSV-Jm66 or RSV-RXRβ were added as indicated to MCF-7 cells transfected with 7.5 μg of the A2 ERE tkCAT reporter in the presence of E_2 (10^{-7} M). Amounts of DNA added to plates were equalized by using pBluescript (Stratagene). (C) Evidence that ERE-*tk* reporter inhibition by RXRβ is ligand independent. MCF-7 cells were transfected with A2 ERE tkCAT and RSV-β-gal or RSV-RXRβ as indicated. Data were normalized and controlled as described above. In addition to estradiol (10^{-7} M), cells were treated with either T_3 (10^{-7} M), all-*trans* RA (10^{-6} M), or 9-*cis* RA (10^{-6} M) as indicated.

A2 gene as a probe and nuclear extracts from MCF-7 cells that were cultured in the presence or absence of E_2. As shown in lanes 1 to 4 of Fig. 1B, nuclear extracts prepared from estrogen-treated MCF-7 cells produced three major bands, all of which were removed by a 200-fold molar excess of unlabeled homologous ERE. In addition, oligonucleotides corresponding to the RXR target sequence, murine *H-2L*^{d} gene (MHC class I) region II (Fig. 1B, lane 3), showed partial competition; these oligonucleotides removed the middle band (arrow) but not the uppermost and lowermost bands. An unrelated competitor (MHC class I, region I) did not affect the mobility of any of these bands. These results indicate that ERE binding activities in MCF-7 cells consist of multiple factors. Since the murine *H-2L*^{d} gene region II binds RXRβ but not the ER (57), it is possible that the upper and lower bands correspond to an ER/ERE complex and the middle band corresponds to an RXRβ/ERE complex. To determine the identity of these bands, supershift experiments were performed with monoclonal antibodies specific for the ER (H222 [31]) or RXRβ (MOK 15.55 [51]) (Fig. 1B, lanes 5 to 11). Addition of anti-ER antibody shifted the position of the uppermost band (open arrowhead to the position marked by the arrow; lane 6) upward. This antibody did not affect the position of the remaining bands. MCF-7 extracts also produced a more rapidly migrating band (asterisk in Fig. 1B) which may represent a proteolytic product of the ER as suggested previously (67). A matched control antibody (lane 7) did not affect the migration of the upper band. In addition, the antibody directed against RXRβ altered the migration of the middle band but did not affect the uppermost band. Matched control antibodies did not affect the position of any of the bands (lanes 10 and 11). These results indicate that the uppermost band represents an ER/ERE complex, while the middle band contains RXRβ. Identical results were observed with nuclear extracts from MCF-7 cells cultured in the absence of estrogen (not shown). These data led us to postulate that RXRβ contributes to ERE binding activity in MCF-7 cell nuclear extracts and may therefore play a role in estrogen-mediated gene regulation in MCF-7 cells. Consistent with prior data generated by using recombinant receptors (46, 51, 81), data in Fig. 1B argue against the existence of an RXR/ER heterodimer.

RXRβ inhibits ERE-*tk* promoter activity in MCF-7 cells. To determine whether RXRβ influenced transcription of an estrogen-responsive gene in MCF-7 cells, we performed cotransfection experiments using a *tk*-CAT reporter driven by the vitellogenin A2 ERE (38, 56) (Fig. 2A) and pRSV-H-2RIIBP, an mRXRβ expression plasmid (62). It should be noted that pRSV-H-2RIIBP has been found to produce a plasmid-specific RXRβ transcript in NTera-2 cells following transient transfection (62). As expected (69), MCF-7 cells

treated with estrogen showed an increase in CAT activity when transfected with the A2 ERE reporter in the presence of a control pRSV vector not expressing RXRβ (Fig. 2A). However, when RXRβ was cotransfected, CAT activity produced by the A2 ERE reporter in E_2-treated cells was 70% lower than that obtained with the RSV control plasmid. Down-regulation was also seen when RXRβ was transfected into cells not treated with estrogen. In these cells, the ERE tkCAT activity was higher than that of the control construct, tkCAT. This finding suggests that in these cells the ERE is slightly activated in the absence of estrogen, possibly as a result of remaining estrogenic activity in the serum. Most importantly, the RXRβ-dependent inhibition was specific for the ERE, since the same *tk* promoter driven by either a GRE or an altered ERE that is unable to respond to estrogen (Δ2 ERE [56]) did not demonstrate down-regulation by cotransfected RXRβ (Fig. 2A). Furthermore, the basal transcription activity was not affected by RXRβ (tkCAT and Δ2 ERE tkCAT constructs), indicating that RXRβ is not inhibiting general transcription. As shown in Fig. 2B, RXRβ down-regulation of the ERE was dose dependent; the greatest suppression observed was 3% of the control value, with higher amounts of DNA than in Fig. 2A. Conversely, the control RSV vector was without any effect at the doses tested. Other RSV vectors containing other unrelated inserts showed no inhibition, further supporting the specificity of the inhibition (data not shown). Since RXRβ has been shown to transactivate a MHC class I promoter in an RA-dependent fashion (62) and since 9-*cis* RA has been shown to be a ligand for RXRβ (36, 47), we tested the effect of all-*trans* RA and 9-*cis* RA on RXRβ inhibition of the A2 ERE-*tk* promoter activity. As seen in Fig. 2C, there was no change in RXRβ inhibition of promoter activity following addition of 9-*cis* RA at 10^{-6} M or all-*trans* RA at 10^{-6} M. It should be noted that a comparable concentration of all-*trans* RA led to *trans* activation of MHC class I promoter activity by RXRβ (62). Together, these results indicate that (i) RXRβ inhibits estrogen-dependent transcription of ERE-*tk* promoter activity in an element-specific fashion without requiring the addition of 9-*cis* RA and (ii) there is no inhibition of basal transcription activity.

