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A Shift in the Ligand Responsiveness of Thyroid Hormone Receptor α Induced by Heterodimerization with Retinoid X Receptor α

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Thyroid hormone (T3) receptors (T3Rs) are ligand-modulated transcription factors that bind to thyroid hormone response elements (T3REs) and mediate either positive or negative transcriptional regulation of target genes. In addition, in response to ligand binding, T3Rs can interfere with AP-1 activity and thereby inhibit transcription of AP-1-responsive genes. T3Rs were recently shown to form heterodimers with retinoid X receptors (RXRs), leading to increased binding to T3REs in vitro and potentiation of transcriptional responses in vivo. Here we demonstrate that T3Rα forms stable heterodimers with RXRα in living cells. Most important, we describe a new role for RXRα in modulating ligand-dependent T3Rα activity: heterodimerization with RXRα greatly increases transcriptional interference with AP-1 activity, augments T3-dependent transcriptional activation, and potentiates the reversal of ligand-independent activation by T3Rα. In all three cases, the responses occur at substantially lower T3 concentrations when elicited by T3Rα plus RXRα than by T3Rα alone. In vitro, the binding of T3 decreases the DNA-binding activity of T3Rα homodimers but does not affect DNA binding by T3Rα:RXRα heterodimers. We provide evidence that increased activities of T3Rα at lower T3 concentrations are not due to changes in its T3 binding properties. Instead, the altered response could be mediated by either RXRα-induced conformational changes, increased stability of heterodimers over homodimers, especially following T3 binding, or both.

Thyroid hormone 3,5,3′-triiodo-L-thyronine (T3) is required for normal growth and development and maintenance of normal metabolism (for a review, see reference 27). The actions of T3 are mediated through binding to T3 receptors (T3Rs), which belong to the nuclear receptor superfamily of transcription factors encoded by the c-*erbA*α and c-*erbA*β genes (36, 41). Nuclear receptors are ligand-activated transcription factors possessing highly conserved DNA-binding domains and moderately conserved ligand-binding domains (7, 15). Together with receptors for retinoic acid (RA) and vitamin D, the T3Rs form a subgroup that recognizes the consensus half-site AGGTCA (7). This sequence can be arranged as a direct repeat, a palindrome, or an inverted repeat, and the spacing between the half sites and their relative orientations determine receptor specificity and the nature of the transcriptional response (26, 34, 39). For example, unliganded T3Rα can repress promoters that contain palindromic T3 response elements (T3REs), and this repression is reversed upon binding of T3, resulting in net activation (13, 37). Unliganded T3Rα can also activate transcription from promoters which contain an inverted repeat of a variant half-site, and this activation is reversed upon T3 binding (34). Whereas the ligand-independent repression of basal promoter activity and the ligand-dependent activation functions are mediated by sequences within the C-terminal ligand-binding domain (4–6, 30, 32), the ligand-independent activation function is mediated through an N-terminal activation domain (34).

The activity of T3Rα is modulated by interactions with other proteins. In vitro, retinoid X receptors (RXRs) form heterodimers with T3Rs that bind to T3REs more efficiently than T3R homodimers (10, 17, 20, 23, 25, 44, 45). In vivo, coexpression of T3Rs with RXRs results in increased ligand-dependent and ligand-independent transcriptional activation (10, 17, 20, 23, 25, 34, 44, 45). On the other hand, AP-1 complexes, composed of either Jun homodimers or Jun/Fos heterodimers (for a review, see reference 1), interfere with ligand-dependent transactivation by T3Rs (46). Conversely, the T3Rs interfere with AP-1-mediated transactivation in a ligand-dependent manner (14, 32, 46). This interference with AP-1 activity is not mediated by direct binding of T3Rs to DNA and requires a short region located at their C termini which is not directly involved in ligand binding (32, 46).

In this report, we directly demonstrate that T3Rα forms a heterodimer with RXRα in vivo. We also show that heterodimerization with RXRα allows ligand-dependent transactivation and ligand-dependent transcriptional interference with AP-1 activity by T3Rα to occur at doses of T3 that are more than 10-fold lower than those required by T3Rα homodimers to elicit responses of similar magnitude. A similar effect on the reversal of ligand-independent transactivation by T3Rα was observed, albeit the shift in dose response was less pronounced. This enhanced ligand responsiveness is not due to a change in the ligand-binding properties of T3Rα. Instead, it could be mediated by RXRα-induced conformational changes, greater stability of heterodimers versus homodimers, or a combination of both mechanisms.

MATERIALS AND METHODS

Cell culture, transient transfection, and CAT assays. CV1 and Cos-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% bovine calf serum (BCS) and 10% fetal bovine serum, respectively, both treated with charcoal and AG-1X8 resin (Bio-Rad). The calcium phosphate coprecipitation method was used to transfect CV1 cells with 1 μg of reporter plasmid, 15 to 1,000 ng of expression vector, and pUC18 to a total of 3 μg of DNA per 60-mm dish. After 5 to 8 h of incubation with the precipitates, cells were washed once with phosphate-buffered saline (PBS) and then maintained in DMEM supplemented with 5% BCS in the presence or absence of

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hormones T3 (10^{-7} M) or 9-*cis*-RA (10^{-8} M) unless otherwise indicated. Where indicated, tetradecanoyl phorbol acetate (TPA; 100 ng/ml) was added 18 to 20 h later, and after an additional 16 h, cells were harvested. Chloramphenicol acetyltransferase (CAT) enzyme activities were determined as previously described (32).

Cos-7 cells were transfected by a liposome-mediated transfection procedure (Lipofectamine; Gibco) with 6 μg of the indicated expression vector per 60-mm dish. After 6 h, cells were washed with PBS once and maintained in DMEM supplemented with 5% BCS. After 24 h, cells were harvested. Nuclear extracts for band shift analysis (34) or whole-cell extracts for immunoprecipitations (11) were prepared.

The reporter plasmids −73 Col-CAT (2), −60 Col-CAT (2), 2XMHC-CAT (22), 2XT3RE-LUC (16), and RSV_{180}-LUC (34) and the expression vectors for T3Rα (34) and RXRα (45) have been described.

Band shift analysis. Preparation of nuclear extracts and recombinant T3Rα and band shift analysis were done as described previously (34). For supershift analysis with the T3Rα-specific antiserum, recombinant T3Rα or nuclear extracts were incubated with the antiserum or nonimmune rabbit serum on ice for 15 min prior to the binding reaction. T3Rα-specific antiserum was previously described (34). The RXRα-specific antiserum used in immunoprecipitation assays was raised against an N-terminal peptide, MDTKHFLPLDFS, of human RXRα coupled to bovine serum albumin. The RXRα-specific antiserum used in band shift analysis was raised against the ligand-binding domain of human RXRα (24a).

