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Recombinant thyroid hormone receptor and retinoid X receptor stimulate ligand-dependent transcription *in vitro*

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ABSTRACT **The thyroid hormone and retinoid X receptors form a heterodimer with each other and mediate thyroid hormone (T3)-dependent transcription. Retinoid X receptor, in addition, forms a homodimer and mediates 9-*cis*-retinoic acid-dependent transcription. Here, recombinant thyroid hormone receptor and recombinant retinoid X receptor β expressed from baculovirus vectors have been studied for ligand-mediated activation of transcription *in vitro*. We show that the two recombinant receptors, most likely as a heterodimer, cooperatively enhance transcription *in vitro* from a template containing functional T3 responsive elements. The enhancement was specific for the T3 responsive element and was greatest when T3 was added to the reaction (≈14-fold increase). Albeit to a lesser degree, the two receptors also directed transcription in the absence of T3. Template competition experiments suggest that the two receptors enhance formation of the preinitiation complex and that activation by T3 occurs when the ligand binds the receptor prior to (or during), but not after, the formation of the preinitiation complex. Although 9-*cis*-retinoic acid had no effect on the T3-dependent transcription, this ligand activated transcription *in vitro* directed by recombinant retinoic X receptor β, most likely as a homodimer. This activation was observed when using nuclear extracts from embryonal carcinoma cells as a source of basal transcription factors, but not those from B lymphocytes. These results demonstrate that transcriptional activation mediated by T3 and 9-*cis*-retinoic acid can be reconstituted *in vitro* with the respective recombinant receptors.**

Thyroid hormone receptors (TRs) and retinoid X receptors (RXRs) belong to a group of ligand-inducible transcription factors, termed the nuclear hormone receptor superfamily (1). TRα and TRβ activate or repress transcription of thyroid hormone (T3) responsive genes by binding to T3 responsive elements (TREs). TREs share a common sequence motif, AGGTCA (2–7). TRs readily heterodimerize with RXRs (subtypes α, β, and γ) *in vitro*, independent of T3 and TREs (8–11). TR–RXR heterodimers bind to TREs at an affinity much higher than that of either receptor alone. When the two receptors are cotransfected into cultured cells, TRE-containing reporters are synergistically activated (8–11). Thus, RXRs are candidates for the TR auxiliary protein (TRAP) that enhances binding of TR to TREs (12, 13). RXRs also heterodimerize with other members of the superfamily and are involved in gene regulation by other ligands (14–16). Moreover, RXR functions as a homodimer and stimulates transcription when bound to its specific ligand, 9-*cis*-retinoic acid (9cRA) (17–19).

Examples of ligand-dependent transcription *in vitro* have been demonstrated by using extracts containing endogenous receptors. Corthésy *et al.* (20) showed that *Xenopus* liver extracts containing the estrogen receptor direct transcription *in vitro* from the vitellogenin gene promoter upon addition of β-estradiol. Bagchi *et al.* (21) showed progesterone-dependent transcription *in vitro* using extracts from breast tumor cells. Suen and Chin (22) demonstrated that nuclear extracts from pituitary cells support T3-dependent transcription *in vitro*. Efforts have been made to reconstitute receptor-mediated transcription *in vitro* by using cloned receptors. Recombinant estrogen receptors (23) and glucocorticoid hormone receptors (24–27) have been shown to activate transcription *in vitro* from respective hormone responsive promoters. Unlike *in vivo* activation, however, specific ligands have no effect on transcription in these systems. Ligand-independent transcription *in vitro* has also been observed with a natural progesterone receptor (28). On the other hand Elliston *et al.* (29) reported that the recombinant progesterone receptor produced from a baculovirus vector directs ligand-dependent transcription *in vitro*. Recently, Fondell *et al.* (30) have shown that a recombinant (r) TRα represses transcription *in vitro* from TRE-containing promoters.