RXRβ inhibits ER action. Because the strongest RXRβ inhibition was seen when cells were treated with estrogen, we sought to determine whether this down-regulation was specific for gene transcription attributable to the ER. Further, we sought to determine whether RXRβ might positively *trans* activate an ERE-*tk* promoter in the absence of the ER. To address these questions, we performed cotransfection experiments with an ER-negative breast tumor cell line, MDA-MB-231. These cells do not express a detectable level of the ER and thus do not respond to E_2 (10). As seen in Fig. 3, the ERE-*tk* promoter activity was low in the absence of the exogenous ER (lanes 1 and 2), and treatment of these cells with E_2 did not significantly change the level of the promoter activity (not shown). As expected, transfection of HEO, an ER expression plasmid (30), resulted in an 8- to 10-fold stimulation of the promoter activity only when cells were treated with E_2 (lanes 3 and 4). However, when RXRβ was cotransfected together with the ER, A2 ERE promoter activity was decreased to 50% of that seen with the control plasmid (compare lane 4 with lane 6). A similar down-regulation of ERE promoter activity was also observed when the same experiment was performed with HeLa cells (not shown). In agreement with the results in Fig. 2, cotransfection of RXRβ in the absence of ER did not significantly affect the basal level of promoter activity, either in the presence or

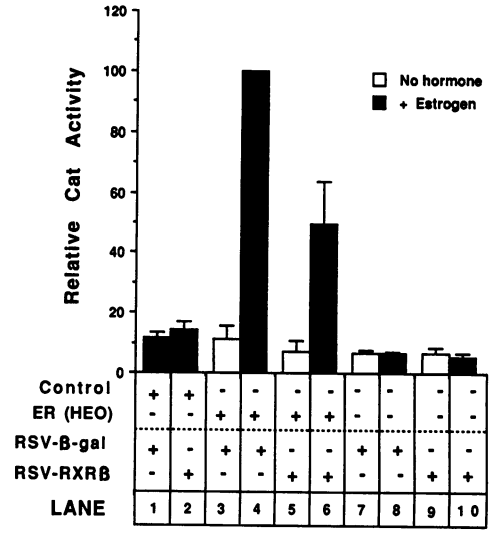

FIG. 3. Evidence that RXRβ inhibits ER-stimulated transcription of an ERE. Twelve micrograms of A2 ERE tkCAT reporter was transfected in the ER-negative cell line MDA-MB-231 with either 5 μg of an ER expression vector (HEO) or 5 μg of a control (pKCR2) in addition to 10 μg of RSV-β-gal or RSV-RXRβ (pRSV-H-2RIIBP) as shown. Mean CAT activity (± standard deviation) of three independent experiments was controlled for luciferase expression and normalized as described in the legend Fig. 2. Cells were treated with E_2 (10^{-7} M) as indicated.

in the absence of E_2 (lanes 9 and 10). These results confirm that the inhibition observed in MCF-7 cells was due to an interference with ER-mediated gene activation caused by RXRβ.