Coimmunoprecipitation assay. For in vivo labeling, proteins were expressed in Cos-7 cells (10^6 cells per 60-mm dish). At 24 h before labeling, cells were transfected with the corresponding expression vectors with Lipofectamine (Gibco). After 5 to 7 h, cells were washed twice with PBS, and fresh medium was added. After 24 h, cells were labeled with [^{35}S]methionine (11), and cell lysates were prepared as described previously (29). Cell lysates were precleared by incubation with preimmune serum (3 to 5 μl) and protein A-Sepharose (Pharmacia) for 60 min. The cleared lysates were incubated further with the appropriate antiserum for 90 min, and protein A-Sepharose was added for another 60 min. After extensive washing, samples were resolved on a 10% polyacrylamide–sodium dodecyl sulfate (SDS) gel.

T3 binding assay. T3 binding assays were done essentially as described before (19). Cell-free translated T3Rα and/or RXRα was incubated on ice for 15 min. ^{125}I-T3 was added in increasing amounts in band shift buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.9], 50 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol), and incubation was continued at room temperature for 20 min. Reactions were filtered through nitrocellulose filters, which were washed three times with 1 ml of binding buffer and counted in a gamma counter.

RESULTS

Stable association of T3Rα and RXRα in vivo. Previously, heterodimerization between T3Rs and RXRs was demonstrated in cell-free systems by immunoprecipitation and band shift analysis (10, 17, 20, 23, 25, 34, 44, 45). Even though analysis of reporter gene activation suggested that heterodimers are also formed in living cells (10, 17, 20, 23, 25, 34, 44, 45), no direct evidence to this effect was provided. To examine whether T3Rα and RXRα are stably associated in vivo, Cos-7 cells were transiently transfected with T3Rα and/or RXRα expression vectors, and extracts from these cells were used in mobility shift and immunoprecipitation analyses. Figure 1A shows a mobility shift assay in which nuclear extracts made from cells transfected with an empty expression vector (pSG5) or expression vectors encoding RXRα and/or T3Rα were used with the myosin heavy chain (MHC) alpha T3RE as a probe. The specific protein-DNA complexes formed were compared with the one formed by recombinant T3Rα produced in *Escherichia coli*. Whereas no specific protein-DNA complexes were detected in extracts of cells transfected with either pSG5 or RXRα, two distinct complexes were found in extracts from T3Rα-transfected cells. The faster-migrating complex had the same electrophoretic mobility as the protein-DNA complex formed by recombinant T3Rα. Only one major complex, corresponding in its mobility to the more slowly migrating complex formed by extracts of T3Rα-transfected cells, was formed by extracts of cells cotransfected with the T3Rα and RXRα expression vectors. Furthermore, the total level of T3RE binding activity exhibited by extracts of T3Rα- plus RXRα-transfected cells was considerably higher than the activity displayed by equal amounts of extracts of cells transfected with T3Rα alone. These results are similar to previous findings with cell-free translated or recombinant proteins (10, 17, 20, 23, 25, 34, 44, 45). The specificities of the DNA-protein complexes were demonstrated by their disappearance in the presence of a 50-fold excess of unlabeled MHC T3RE in the binding reaction. The presence of T3Rα in the two complexes was confirmed by supershift analysis with an anti-T3Rα antiserum raised against a C-terminal peptide (34) but not with nonimmune serum (Fig. 1B). The presence of RXRα in the more slowly migrating complex was confirmed by incubation of the extract with an anti-RXRα antiserum raised against the ligand-binding domain of RXRα, which abolished all binding, whereas nonimmune serum had no effect (Fig. 1C). This inhibition was specific, since anti-RXRα did not affect binding of recombinant T3Rα to the MHC T3RE.

We have also examined the binding activities in the Cos-7 cell extracts used in the above analysis to a specialized T3RE found in the long terminal repeat (LTR) of the Rous sarcoma virus (RSV), called the RSV T3RE, which mediates ligand-independent transactivation by T3Rα (34). Whereas no specific protein-DNA complexes were detected in extracts prepared from cells transfected with pSG5, T3Rα, or RXRα (data not shown), one major band was formed by extracts of T3Rα- plus RXRα-transfected cells (Fig. 1D). This interaction was specific, since it was abolished in the presence of a 50-fold excess of unlabeled RSV T3RE. The presence of both T3Rα and RXRα in this complex was demonstrated by its disappearance in the presence of anti-T3Rα or anti-RXRα antiserum but not with nonimmune serum (Fig. 1E).

The interaction between T3Rα and RXRα was examined more directly by coimmunoprecipitation analysis. When lysates of T3Rα-transfected Cos-7 cells metabolically labeled with [^{35}S]methionine were immunoprecipitated with anti-T3Rα antiserum, a 45-kDa band corresponding to the expected size of T3Rα was detected (Fig. 2A). This band was not precipitated by an anti-RXRα antiserum. This 45-kDa polypeptide was not detected following immunoprecipitation of lysates of cells transfected with either the empty expression vector pSG5 or the RXRα expression vector. When lysates of RXRα-transfected cells were analyzed, a 55-kDa band corresponding to the expected size of RXRα was detected following immunoprecipitation with anti-RXRα but not with anti-T3Rα. Similar immunoprecipitations with lysates from cells transfected with both the T3Rα and RXRα expression vectors revealed two bands corresponding in size to T3Rα and RXRα, detected by either anti-T3Rα or anti-RXRα antiserum. The two bands precipitated by anti-RXRα were of approximately equal intensities, and most important, the precipitation of the 45-kDa T3Rα polypeptide was as efficient as that by anti-T3Rα. Although some association between T3Rα and RXRα was observed when extracts expressing either protein alone were mixed prior to immunoprecipitation, the efficiency of heterodimerization was much lower than that observed after the coexpression of both proteins (Fig. 2B). These results directly demonstrate that T3Rα and RXRα are associated in vivo.