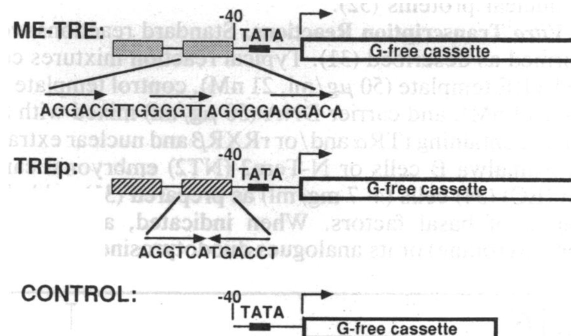

FIG. 1. Templates used in this work. The ME-TRE and TREp templates had two copies of the respective TREs connected to the 40-bp basal promoter with a TATA box. The control template had a shorter G-less cassette.

MATERIALS AND METHODS

Templates. pL[d]40GF was constructed from pL[d]60GF (31) by overlap amplification. The ME-TRE and TREp templates (Fig. 1) were constructed from pL[d]40GF. Oligonucleotides containing the palindromic TRE (TREp, 5) or the TRE of the

Abbreviations: TR, thyroid hormone receptor; RXR, retinoid X receptor; T3, thyroid hormone; RA, retinoic acid; 9cRA, 9-*cis*-RA; TRE, T3 responsive element; PIC, preinitiation complex; r, recombinant; EC, embryonal carcinoma; DIT, diiodotyrosine; T4, thyroxine; TRIAC, triiodothyroacetic acid.

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malic enzyme gene (4, 10) (ME-TRE) were cloned into the *Bgl* II site of $pL^{d}40GF$.

Recombinant Receptor Preparations. rTRα and rRXRβ in baculovirus vectors have been described (10, 32). Nuclei from Sf9 cells (33) were resuspended in buffer D [25 mM Hepes, pH 7.8/15% (vol/vol) glycerol/40 mM KCl/0.1 mM EDTA/0.1 mM EGTA/0.1 mM spermidine/1 mM phenylmethylsulfonyl fluoride/aprotinin (10 μg/ml)/E64 (5 μg/ml)/ pepstatin A (10 μg/ml)/leupeptin (10 μg/ml)/2 mM dithiothreitol]. $(NH_4)_2SO_4$ (4 M) was then added to a final concentration of 0.37 M. Pellets were resuspended in buffer D and dialyzed. Receptor concentrations ranged from 10 to 30% of total nuclear proteins (32).

***In Vitro* Transcription Reactions.** Standard reactions were performed as described (31). Typical reaction mixtures contained TRE template (50 μg/ml, 21 nM), control template (30 μg/ml, 13 nM), and carrier DNA (50 μg/ml) mixed with Sf9 extracts containing rTRα and/or rRXRβ and nuclear extracts from Namalwa B cells or N-Tera2 (NT2) embryonal carcinoma (EC) (34) cells (4–7 mg/ml) as prepared (31), added as a source of basal factors. When indicated, a ligand, T3 (triiodothyronine) or its analogues diiodotyrosine (DIT), thyroxine (T4), triiodothyroacetic acid (TRIAC) (0.2 μM) (Sigma), 9cRA (1^{-5}–10 μM) (gift from J. Grippo, Hoffmann-La Roche), or all-*trans*-retinoic acid (RA; 1–10 μM; Sigma) was added. Transcription was performed (31). To some reaction mixtures, 0.05% sarkosyl was added 15 sec before addition of NTPs to allow a single round of transcription (35). Transcripts were quantified using the IMAGEQUANT software after PhosphorImager analysis (Molecular Dynamics).