Domain requirements for RXRβ-mediated ERE inhibition. Similar to other nuclear hormone receptors, RXRβ is composed of a modular domain structure: the least conserved N-terminal domain, the highly conserved DNA binding domain, and the moderately conserved C-terminal domain that is responsible for dimerization and ligand binding (49, 51). In agreement with studies performed on other receptors (23), we have found that the C-terminal domain of mRXRβ is essential for both homodimer and heterodimer formation (51). To assess domains involved in RXRβ down-regulation of the ERE-*tk* promoter, we tested three RXRβ deletion mutants deficient in each of the three modular domains but possessing an intact nuclear localization domain. Figure 4A shows a schematic representation of the deletion constructs and results of cotransfection assays performed in MCF-7 cells. To exclude the trivial explanation that abnormal translation or protein transport of the deletion mutants could influence the findings, a monoclonal antibody directed against a conserved region of RXRβ was used to perform immunofluorescence studies of MCF-7 cells transfected with the described deletion mutants. Those studies support the conclusion that the described deletion mutants are expressed and transported to the nucleus of transfected MCF-7 cells in a manner similar to that for intact RXRβ (data not shown). As previously shown (Fig. 2A), the intact RXRβ caused a 70% reduction in the ERE-*tk* promoter activity. In contrast, deletion of the N-terminal domain resulted in a loss of down-regulation. The inability of the N-terminal deletion to inhibit ERE-*tk* promoter activity was not likely due to gross alteration of RXRβ tertiary structure because the same mutant has been shown to retain the ability to *trans* activate

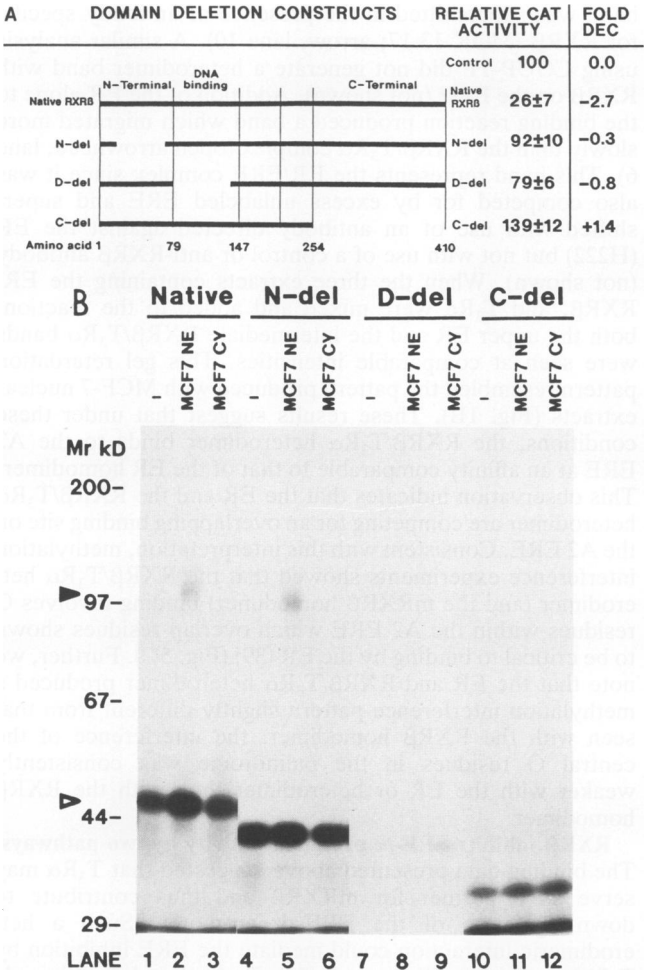

FIG. 4. (A) Deletion analysis of RXRβ inhibition of ERE-dependent *trans* activation. Structures of RXRβ domain deletion constructs are shown. Cotransfection experiments were performed in MCF-7 cells as described for Fig. 2A but using 10 μg of the domain deletion constructs instead of the native RXRβ. Relative CAT activity (mean ± standard error of the mean) of three experiments normalized as described for Fig. 2A and controlled for protein is indicated for each construct. Deletion constructs were designed to conserve the putative nuclear localization signal. Values obtained with the estrogen-stimulated control represent a 3.7-fold stimulation over CAT activity in the absence of ligand and are shown as 100% relative CAT activity. Fold reduction relative to stimulated control is also shown. (B) Evidence that RXRβ binding to the ERE involves an endogenous protein present in MCF-7 nuclear extracts. ^{35}S-labeled in vitro-translated native RXRβ or deletion construct was incubated alone or in combination with nuclear (MCF7 NE) or cytoplasmic (MCF7 CY) extracts prepared from estrogen-treated MCF-7 cells as indicated. After mixing with a biotinylated oligonucleotide corresponding to the A2 ERE, protein-DNA complexes were chemically cross-linked, precipitated with streptavidin-agarose, and resolved by SDS-PAGE (10% polyacrylamide gel). A retarded band (black arrowhead) was observed only when MCF-7 nuclear extracts were combined with ^{35}S-labeled native RXRβ (lane 2) or N-terminally deleted RXRβ (lane 5). No similar retarded band was observed in the presence of a nonspecific biotinylated oligonucleotide (data not shown).

a MHC class I promoter (62). Similarly, deletion of the DNA binding domain or the C-terminal domain resulted in a loss of ERE promoter inhibition. It should be noted that the mutant lacking the DNA binding domain was capable of heterodimerizing with the T_3R (53). Also, the identical C-terminal deletion construct is capable of binding to the ERE in vitro (Fig. 4B). These results suggest that in addition to the DNA binding domain, the N-terminal and C-terminal domains are involved in the repression of ERE-*tk* promoter activity.