RXRα enhances ligand-dependent interference with AP-1 activity by T3Rα. To determine the effect of RXRα on T3Rα-mediated repression of AP-1 transcriptional activity, the AP-1-dependent reporter −73 Col-CAT (2) was cotransfected into CV-1 cells with either an empty expression vector (pSG5) or expression vectors encoding T3Rα and/or RXRα. As shown previously (32), −73 Col-CAT expression was modestly stimulated by expression of T3Rα, and this activity was repressed three- to fourfold upon T3 addition (Fig. 3A). In the presence

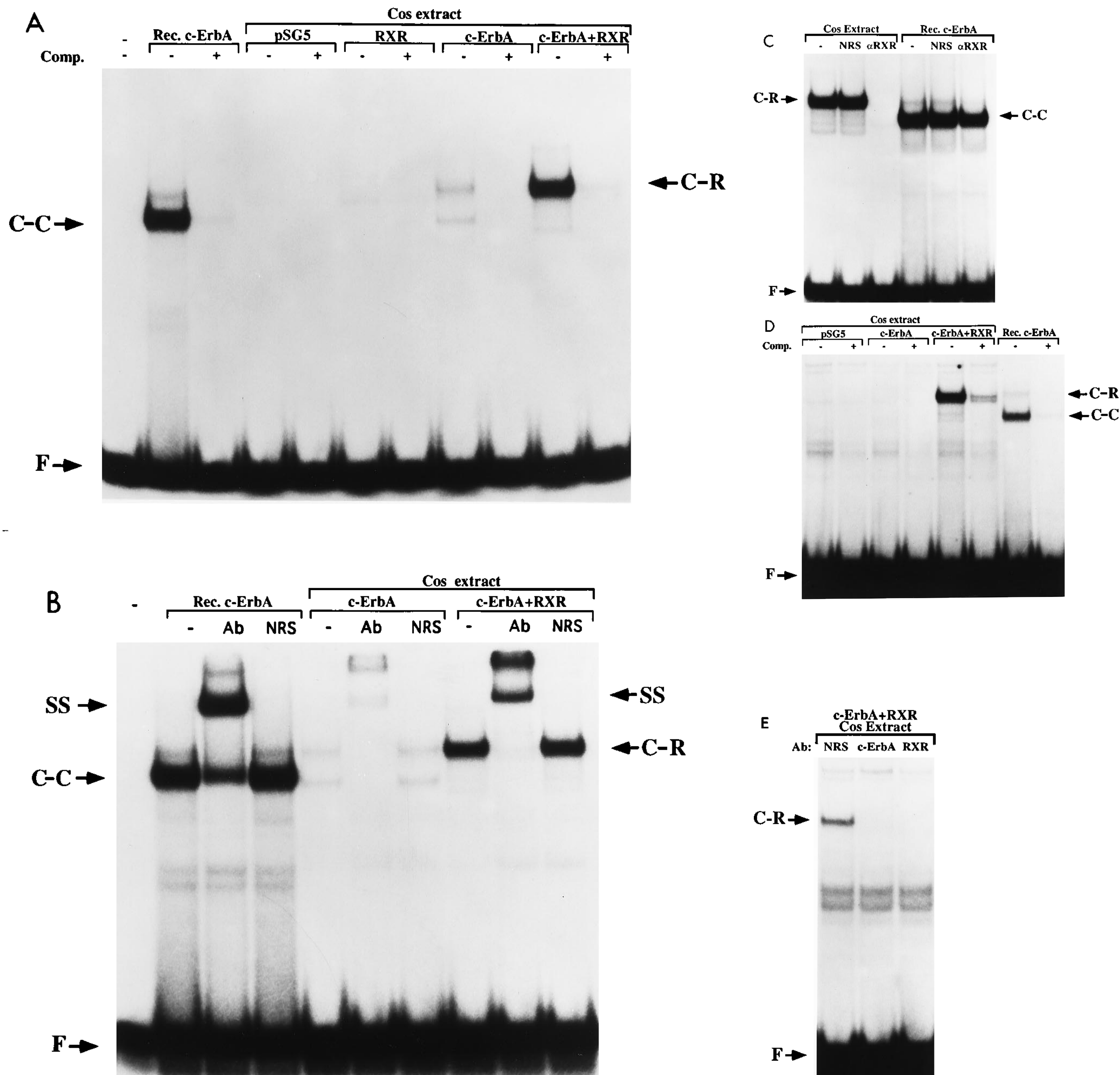


FIG. 1. T3Rα and RXRα form heterodimers in vivo. (A) Cos-7 cells were transfected with an empty expression vector (pSG5) or with expression vectors encoding T3Rα and/or RXRα as indicated. At 24 h after transfection, cells were harvested and nuclear extracts were prepared. These extracts (2 μg per reaction) were used in a mobility shift assay with ^{32}P-labeled MHC T3RE as the probe. C-C, T3Rα homodimer; C-R, T3Rα/RXRα heterodimer; F, free probe; Comp., competitor. (B) Presence of T3Rα in complexes formed from Cos-7 extracts with the MHC T3RE. The extracts from panel A were used in a mobility shift assay in the presence of anti-T3Rα antibody (Ab) or nonimmune serum (NRS) or left untreated (—). To the left is the assay with recombinant T3Rα produced in *E. coli* (Rec. c-ErbA), and to the right are Cos-7 extracts (Cos extract) as indicated. SS, supershifted complex. (C) Presence of RXRα in the complexes formed on the MHC T3RE. Cos7 cell extracts containing T3Rα and RXRα or recombinant T3Rα were used in a mobility shift assay in the presence and absence of nonimmune serum (NRS) or an antiserum against RXRα, as indicated. (D) Interaction of T3Rα and RXRα expressed in Cos-7 cells, determined as in panel A, with the RSV T3RE. (E) Specificity of the interaction of Cos-7 extracts with the RSV T3RE. Cos-7 cell extracts containing T3Rα and RXRα were used in a mobility shift assay in the presence and absence of nonimmune serum (NRS), an antiserum against RXRα (RXR), or an antiserum against T3Rα (c-ErbA), as indicated.

of RXRα, the ligand-dependent repression of −73 Col-CAT expression by T3Rα was greatly enhanced, resulting in 13- to 14-fold repression (Fig. 3A). In contrast, expression of −60 Col-CAT, in which the AP-1 site is deleted (2), was neither stimulated nor repressed by T3Rα in the presence or absence of T3 and/or RXRα (Fig. 3A). This indicates that, as previously shown for T3Rα (32), the target for ligand-dependent repression by the T3Rα:RXRα heterodimer is the AP-1 binding site. RXRα by itself had no significant effect on −73 Col-CAT expression even in the presence of its ligand 9-*cis*-RA. Although 9-*cis*-RA treatment did not stimulate the repressive effect of the T3Rα:RXRα heterodimer, it did stimulate expression of 2XT3RE-LUC, a reporter which contains two copies of the consensus palindromic T3RE (16), by eightfold