RESULTS

Fig. 1 depicts the templates used in this study. The ME-TRE template contained the malic enzyme gene TRE (4, 10) placed in front of the 40-bp basal promoter fused to a G-free cassette. The ME-TRE contains direct repeats of the AGGTCA motif and elicits T3-dependent reporter activity in transfected cells (10, 36). Another template, TREp, contained palindromic AGGTCA sequences (5, 7). Reporters containing a TREp are activated by T3 or RA after transfection with TR, RA receptor, or RXR (5, 7, 11, 37). The control template had only the basal promoter and a G-free cassette 101-bp shorter than that used for the TRE templates. *In vitro* transcription assays

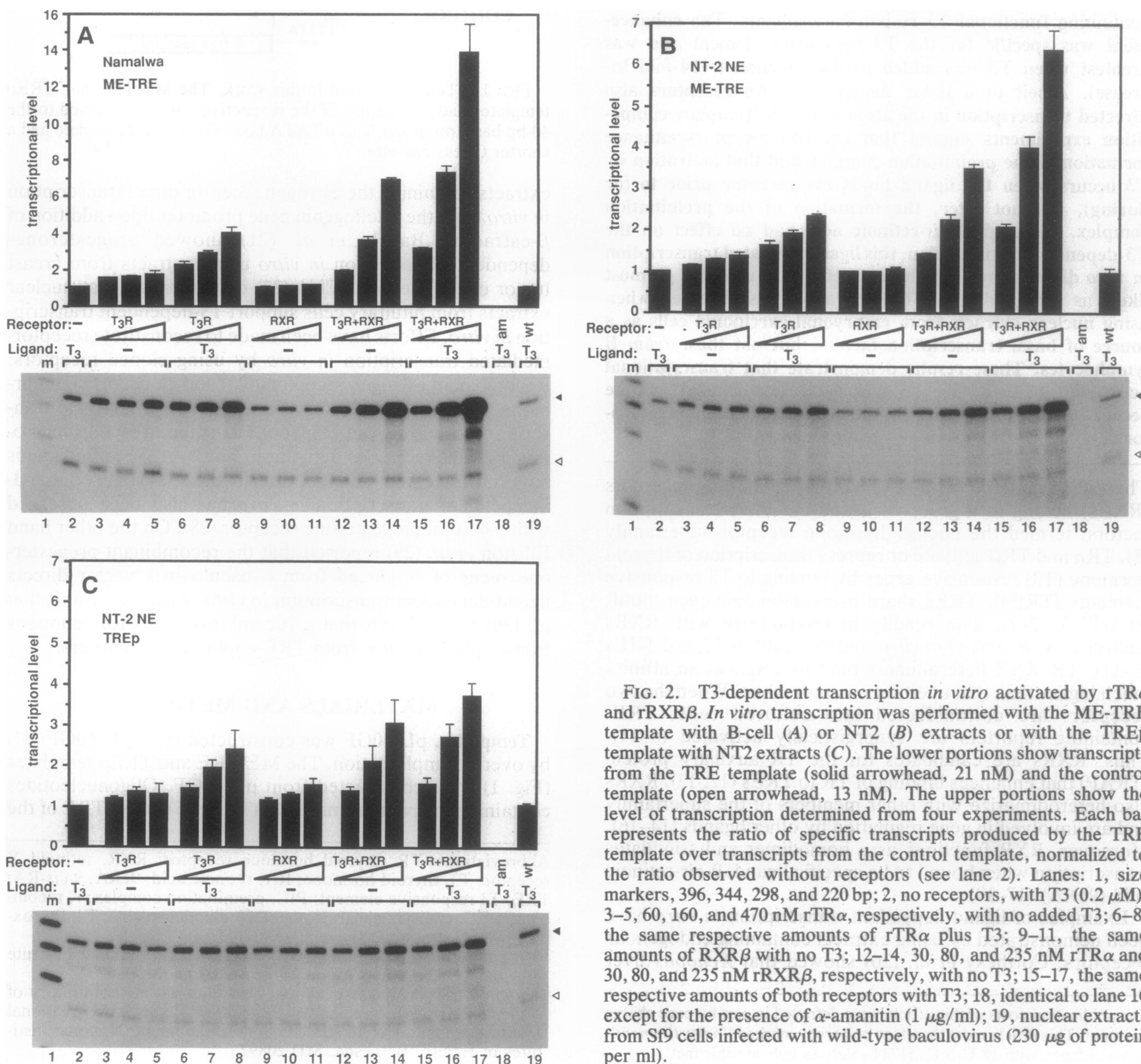

FIG. 2. T3-dependent transcription *in vitro* activated by rTRα and rRXRβ. *In vitro* transcription was performed with the ME-TRE template with B-cell (*A*) or NT2 (*B*) extracts or with the TREp template with NT2 extracts (*C*). The lower portions show transcripts from the TRE template (solid arrowhead, 21 nM) and the control template (open arrowhead, 13 nM). The upper portions show the level of transcription determined from four experiments. Each bar represents the ratio of specific transcripts produced by the TRE template over transcripts from the control template, normalized to the ratio observed without receptors (see lane 2). Lanes: 1, size markers, 396, 344, 298, and 220 bp; 2, no receptors, with T3 (0.2 μM); 3–5, 60, 160, and 470 nM rTRα, respectively, with no added T3; 6–8, the same respective amounts of rTRα plus T3; 9–11, the same amounts of RXRβ with no T3; 12–14, 30, 80, and 235 nM rTRα and 30, 80, and 235 nM rRXRβ, respectively, with no T3; 15–17, the same respective amounts of both receptors with T3; 18, identical to lane 16 except for the presence of α-amanitin (1 μg/ml); 19, nuclear extracts from Sf9 cells infected with wild-type baculovirus (230 μg of protein per ml).

were performed using rTRα and its heterodimer partner rRXRβ, both contained in nuclear extracts from Sf9 cells infected with the respective recombinant baculovirus (11, 32). These recombinant receptors have been shown to form heterodimers with each other and to bind to both the ME-TRE and the TREp (9, 10, 32). Nuclear extracts from Namalwa B cells and from NT2 EC cells were tested as sources of basal factors.