ERE binding by RXRβ involves an auxiliary protein present in MCF-7 cells. Previous studies of RXR function support the postulate that mRXRβ functions predominantly as a heterodimer rather than a homodimer (or a monomer) in the intracellular environment (8, 40, 46, 51, 80, 81). If this postulate is correct, the inhibition observed above may be caused by RXRβ acting as a heterodimer. To test this postulate, we used chemical cross-linking experiments to examine MCF-7 nuclear extracts for the presence of proteins capable of interacting with mRXRβ. Figure 4B shows results of a streptavidin-biotin-coupled DNA/cross-linking assay in which in vitro-translated ^{35}S-labeled mRXRβ was mixed with MCF-7 nuclear extracts in the presence of a biotinylated ERE and subjected to chemical cross-linking, and the bound materials were then precipitated with streptavidin-agarose beads. When labeled native RXRβ was cross-linked with MCF-7 cell nuclear extracts (lane 2) and precipitated with the ERE, a high-molecular-size band (100 kDa; black arrowhead) was seen in addition to the monomeric RXR (44 kDa; white arrowhead). A high-molecular-size band was not observed when MCF-7 cytoplasmic extracts were substituted for the nuclear extracts (lanes 3 and 6). Similar to native RXRβ, a high-molecular-size band (lane 4) was observed when the labeled N-deletion mutant was combined with MCF-7 nuclear extracts. As expected, the C-deletion mutant lacking the dimerization domain failed to produce a high-molecular size band. The labeled DNA binding domain mutant failed to bind the ERE and thus could not be precipitated in this assay (lanes 7 to 9). Control experiments using a biotinylated DNA sequence which fails to bind RXRβ showed neither precipitation of monomeric RXRβ nor the high-molecular-size band (not shown). These results indicate that MCF-7 nuclear extracts, but not cytoplasmic extracts, contain an auxiliary factor (likely a heterodimer partner and not a homodimer; see below) that is capable of associating with RXRβ to bind the A2 ERE.

RXRβ heterodimerizes with T_3Rα to bind the ERE. To look for heterodimer partners contributing to ERE binding of RXRβ, we tested the ability of T_3Rα (34), COUP-TF (1, 78), and the ER (7) to dimerize with RXRβ and bind to the ERE. It has been shown that T_3R not only binds to the ERE (27) but also is capable of forming a heterodimer with RXRβ (46, 80) to bind various hormone response elements, including the "imperfect" vitellogenin B1 ERE (68), with strikingly higher affinities than do either RXRβ or T_3Rα homodimers (51). Gel retardation assays were performed with labeled A2 ERE oligonucleotides and extracts prepared from Sf9 cells infected with recombinant baculovirus encoding proteins for the ER, mRXRβ, COUP-TF, or T_3Rα. These extracts were estimated to contain similar levels of receptor proteins (not shown). Addition of extracts containing either T_3Rα (Fig. 5A, lane 1) or mRXRβ (lane 9) alone to the binding reaction produced a weak, barely detectable band. However, when extracts containing both RXRβ and T_3Rα were combined, a new band of much greater intensity was seen (lane 2; black arrowhead). The newly formed band was specifically competed for by the A2 ERE (lane 3) but not by a control AP-1 element (lane 4) and was not produced using control extracts prepared from Sf9 cells infected with wild-type baculovirus (not shown). This new complex was most likely a RXRβ/T_3Rα heterodimer bound to the ERE, since much of this