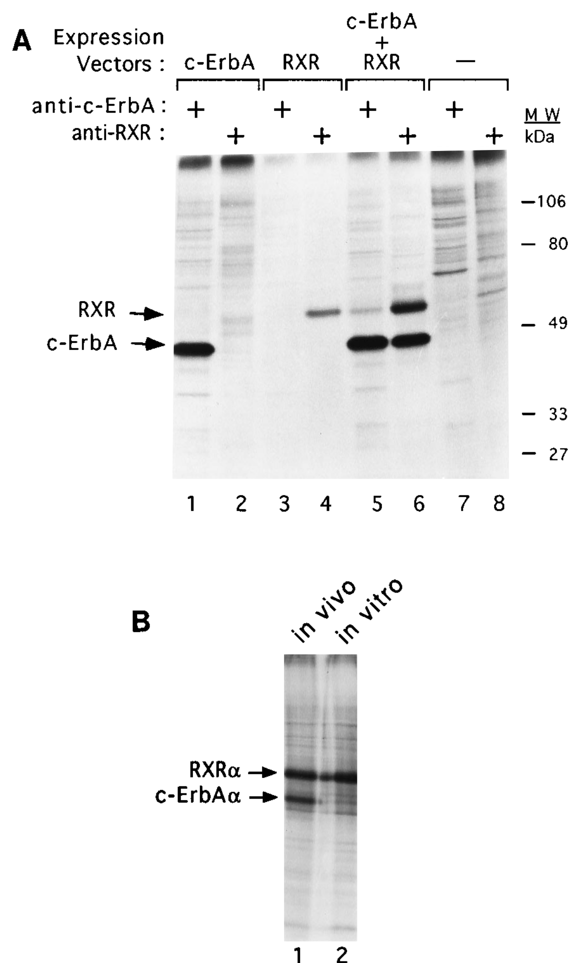


FIG. 2. Immunoprecipitation analysis of T3Rα:RXRα heterodimers. (A) Cos-7 cells were transfected with expression vectors encoding T3Rα and/or RXRα or an empty expression vector (pSG5) as indicated. After 24 h, cells were metabolically labeled with [^{35}S]methionine, lysed under native conditions, and subjected to immunoprecipitation. Final immunoprecipitates were boiled in SDS sample buffer and fractionated on a 10% polyacrylamide–SDS gel. Molecular mass standards are indicated to the right, and the specifically immunoprecipitated proteins are indicated to the left. (B) [^{35}S]methionine-labeled protein extracts containing T3Rα and RXRα were coexpressed (lane 1) or expressed separately and then mixed in vitro (lane 2) and immunoprecipitated as described above with the anti-RXRα antiserum.

(Fig. 3B). These results are consistent with previous reports (20, 45).

The results described above suggested that the T3 response curve of T3Rα may be altered in the presence of RXRα. To compare the anti-AP-1 activities of T3Rα homodimers with those of T3Rα:RXRα heterodimers at various T3 concentrations, we carried out titration experiments. Half-maximal inhibition of −73 Col-CAT activity by T3Rα alone occurred at 2×10^{-8} M T3 (Fig. 3C). In the presence of both T3Rα and RXRα, however, half-maximal inhibition of −73 Col-CAT activity occurred at 5×10^{-10} M T3, and maximal inhibition was achieved at slightly above 10^{-7} M. On the other hand, RXRα did not affect −73 Col-CAT activity in the presence or absence of 9-*cis*-RA. This indicates that T3Rα:RXRα heterodimers respond to T3 treatment much more efficiently than the T3Rα homodimers.

Effect of RXRα on ligand-dependent transactivation and relief of ligand-independent transactivation by T3Rα. It was previously shown that coexpression of T3Rs and RXRs potentiates ligand-dependent transactivation in comparison with activation by T3Rs alone (10, 17, 20, 23, 25, 34, 44, 45). We examined whether heterodimerization with RXRα alters the dose-response curve for T3-dependent activation of a 2X MHC-CAT reporter, which contains two copies of the MHC T3RE (22). Half-maximal activation of 2XMHC-CAT by T3Rα occurred at approximately 2×10^{-8} M T3 (Fig. 4A). In the presence of both T3Rα and RXRα, however, half-maximal activation of 2XMHC-CAT occurred at 8×10^{-10} M T3. Maximal 2XMHC-CAT activation by T3Rα homodimers was achieved at 10^{-7} M T3. At this dose, T3Rα alone had only a marginal effect on reporter gene expression. The same level of activation by the T3Rα:RXRα heterodimer was already achieved at 5×10^{-10} M T3. Maximal activation by the heterodimers was fourfold greater than maximal activation by the homodimers. Thus, in addition to augmentation of ligand-dependent transcriptional activation by T3Rα at saturating T3 concentrations, as reported previously (10, 17, 20, 23, 25, 34, 44, 45), heterodimerization with RXRα increases the sensitivity of T3Rα to T3.

Coexpression of RXRα also increases the ability of T3Rα to activate transcription in a ligand-independent manner (34). As this activity is reversed by addition of ligand, we examined the effect of heterodimerization with RXRα on the sensitivity of this response to T3. The RSV_{180}-LUC reporter (34) was cotransfected into CV-1 cells with a T3Rα expression vector in the absence or presence of an RXRα expression vector. Cells were either left untreated or treated with increasing amounts of T3. Figure 4B shows that in the absence of RXRα, 50% inhibition of the ligand-independent activation of RSV_{180}-LUC was achieved at approximately 5×10^{-11} M T3, while in the presence of RXRα, 50% inhibition was achieved at just below 1×10^{-11} M T3. Thus, although ligand-dependent inactivation of RSV_{180}-LUC expression is also more pronounced in the presence of RXRα, the difference in ligand sensitivity of the heterodimer versus the homodimer is not as great as the differences seen for the other two responses. Both the ligand-dependent activation of the 2XMHC-CAT reporter and the ligand-dependent inhibition of the −73 Col-CAT reporter required 25- to 40-fold less T3 when mediated by the heterodimer instead of the homodimer.