T3-Dependent Transcriptional Activation *in Vitro* Directed by rRXRβ and rTRα. The ME-TRE and the control templates were incubated with increasing amounts (60–450 nM) of rTRα or rRXRβ alone or together, in the presence or absence of T3 (0.2 μM), followed by addition of nuclear extracts. Results obtained with B-cell nuclear extracts are shown in Fig. 2*A*. Both the ME-TRE and control templates produced correctly initiated transcripts of the expected sizes (377 nt and 276 nt, respectively). In the absence of receptors, levels of transcription from the ME-TRE template were only slightly higher than those from the control template and were not affected by addition of T3 (lane 2). Addition of rTRα alone without T3 (lanes 3–5) or of control Sf9 extracts infected with the wild-type virus (lane 19) had no effect on transcription from either template. When T3 was added to reaction mixtures containing rTRα alone, transcription from the ME-TRE template but not from the control template was increased modestly over basal levels (3.5-fold, lanes 6–8). Addition of rRXRβ alone had no effect (lanes 9–11). However, when rTRα and rRXRβ were added to the reaction mixture together, transcription from the ME-TRE template (but not from the control template) was significantly increased even in the absence of T3 (lanes 12–14). Total amounts of receptors added were comparable whether each receptor was added alone or together. Most significantly, when T3 was added to the reaction mixtures containing both rTRα and rRXRβ (lanes 15–17), transcription from the ME-TRE template was increased to the highest level, reaching up to a 14-fold increase relative to the levels seen by control reaction mixtures (lane 19 vs. lane 2). This increase was dependent on the dosage of added receptors and specific for the ME-TRE template. At higher receptor concentrations, the increase was synergistic; levels of transcripts generated by rTRα plus rRXRβ were greater than the sum of transcripts by each receptor alone (compare lane 16 to lanes 7 and 10 and lane 17 to lanes 8 and 11). A titration analysis revealed that T3 from 0.1 to 10 μM was effective in enhancing transcription, but T3 at 10 nM was only weakly effective and T3 at 1 nM was not effective at all (data not shown). No transcripts were produced in these reaction mixtures when α-amanitin at 1 μg/ml was included (lane 18), indicating that this transcription is mediated by RNA polymerase II. Nuclear extracts from NT2 EC cells (34) gave essentially the same results (Fig. 2*B*). Thus, rRXRβ and rTRα cooperatively activate transcription from the ME-TRE template in a ligand-dependent and -independent fashion. Under comparable conditions, rTRα and rRXRβ also activated transcription from the TREp templates although the activation was additive rather than synergistic (Fig. 2*C*). To our knowledge, this is the first demonstration of transcriptional activation *in vitro* by two exogenous recombinant receptors that heterodimerize with each other.