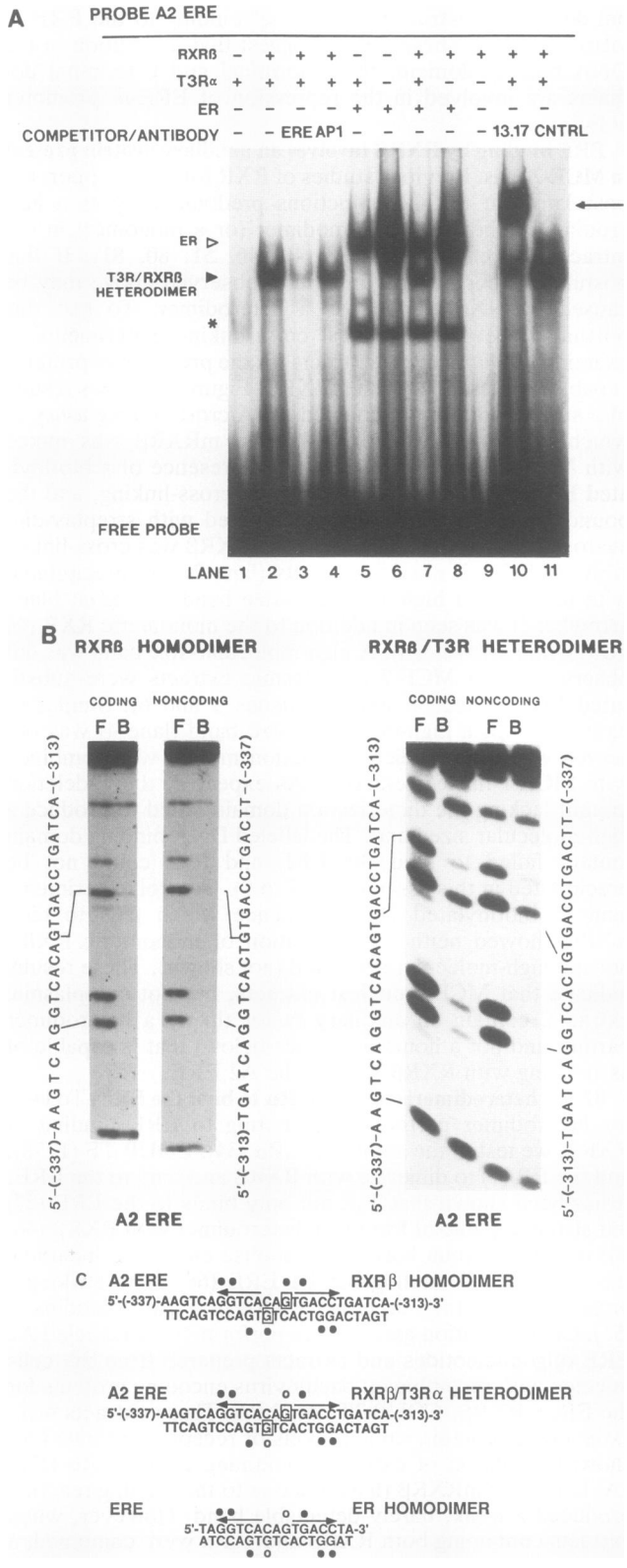

FIG. 5. Evidence that RXRβ heterodimerization with $T_3R\alpha$ results in increased A2 ERE binding. (A) Gel retardation assay. ^{32}P-labeled A2 ERE oligonucleotide was mixed with extracts prepared from Sf9 cells infected with recombinant baculovirus expressing RXRβ, $T_3R\alpha$, or ER, alone or in combination, as indicated. Coincubation of $T_3R\alpha$ and RXRβ generated a complex of mobility intermediate between bands generated with either $T_3R\alpha$ or RXRβ alone (lane 2, black arrowhead). This new complex was competed for with a 200-fold molar excess A2 ERE but not with a nonspecific oligonucleotide (AP-1). Coincubation of RXRβ with the ER (lane 8) or COUP-TF (not shown) did not generate a new complex. Addition of 1 μl of antibody (MOK 13.17) directed against RXRβ for 15 min at 30°C further shifted this band (black arrowhead) to the position noted by the arrow. One microliter of an antibody of unrelated specificity (MOK 14.44; CNTRL) had no such effect. (B) Methylation interference. DNA isolated from free (F) and bound (B) protein/DNA complexes was resolved on a 12% denaturing polyacrylamide gel. G residues whose methylation completely abolishes DNA binding appear as missing bands in the bound lanes and are summarized as black dots in panel C. Partially interfering residues are depicted as open circles. The published pattern of the ER on the same sequence (ERE) is provided for comparison (39). Central G residues showing differential methylation between the receptors are boxed.

band was supershifted in the presence of antibody specific for RXRβ (MOK 13.17; arrow, lane 10). A similar analysis using COUP-TF did not generate a heterodimer band with RXRβ on the ERE (not shown). Addition of the ER alone to the binding reaction produced a band which migrated more slowly than the RXRβ/$T_3R\alpha$ complex (open arrowhead; lane 6). This band represents the ER/ERE complex since it was also competed for by excess unlabeled ERE and supershifted with use of an antibody directed against the ER (H222) but not with use of a control or anti-RXRβ antibody (not shown). When the three extracts containing the ER, RXRβ, and $T_3R\alpha$ were mixed and added to the reaction, both the upper ER and the intermediate RXRβ/$T_3R\alpha$ bands were seen at comparable intensities. This gel retardation pattern resembled the pattern produced with MCF-7 nuclear extracts (Fig. 1B). These results suggest that under these conditions, the RXRβ/$T_3R\alpha$ heterodimer binds to the A2 ERE at an affinity comparable to that of the ER homodimer. This observation indicates that the ER and the RXRβ/$T_3R\alpha$ heterodimer are competing for an overlapping binding site on the A2 ERE. Consistent with this interpretation, methylation interference experiments showed that the RXRβ/$T_3R\alpha$ heterodimer (and the mRXRβ homodimer) binding involves G residues within the A2 ERE which overlap residues shown to be critical to binding by the ER (39) (Fig. 5C). Further, we note that the ER and RXRβ/$T_3R\alpha$ heterodimer produced a methylation interference pattern slightly different from that seen with the RXRβ homodimer; the interference of the central G residues in the palindrome was consistently weaker with the ER or heterodimer than with the RXRβ homodimer.