Heterodimerization with RXRα does not change the ligand-binding properties of T3Rα. A plausible explanation for these results is that RXRα increases the affinity and/or capacity of ligand binding by T3Rα. To explore this possibility, T3 binding assays with cell-free-translated T3Rα in the absence or presence of cell-free-translated RXRα were carried out. Binding reactions were performed under conditions which favor heterodimer formation, as determined by electrophoretic mobility shift analysis of the same extracts (data not shown). As shown in Fig. 5, the T3 binding profile of the T3Rα homodimers was similar to that of the T3Rα:RXRα heterodimers, with a K_d of approximately 2×10^{-10} M. The binding assay was repeated under a variety of binding buffer conditions, in the presence and absence of oligonucleotides to which T3Rα and RXRα bind as heterodimers, and at various temperatures, but no appreciable differences were detected in the K_d or B_{max} of T3 binding between the homodimer and the heterodimers (data not shown). T3 binding assays carried out on Cos-7 cells transiently transfected with the T3Rα and/or RXRα expression vector or recombinant T3Rα produced in *E. coli* and cell-free-translated RXRα also did not yield any detectable differences (data not shown). These findings suggest that the observed effects of heterodimerization with RXRα on the different activities of T3Rα are not due to an altered T3 binding property of T3Rα:RXRα heterodimers.

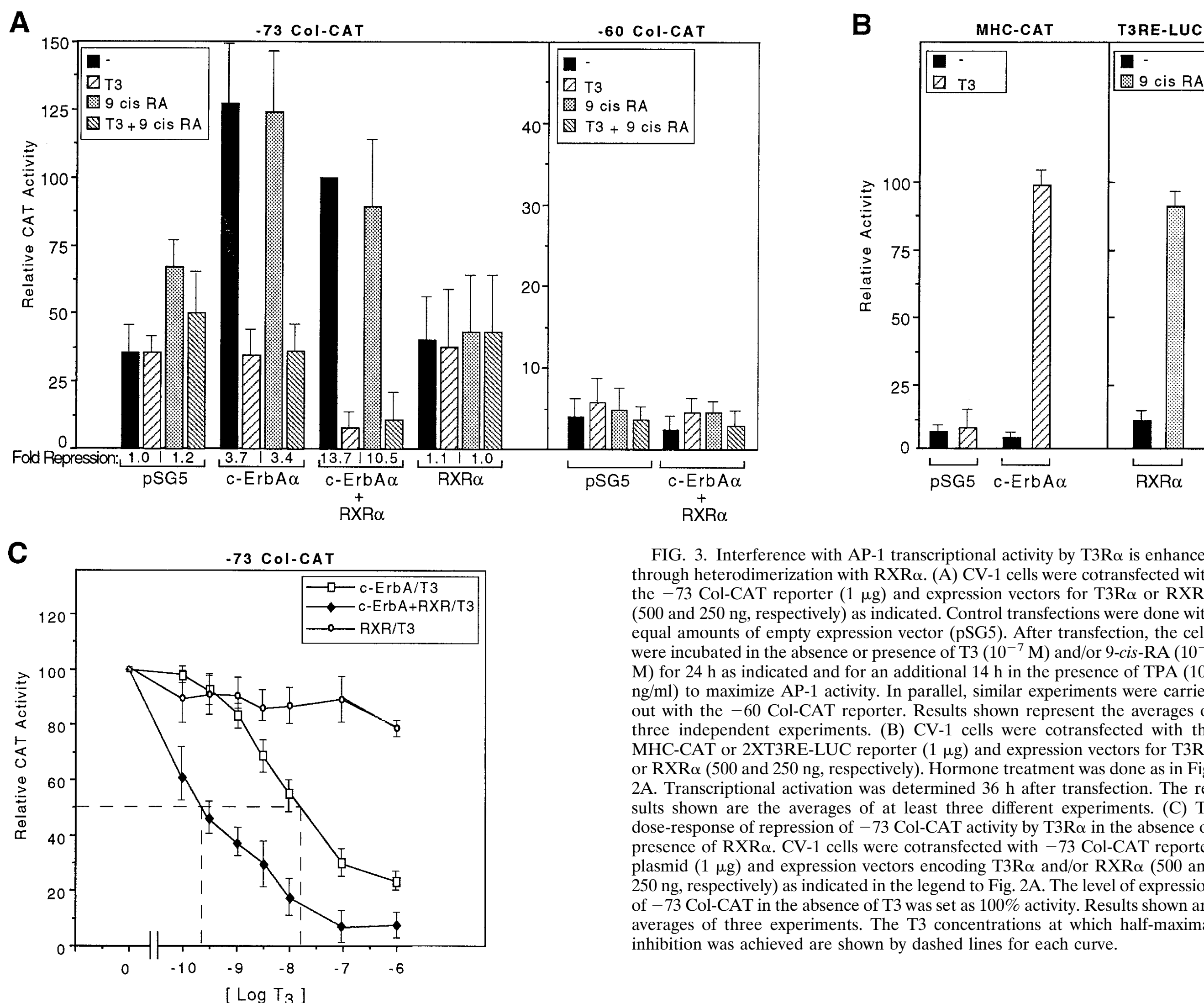


FIG. 3. Interference with AP-1 transcriptional activity by T3Rα is enhanced through heterodimerization with RXRα. (A) CV-1 cells were cotransfected with the −73 Col-CAT reporter (1 μg) and expression vectors for T3Rα or RXRα (500 and 250 ng, respectively) as indicated. Control transfections were done with equal amounts of empty expression vector (pSG5). After transfection, the cells were incubated in the absence or presence of T3 (10^{-7} M) and/or 9-*cis*-RA (10^{-8} M) for 24 h as indicated and for an additional 14 h in the presence of TPA (100 ng/ml) to maximize AP-1 activity. In parallel, similar experiments were carried out with the −60 Col-CAT reporter. Results shown represent the averages of three independent experiments. (B) CV-1 cells were cotransfected with the MHC-CAT or 2XT3RE-LUC reporter (1 μg) and expression vectors for T3Rα or RXRα (500 and 250 ng, respectively). Hormone treatment was done as in Fig. 2A. Transcriptional activation was determined 36 h after transfection. The results shown are the averages of at least three different experiments. (C) T3 dose-response of repression of −73 Col-CAT activity by T3Rα in the absence or presence of RXRα. CV-1 cells were cotransfected with −73 Col-CAT reporter plasmid (1 μg) and expression vectors encoding T3Rα and/or RXRα (500 and 250 ng, respectively) as indicated in the legend to Fig. 2A. The level of expression of −73 Col-CAT in the absence of T3 was set as 100% activity. Results shown are averages of three experiments. The T3 concentrations at which half-maximal inhibition was achieved are shown by dashed lines for each curve.