Element and Ligand Specificity. To confirm specificity, excess oligonucleotides corresponding to the ME-TRE, TREp, or NF-κB (38) were added to reaction mixtures and transcription was tested in the presence of T3 using the ME-TRE template. As seen in Fig. 3*A*, addition of ME-TRE or TREp oligonucleotides inhibited transcriptional activation by >70%, while NF-κB oligonucleotides gave little inhibition. Results in Fig. 3*B* show that transcription from the ME-TRE template is activated not only by T3 but also by its analogues, T4 and TRIAC, while DIT (all tested at 0.2 μM) had no effect. These results are consistent with the reported relative affinity and biological potency of these analogues (22, 39). In Fig. 3*C*, effects of 9cRA were tested on transcription *in vitro* from the ME-TRE template. Addition of 9cRA at 1 μM failed to activate transcription by rTRα or rRXRβ alone or together. When 9cRA and T3 were added together, levels of activation by TRα alone or by TRα plus rRXRβ were similar to those by T3 alone. These results are generally in agreement with previous *in vivo* data (36). In Fig. 3*D*, effects of the timing of T3 addition on transcription were analyzed. To allow a single round of transcription (35), 0.05% sarkosyl was added before addition of NTPs. T3-dependent activation was observed when the ligand was added to reaction mixtures prior to addition of nuclear extracts, but T3 had no effect when added after the template and receptors had been incubated with nuclear extracts. These results indicate that activation by the receptors requires ligand binding prior to the formation of the preinitiation complex.

rRXRβ and rTRα Affect Formation of the Preinitiation Complex (PIC). To address mechanisms by which rRXRβ and rTRα activate TRE-specific transcription *in vitro*, template