RXRβ inhibits ERE-*tk* promoter activity by two pathways. The binding data presented above suggested that $T_3R\alpha$ may serve as a partner for mRXRβ and thus contribute to down-regulation of the ERE-*tk* promoter. Such a heterodimeric interaction could mediate the ERE inhibition by T_3R described by Glass et al. (28). To further define the role of $T_3R\alpha$ in the inhibitory effect, cotransfection experiments were performed with MDA-MB-231 cells, using expression plasmids for the ER, $T_3R\alpha$, and mRXRβ. MDA-MB-231 cells were used for these experiments since they permitted adjustment of the amounts of RXRβ and $T_3R\alpha$ vectors relative to the ER expression vector. Cells were cotransfected with HEO (ER) and either 0.2 or 2.0 μg of pRSV-H-2RIIBP or pRSV-$T_3R\alpha$, either alone or in combination (Fig. 6). Cotransfection of increasing amounts of RXRβ, as expected, resulted in inhibition of the ERE promoter activity

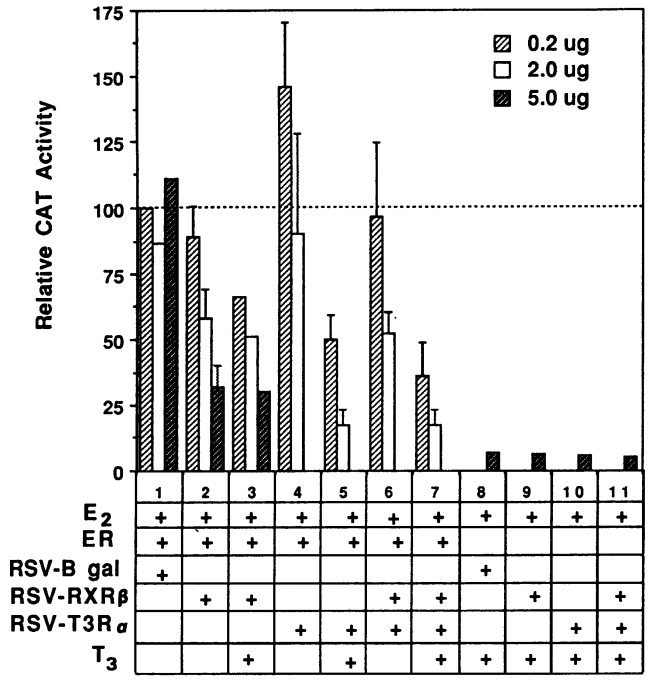

FIG. 6. Evidence that inhibition of estrogen-responsive gene transcription by RXRβ involves two pathways. Cotransfection experiments were performed with MDA-MB-231 cells, and data were controlled and normalized as described for Fig. 3. All transfections were performed in the presence of E_2 (10^{-7} M) and 3.5 μg of ER (HEO) as indicated. Expression vectors were added for RXRβ (RSV-RXRβ), T_3Rα (RSV-T_3R), or control (RSV-β-gal) at either 0.2, 2.0, or 5.0 μg as indicated. T_3 was added at 10^{-7} M as indicated. Plates transfected with both T_3Rα and RXRβ received 0.1 or 1.0 μg (total of 0.2 and 2.0 μg of expression vector, respectively) of each vector. pBluescript was used to equalize amounts of DNA added to cells.