T3 has distinct effects on T3Rα homodimer and T3Rα:RXRα heterodimer binding to DNA. Another possible explanation for the observed effects of heterodimerization with RXRα on the biological activities of T3Rα is an induced conformational change. By using limited protease digestion, several recent studies have demonstrated that T3 binding has distinct effects on T3R homodimers and T3R:RXR heterodimers in solution (8, 9, 23, 38). By using the mobility shift assay, it was also shown that T3 binding differentially affects homodimer and heterodimer binding to various T3REs (1, 31, 42, 43). We have used the mobility shift assay to assess the effect of T3 on binding of T3Rα homodimers and T3Rα:RXRα heterodimers to the MHC T3RE and RSV T3RE. For homodimer and heterodimer analysis, recombinant T3Rα or extracts of Cos-7 cells transfected with T3Rα and RXRα, respectively, were incubated with increasing amounts of T3 and then used in the mobility shift assay. T3 efficiently inhibited homodimer binding to both the MHC T3RE (Fig. 6A) and RSV T3RE (Fig. 6B) in a dose-dependent manner. On the other hand, no significant change was observed in heterodimer binding to either probe in the presence of T3.

DISCUSSION

T3Rs bind to specific DNA sequences (T3REs) and mediate changes in transcription of specific genes. Binding of T3Rα to T3REs in vitro is much more efficient in the presence of RXRs (10, 17, 20, 23, 25, 34, 44, 45). While heterodimerization between T3Rs and RXRs was previously demonstrated in vitro, these factors are also believed to undergo such interactions in vivo, based on reporter gene activation assays (10, 17, 20, 23, 25, 34, 44, 45). However, these analyses are confounded by the ubiquitous presence of RXRs in the cell lines commonly used in these studies. Here, we provide further and more direct evidence that T3Rα and RXRα form hetero-oligomers in vivo. Stable T3Rα:RXRα complexes can be coimmunoprecipitated from cell extracts expressing both proteins by using antiserum against either protein. Mixing of extracts expressing either protein alone under our immunoprecipitation conditions results in only a low level of complex formation. Therefore, the majority of the T3Rα:RXRα hetero-oligomers thus detected must have formed in vivo.

The heterodimerization with RXRα affects T3Rα activity in three different ways, as it augments not only ligand-dependent

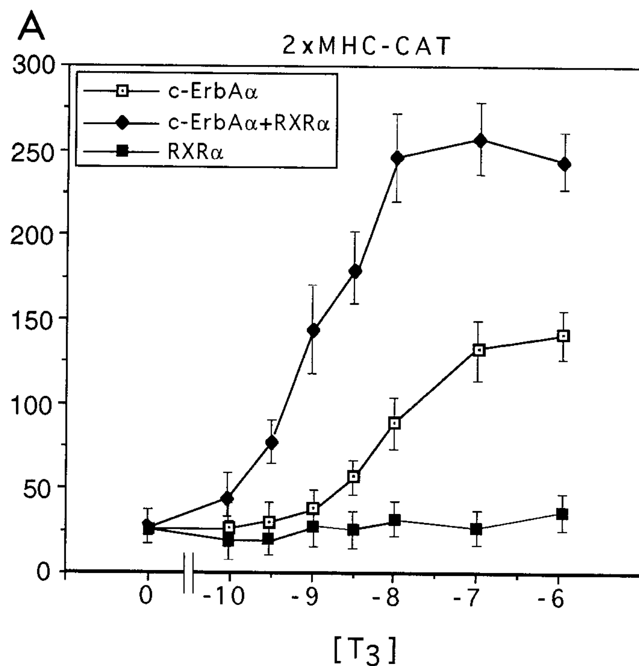
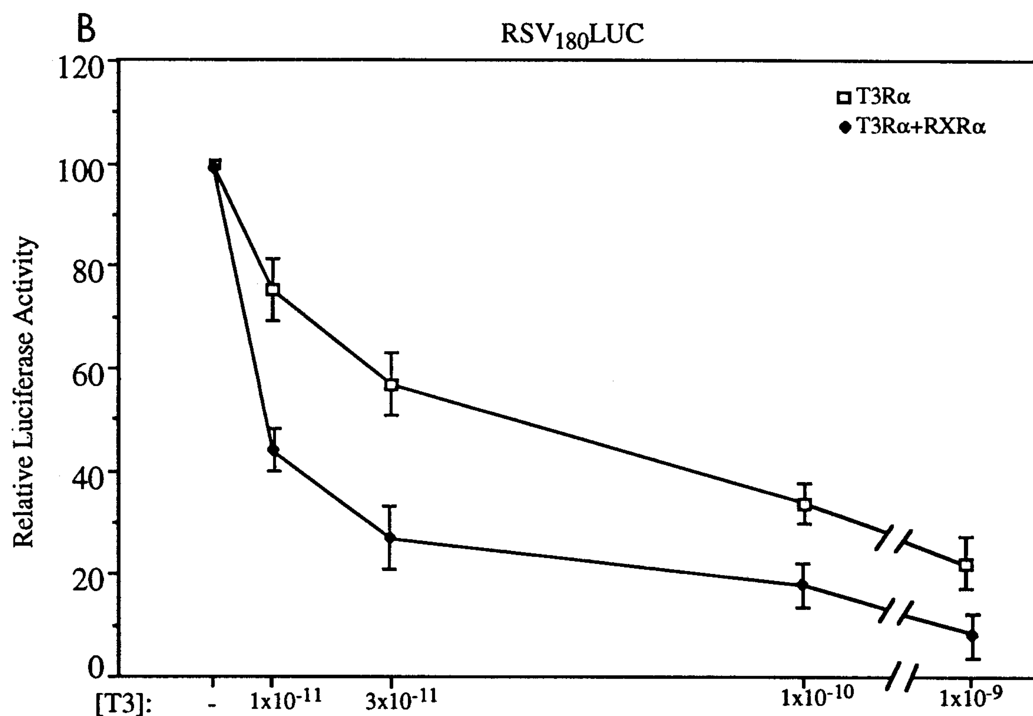


FIG. 4. Ligand-dependent transcriptional activation and relief of ligand-independent transactivation by T3Rα are enhanced through heterodimerization with RXRα. (A) CV-1 cells were cotransfected with 2XMHC-CAT (1 μg) reporter plasmid and expression vectors encoding T3Rα and/or RXRα (15 and 50 ng each, respectively) by the calcium phosphate transfection procedure. Cells were treated twice with increasing amounts of T3, as indicated, 0 and 12 h after the excess calcium phosphate precipitates were removed and harvested at 24 h. The total amount of expression vector in each transfection was kept constant by addition of the empty expression vector pSG5. (B) CV-1 cells were cotransfected with RSV_{180}-LUC reporter plasmid (1 μg) and an empty expression vector (pSG5) or expression vectors encoding T3Rα and/or RXRα (200 ng each), as indicated. After transfection, cells were either left untreated or treated with the indicated amounts of T3 for 36 h and harvested, and luciferase activities were determined. Results shown are averages of three independent experiments.



transactivation but also transcriptional interference with AP-1 activity and, to a lesser extent, reversal of ligand-independent transactivation. The potentiation of T3-dependent reporter gene activation by T3Rs upon heterodimerization with RXRs is well established (10, 17, 20, 23, 25, 34, 44, 45). We have extended these previous results, obtained at saturating T3 concentrations, by demonstrating that in addition to higher overall levels of transcriptional activity, there is also a marked increase in the responsiveness to T3 upon heterodimer formation. Half-maximal activation of the T3R-dependent reporter 2XMHC-CAT by T3Rα occurred at approximately 25-fold-lower T3 concentrations in the presence of RXRα than in its absence. Furthermore, we have found that T3 inhibits T3Rα homodimer binding to the MHC T3RE in a mobility shift assay, whereas T3Rα:RXRα heterodimer binding was not diminished under the same conditions. As discussed below, these differences may account, at least in part, for the enhanced T3 sensitivity of the heterodimer.