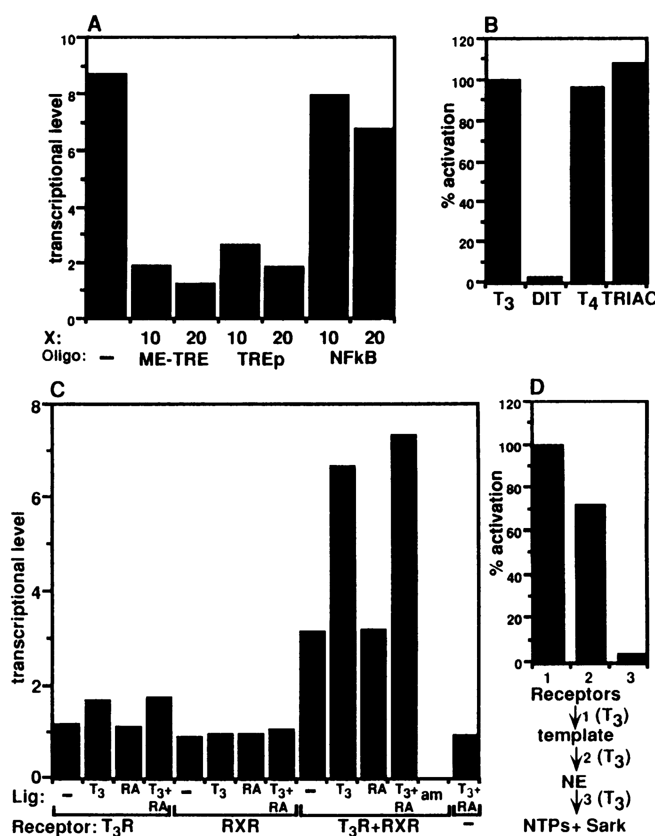

FIG. 3. Element and ligand specificity. (*A*) Competition by oligonucleotides. Transcription by the ME-TRE template was performed as in Fig. 2*A* using both receptors (each at 225 nM) and T3 in the presence of a 10- or 20-fold molar excess of oligonucleotides. The level of transcriptional activation is expressed as in Fig. 2. (*B*) Effects of T3 analogues. Reactions were performed with various analogues at 0.2 μM as in Fig. 2*A*. The level of activation was estimated by taking that by T3 activation as 100%. (*C*) Effects of 9cRA on T3-dependent transcription. Reactions were performed as in Fig. 2*A* using each receptor alone at 450 nM or both receptors (each at 225 nM) and 9cRA (RA) was added at 1 μM when indicated. (*D*) Timing of T3 addition. Reactions were performed as in Fig. 2*A* using both receptors (each at 225 nM). Bars: 1, T3 was added to the rTRα and rRXRβ before addition to the template and then nuclear extract (NE); 2, T3 was added after preincubation of rTRα and rRXRβ and the template, but before addition of nuclear extracts; 3, T3 was added after preincubation of rTRα and rRXRβ, templates, and nuclear extracts. Sark, sarkosyl.

competition experiments were performed in which the ME-TRE and the control templates were added at different times during the reaction, and the relative amounts of transcripts produced by the two templates were assessed (Fig. 4). Simultaneous addition of the two templates in the presence of the receptors produced a >5-fold higher level of ME-TRE transcripts over control transcripts (lane 4). However, when the control template was added first, the level of ME-TRE transcripts was greatly reduced, and the level of control transcripts was increased to a level higher than that obtained by the simultaneous template addition (lane 5). Conversely, when the ME-TRE template was first added to the reaction, the level of ME-TRE transcripts was higher than that seen by simultaneous template addition, while control transcripts were reduced to a negligible amount (lane 6). When experiments were performed using control Sf9 extracts (lanes 1–3), the ratios of transcripts produced by the two templates differed only slightly. These results indicate that in the presence of rTRα and rRXRβ the ME-TRE template competes with the control template for basal factors more efficiently than in their absence and that the binding of receptors to the TRE promotes formation and/or stability of the PIC.

9cRA-Dependent Activation of Transcription Directed by rRXRβ. The RXRβ homodimer binds TREp (32), which is increased upon 9cRA treatment (17). Furthermore, RXR transfected into yeast cells activates a TREp reporter upon 9cRA addition without a heterodimer partner (40). We tested whether rRXRβ, upon binding to 9cRA, activates transcription from the TREp template. Results with nuclear extracts from NT2 EC cells are shown in Fig. 5*A*. Without ligand, rRXRβ (from 150 to 900 nM) had little effect on transcription (lanes 2–4). However, when 9cRA was added, transcription from the TREp template (but not from the control template) was enhanced in a dose-dependent fashion (lanes 5–7); the greatest enhancement ($\approx$3-fold) was detected with the greatest amount of rRXRβ added. 9cRA had no effect on transcription when added to control Sf9 extracts (lanes 11–13). Furthermore, all-*trans*-RA, a ligand for RA receptors but not for RXRs (18, 19), failed to enhance transcription (lanes 8–10). Fig. 5*B* shows that this transcription is observed over a relatively wide range of template and NT2 nuclear extract concentrations.

In contrast to NT2 extracts, no transcriptional activation by rRXRβ was observed with multiple preparations of B-cell nuclear extracts in the presence or absence of 9cRA (data not shown). These results indicate that rRXRβ activates transcription in a ligand-dependent manner, when using extracts from NT2 EC cells.