(blocks 2 and 3) compared with transfection of the RSV-β-gal control (block 1). Transfection of increasing amounts of T_3Rα alone in the absence of thyroid hormone resulted in either a slight stimulation or no reduction of ERE-*tk* promoter activity (block 4). Consistent with prior reports (28), addition of thyroid hormone to plates transfected with T_3Rα alone did result in down-regulation of CAT activity (block 5). Cotransfection of RXRβ and T_3Rα together caused a significant reduction in promoter activity in the presence of thyroid hormone. This down-regulation was dependent on the amounts of pRSV-H-2RIIBP and pRSV-T_3Rα added to the cells. Similar T_3-dependent inhibition of ERE-*tk* promoter activity was observed following cotransfection of RXRβ and T_3Rα in MCF-7 cells (not shown). It is noteworthy that in the absence of thyroid hormone, but in the presence of T_3Rα and RXRβ, the reduction was similar to that seen with RXRβ alone. It should be noted that in the absence of added ER, we did not observe stimulation of reporter activity following addition of RXRβ or T_3Rα, either alone or in combination (blocks 9 to 11). Thus, there appear to be two distinct pathways leading to an inhibition of ERE-*tk* promoter activity: (i) a thyroid hormone-independent mechanism, seen with addition of mRXRβ only, and (ii) a thyroid hormone-dependent mechanism, seen with T_3Rα alone or in combination with RXRβ. These results suggest that since the down-regulation of ERE-*tk* promoter activity seen with RXRβ alone did not require addition of T_3 (Fig. 2 to 4), it is likely caused by the interaction of RXRβ with an unidentified heterodimer partner and not by heterodimerization with T_3Rα.

DISCUSSION

We have shown that cotransfection of RXRβ leads to strong element-specific inhibition of ERE-*tk* reporter activity in MCF-7 cells. This inhibition results from a block in ER-mediated activation because the inhibition was not observed in MDA-MB-231 cells, which do not express the ER, but became detectable following cotransfection of the ER (Fig. 3). Further, MCF-7 cells were shown to express RXRβ and an auxiliary protein which contributed to ERE binding activity. To determine whether a known heterodimer partner of RXRβ could contribute to the observed inhibition, we studied the role of T_3Rα, which dimerizes with RXRβ and binds to the A2 ERE (Fig. 5). We found that transfection of T3Rα alone or in combination with RXRβ also inhibited ERE-*tk* reporter activity. In contrast to the inhibition caused by RXRβ alone (which was observed in the absence of a receptor-specific ligand), T_3Rα-mediated inhibition was dependent upon addition of thyroid hormone. These results indicate that estrogen-responsive genes are not only activated by the ER, but are also repressed by RXRβ. Because of the multiple partners with which RXRβ is known to dimerize, such negative regulation could involve multiple nuclear hormone receptors. These findings suggest that the net ERE activity measured in MCF-7 cells represents a balance between a positive effect produced by the endogenous ER and a negative effect produced by the endogenous RXRβ.

Feavers et al. (21) have reported the existence of a non-ER protein present in MCF-7 cells that can bind to EREs. Based solely on molecular size, this protein (70 kDa) is unlikely to be RXRβ (44 kDa; Fig. 1) but may represent an RXR heterodimer partner. Other members of the RXR gene family (i.e., RXRα and RXRγ [49]) may represent the protein described by Feavers et al. (21), although an ERE-modulating role for these RXR proteins has yet to be demonstrated. Involvement of a non-ER protein in transcriptional inhibition of an ERE reporter in vitro has been reported by Kaling et al. (37); however, binding of that protein to the ERE has not been demonstrated. Similar to our findings, Sharif and Privalsky (69) reported that v-ErbA, an oncogenic variant of T_3R, is capable of binding to the ERE and suppressing ERE-driven CAT activity. These authors concluded that the suppression was caused by interference with ER action by v-ErbA. The inhibition caused by RXRβ may operate through a similar mechanism. However, since v-ErbA is not normally a constituent of mammalian cells, the significance of v-ErbA repression in the physiologic regulation of estrogen-responsive genes is less clear.

ERE inhibition by RXRβ involves an auxiliary heterodimer partner. The inhibition of ERE-*tk* promoter activity caused by RXRβ and T_3Rα, either alone or in combination, may be assumed to be mediated by a heterodimeric complex of these receptors. This assumption is supported by the demonstration that RXR has a strong propensity to form heterodimeric complexes with other nuclear hormone receptors (46, 51, 80). Consistent with the involvement of RXR heterodimers in the inhibition of ERE-*tk* promoter activity, we show that ERE binding by RXRβ involves an auxiliary factor present in MCF-7 cell extracts (Fig. 4B). This finding resembles the demonstration that T_3Rs (and also VDRs and RARs) require an accessory protein present in mammalian nuclei that

dimerizes with them (i.e., RXRs) to result in optimal target DNA binding and *trans* activation of target genes (27, 40, 44–46). The poor binding of the RXRβ homodimer to the A2 ERE (Fig. 5A) further supports the role of a heterodimer partner. On the basis of these results, we favor the interpretation that the inhibition observed when RXRβ alone is transfected (Fig. 2 and 4A) is caused by interaction with an unidentified heterodimeric partner rather than an RXRβ homodimer.