It was previously shown that T3Rs strongly inhibit AP-1 activity in a ligand-dependent manner (14, 32, 46). In this report, we show that overexpression of RXRα greatly potentiates T3Rα-mediated inhibition of AP-1 activity. Not only is

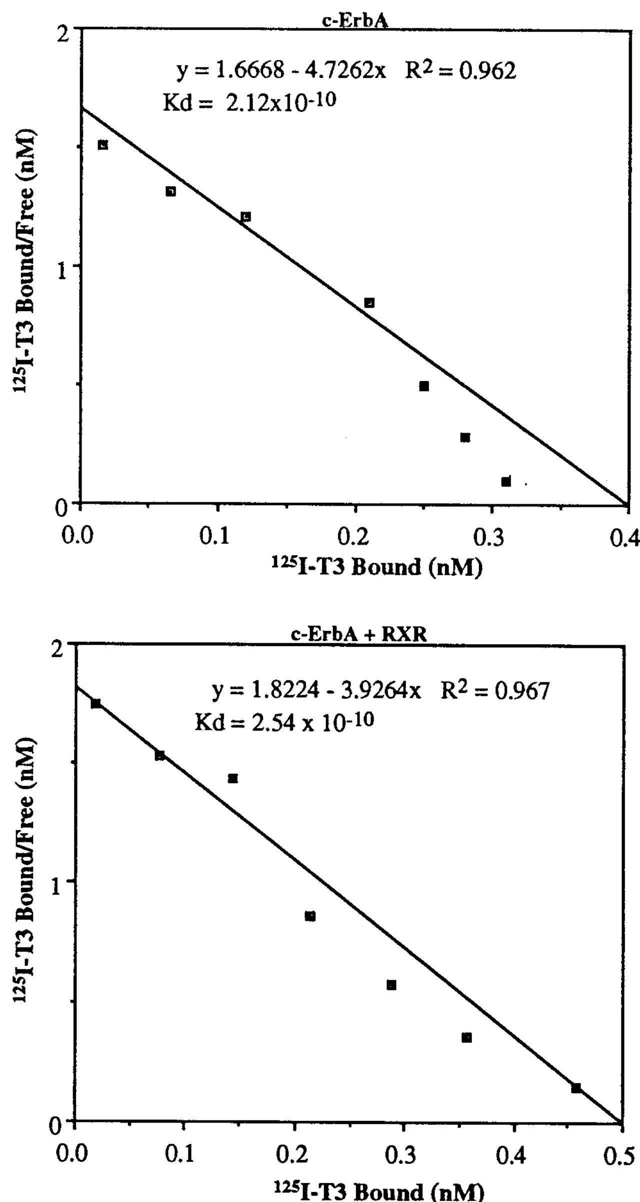


FIG. 5. T3 binding characteristics of T3Rα are not altered by heterodimerization with RXRα. T3Rα and RXRα proteins were synthesized in vitro in a reticulocyte lysate system (45). Lysates containing T3Rα were incubated for 15 min on ice with either unprogrammed lysate or lysates containing RXRα. Proteins were then mixed with ^{125}I-T3 at various concentrations, incubated at 24°C for 20 min, and filtered through nitrocellulose filters. After extensive washing, filters were counted in a gamma counter. Nonspecific T3 binding of unprogrammed lysate was determined and subtracted from total binding with programmed lysate to get specific binding, which is shown. The results are shown as Scatchard plots. Each experiment was repeated four to six times. Results shown are from a representative experiment done in duplicate.

the maximal inhibitory activity achieved in the presence of RXRα three- to fourfold higher than in its absence, but half-maximal inhibition of AP-1 activity is achieved at approximately 40-fold lower levels of T3 when T3Rα is coexpressed with RXRα. These findings differ from those recently reported by Salbert et al. (35), who studied the effect of RARα and RXRα on the AP-1-responsive transforming growth factor beta 1 (TGFβ$_1$) promoter. RARα or RXRα, when expressed alone, inhibited the TPA-induced activity of a TGFβ-CAT reporter in the presence of all-*trans*-RA and 9-*cis*-RA, respectively (35). However, when RARα and RXRα were coexpressed, no further repression of TGFβ-CAT activity was observed. These authors found that T3Rα also inhibited TGFβ-CAT activity, but the effect of RXRα in conjunction with T3Rα was not tested. Consistent with our findings, in CV-1 cells, RXRα alone had no effect on the collagenase promoter activity, but in HepG2 cells, it did give rise to a modest repression (35). This suggests that cell type-specific factors may be involved in this process.

A model proposed to explain transcriptional interference between nuclear receptors and AP-1 that is most consistent with the current data is competition between the two transcription factors for a common cofactor required for efficient transactivation (for reviews, see references 28 and 33). According to this model, in the absence of hormones, AP-1 interacts with this cofactor and activates expression of target genes containing AP-1 binding sites. In the presence of hormone, however, the nuclear receptor gains the ability to interact with the same cofactor, titrating it away from AP-1 and thereby inhibiting AP-1-mediated transcription. The final outcome would also depend on the relative levels of AP-1 and receptor in the cell nucleus. The results described above are consistent with this model. In addition to being a more effective transactivator of T3RE-containing promoters at lower T3 levels, the T3Rα:RXRα heterodimer is a more efficient inhibitor of AP-1 activity. Thus, there is good correlation between enhanced transcriptional efficacy and increased ability to interfere with AP-1 activity. Similar correlations were made by analysis of amino acid substitution mutations affecting the C-terminal activation domain of T3Rα (32).