DISCUSSION

rTRα and rRXRβ cooperatively activated transcription *in vitro* from a template containing a functional TRE in a T3-dependent fashion. Results indicate that receptor preparations used in the present work were capable of productively participating in transcription without requiring additional components or modifications *in vivo*.

Template competition experiments (Fig. 4) suggest that the two receptors stimulate transcription by enhancing the formation of the PIC. In agreement, enhanced PIC formation has been demonstrated in transcription *in vitro* directed by the progesterone receptor (28). Activation was consistently higher when rTRα and rRXRβ were added together than when they were added separately; the two receptors together gave synergistic and additive enhancement in transcription from the ME-TRE and TREp templates, respectively, both with and without T3. Thus rTRα and rRXRβ probably acted as a heterodimer to stimulate transcription, since the two receptors form heterodimers and tightly bind to the TREs (9, 10). These results are consistent with *in vivo* transfection data showing that reporters containing TREs are cooperatively activated by RXRβ and TRα (9, 10). Addition of T3 to the two receptors resulted in the greatest increase in transcription from both ME-TRE and TREp templates (Fig. 2). It has been reported that binding of T3 alters conformation of TR without significantly affecting TRE binding or heterodimer formation

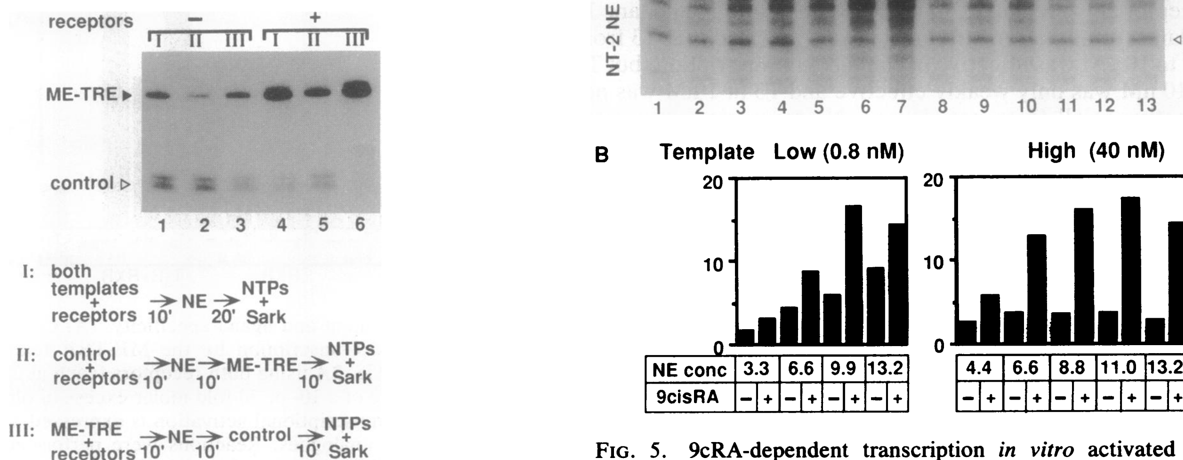

FIG. 4. Template competition assay. Templates were added to reactions containing B-cell extracts at varying times. In experiment I, the two templates were simultaneously incubated with the receptors prior to addition of B-cell nuclear extracts (NE). Reaction mixtures were then incubated with 0.05% sarkosyl (Sark) and NTPs. In experiment II, the control template was first incubated with receptors and nuclear extracts, after which the ME-TRE template was added. In experiment III, conversely, the ME-TRE template was first incubated with the receptors and nuclear extracts. Lanes 1–3 present data obtained with control Sf9 extracts, and lanes 4–6 are results with the two receptors.