The inhibition associated with transfection of T_3Rα in the presence of thyroid hormone (Fig. 6) is likely caused by T_3Rα acting as a heterodimer rather than as a homodimer (or monomer). Interestingly, the interaction of the RXRβ/T_3Rα heterodimer with an ERE appears quite specific, since the closely related RXRβ/T_3Rα heterodimer has been reported to bind poorly, if at all, to the ERE (81). In view of the tendency of T_3Rα to function as a heterodimer, and the inability of the RXRα/T_3Rα heterodimer to bind the ERE, the T_3-induced T_3Rα-mediated inhibition of ERE *trans* activation reported by Glass et al. (28) may be produced by RXRβ/T_3Rα heterodimers. Similarly, we observed T_3Rα-dependent inhibition to be T_3 dependent. However, since the RXRβ-induced inhibition of ERE-*tk* promoter activity in MCF-7 cells was not dependent upon T_3 treatment, it may be concluded that the auxiliary endogenous RXRβ heterodimer partner (mentioned above) is not T_3Rα.

The mechanism of inhibition. Our finding that the RXRβ/T_3Rα heterodimer contacts the ERE through a the same set of G residues as does the ER suggests that ER displacement from the ERE is required for the observed inhibition. A requirement for DNA binding is further supported by the finding that deletion of either the DNA binding domain or the C-terminal dimerization domain abolished the inhibition. This finding contrasts with reports describing inhibition of ERE-driven reporter activity mediated by the glucocorticoid receptor (74) or c-*fos*/c-*jun* (17), which did not require ERE binding. Even though binding of RXRβ to the ERE is a prerequisite, mere binding to the ERE was insufficient to cause the observed inhibition. This is evident from the finding that deletion of the N-terminal domain of RXRβ abolished the inhibitory activity. Of note, we show that deletion of the N-terminal domain did not prevent ERE binding in vitro (Fig. 4B). Furthermore, the deletion mutant used in this work was capable of *trans* activating an MHC class I promoter as efficiently as did intact RXRβ, leading us to conclude that the N-terminal domain is not essential for activating the MHC class I promoter (62). Collectively, these data suggest the mechanism of ERE repression by RXRβ involves dimerization with an auxiliary protein, association with DNA, and interaction of other factors with an intact N-terminal domain.

It is noteworthy that the inhibition observed in the presence of RXR alone did not require the RXR-specific ligand 9-*cis* RA (36, 47). This finding indicates that RXRβ can function as a transcription factor in the absence of a specific ligand (Fig. 2C). In support of these results, we found that RXRβ and T_3Rα can synergistically enhance transcription of malic enzyme thyroid response element and MHC class I reporters in the absence of 9-*cis* RA (34, 51). A similar unidirectional hormone requirement has been reported for the VDR/RXR heterodimer (80). This finding is consistent with the cross-inhibition of T_3Rs and RARs in the absence of a ligand (23).

Näär et al. (61) and Lipkin et al. (48) proposed that the inhibitory effects of nuclear hormone receptors associated with binding to several hormone-responsive elements are caused by conformational constraints of receptor binding imposed by the target elements. It is possible that such conformational restraints induced by RXRβ binding to the ERE might result in an inhibitory effect mediated by the previously described N-terminal or C-terminal transcriptional activation domains (TAF-1 and TAF-2 [60, 75, 79]). If such a mechanism exists, it may be variably dependent on the specific heterodimer partner. It will be of interest to determine whether RXRα and RXRγ, which are highly divergent from RXRβ in the N-terminal domain but share homology in the DNA and ligand binding domain, can also elicit inhibition similar to that demonstrated for RXRβ. It should be mentioned that under no condition tested to date has RXRβ (with or without the T_3R) been observed to function as a positive transcription factor on an ERE.

Physiologic implications. Physiologic and experimental states have been described which may involve RXRβ-mediated inhibition of estrogen-responsive gene transcription such as described here. For instance, during normal mammary development of the rat, cellular differentiation proceeds to a point at which, despite adequate estrogen exposure and the presence of ER, the tissue becomes refractory to the effect of estrogen (71). Similarly, the modulation of luteinizing hormone synthesis in the pituitary by steroids is complex and cannot be explained solely on the basis of estrogen and ER concentrations (70). Because the ER acts only as a transcriptional activator, the existence of a specific opposing factor has been proposed to explain such data. Since RXRβ has the capability of interfering with estrogen-induced transcription, RXRβ may play a role in these and similar instances.

In view of the antagonistic effect of RXRβ upon ER function, it is possible that RXRβ plays a role in the antiproliferative effects of retinoids observed in hormonally responsive breast cancer cells (4, 22). A more thorough understanding of the antiestrogenic action of RARs in breast cancer may be clinically relevant since the antiproliferative effects of retinoids are enhanced by the anticancer agent tamoxifen (references 2, 66, and 73 and references therein). Further studies are needed to more clearly define the role of RXRβ in modulation of estrogen-dependent gene transcription in these physiologic, developmental, and neoplastic states.

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