The third activity of T3Rα that we have examined is its ability to activate the RSV LTR through binding to the RSV T3RE in the absence of ligand (34). Previously, we found that overexpression of RXRs modestly augmented this response, which is reversed upon T3 addition (34). Although the reversal of ligand-independent transactivation by T3Rα:RXRα heterodimers required lower levels of T3 than the reversal of activation by T3Rα alone, the enhancement of T3 sensitivity by RXRα was much lower in this case (only 5-fold) than the enhancement of ligand-dependent activation (25-fold) or interference with AP-1 (40-fold). These differences may be due to the involvement of a different activator domain in ligand-independent activation, which requires only the N-terminal activation domain (34). In contrast, ligand-dependent activation and interference with AP-1 activity are both mediated solely by the C-terminal activation domain, which is also involved directly in T3 binding.

Previous work has shown that ligand binding induces a conformational change in T3Rs in solution (9, 24, 38), and heterodimerization with RXR enhances this ligand-dependent conformational change (8, 24). Such changes in conformation may provide a mechanism for the shift in the response to T3 for both ligand-dependent activation and interference with AP-1 activity. As pointed out above, competition for a common cofactor that is required for both AP-1 and liganded T3Rα for efficient transactivation may be the basis for this case of transcriptional interference. If the T3Rα:RXRα heterodimer is more capable of interacting with this putative cofactor than the T3Rα homodimer, because of a heterodimerization-induced conformational change or simply increased dimer stability (21), heterodimers would interfere with AP-1 activity more effectively than homodimers. The increased stability of the heterodimer may also explain the increased sensitivity to T3 seen for ligand-dependent activation. It was previously shown for some T3REs (1, 31, 42, 43), and confirmed here for

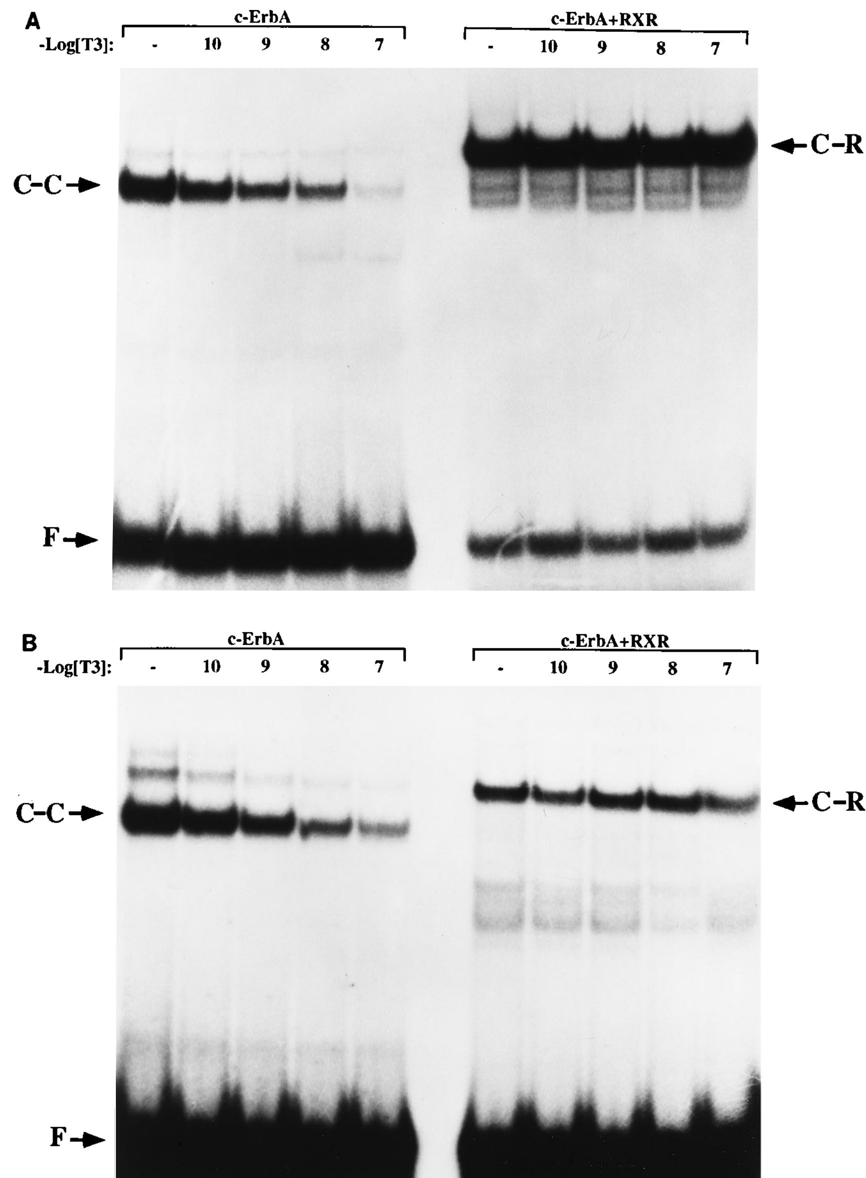


FIG. 6. T3 binding has distinct effects on T3Rα homodimer and T3Rα:RXRα heterodimer binding to DNA. (A) Either recombinant T3Rα produced in *E. coli* or extracts of Cos-7 cells transfected with T3Rα plus RXRα were incubated with the indicated amounts of T3 for 10 min at room temperature and then used in the mobility shift assay with ^{32}P-labeled MHC T3RE as described in the text. The locations of the homodimers (C-C), heterodimers (C-R), and free probe (F) are indicated by arrows. (B) Same as panel A, except that ^{32}P-labeled RSV T3RE was used as the probe.

both the MHC T3RE and RSV T3RE, that T3 decreases the binding of the homodimer to DNA but has no effect on DNA binding by the heterodimer. The decreased DNA-binding activity of the homodimer upon ligand binding was suggested to be due to decreased dimer stability (1, 31, 42, 43). Thus, the binding of T3, which allows the C-terminal activation domain to become active, at the same time renders the homodimer less stable, resulting in a smaller number of functional receptors. The heterodimer, on the other hand, is not subject to this negative effect, and therefore its activity is stimulated much more effectively by ligand binding. These differences in the effect of T3 binding on dimer stability also explain the increased interference with AP-1 activity, even though this response does not involve the binding of receptor to DNA (32, 46).

Regardless of the exact mechanism, the RXR-induced leftward shift in T3 responsiveness is expected to have important consequences for the cell and the entire physiology. Depending on the amount of RXR present, small changes in T3 levels may help target different tissues. It is also possible that by regulating the levels of RXR, the response to T3 can be modulated actively by the cells within the tissue by responding to stimuli other than T3.

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