FIG. 5. 9cRA-dependent transcription *in vitro* activated by rRXRβ. (*A*) Reactions were performed with rRXRβ with the TREp template (40 nM) and control template (12 nM) using nuclear extracts (NEs) from NT2 EC cells (7 mg/ml). Lanes: 1, 9cRA at 1 μM, but no added receptor; 2–4, 16, 450, and 900 nM rRXRβ, but no ligand; 5–7, same amounts of RXRβ and 9cRA; 8–10, same amounts of RXRβ with 4 μM all-*trans*-RA; 11–13, nuclear extracts from control Sf9 cells. (*B*) Reactions were performed with 0.8 nM (plus carrier DNA at 2 μg/ml) or 40 nM TREp template and control template (0.8 nM or 20 nM, respectively). Increasing concentrations of nuclear extracts from NT2 cells (indicated as mg/ml) were tested. To adjust the template-to-receptor ratio, reactions were carried out with 160 nM (*Left*) or 620 nM (*Right*) rRXRβ.

(6, 41). A ligand-induced conformational change may potentiate the ability of the receptors to enhance the formation of the PIC, since T3-dependent increase in transcription was observed only when the ligand was added before or simultaneously with nuclear extracts, but not after (Fig. 3*D*). This enhancement may involve transcription factor TFIIB, since this basal factor is shown to directly bind to TR (30, 42).

Fondell *et al.* (30) reported that a rTRα produced in a bacterial vector represses transcription from the growth hormone gene and other TRE promoters *in vitro* and that this repression is relieved by addition of T3, even though the growth hormone gene and its promoter are activated by T3 *in vivo* (2, 3) and *in vitro* (22). The basis of the difference between their results and those in the present work is not clear. It is possible that the difference stems from the use of different recombinant receptors. Lin *et al.* (43) reported that a bacterially produced TRβ acquires high-affinity TRE binding activity only upon its phosphorylation *in vitro*. Some baculovirus recombinant receptors are constitutively phosphorylated and show avid TRE binding (refs. 9 and 10 and J.A.M., unpublished data). This may be relevant to the noted difficulties in obtaining functionally active recombinant steroid receptors from bacterial vectors (25), while several baculovirus receptors are shown to readily elicit transcriptional activation *in vitro* (23, 29). Differences in nuclear extract components or in promoter context of the TREs (22, 32) may also explain the discrepancy.

It is of note that even in the absence of T3, rTRα and rRXRβ activated transcription from both ME-TRE and TREp templates. The ligand-independent transcriptional enhancement *in vitro* has been reported for several steroid receptors (22–29, 44). The following are possible reasons for the observed T3 independence. (*i*) TRα *in vivo* may be associated with an inhibitor that inhibits constitutive activation and is present in low concentration in our receptor preparations (and nuclear extracts). (*ii*) Receptors may undergo a structural modification *in vitro* that converts them into a constitutively active form but does not take place *in vivo*. (*iii*) There may be an *in vivo*-specific mechanism that inhibits unliganded receptors from activating transcription but is not fully reconstituted in our *in vitro* transcription system. In NT2 cell nuclear extracts, addition of rRXRβ resulted in 9cRA-dependent transcriptional enhancement (Fig. 5). Similar 9cRA-dependent transcription *in vitro* was observed from a CRBPII promoter (J.A.M., unpublished data). It is reasonable to assume that this activation is caused by rRXRβ homodimers, since (*i*) all-*trans*-RA had no effect, (*ii*) RXRβ homodimer binds to TREp (32) and the CRBPII element upon 9cRA addition (J.A.M., unpublished data), and (*iii*) TREp reporter activity is enhanced by transfection of RXR alone in yeast (40). It is noteworthy that 9cRA-dependent RXRβ-directed transcription was observed only when nuclear extracts from NT2 EC cells (but not from B cells) were used. These data suggest that a factor present in NT2 nuclear extracts, but not B-cell extracts, is necessary for 9cRA-dependent transcription *in vitro*. Our data are reminiscent of the report (45) showing that EC cells express an E1A-like factor that supports RA responsive transcription. In summary, this work demonstrates that rRXRβ itself or in combination with rTR activates transcription *in vitro* from appropriate templates in a ligand-dependent fashion.

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