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Unliganded Thyroid Hormone Receptor α Can Target TATA-Binding Protein for Transcriptional Repression

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Unliganded human thyroid hormone receptor α (hTRα) can repress transcription by inhibiting the formation of a functional preinitiation complex (PIC) on promoters bearing thyroid hormone receptor (TR)-binding elements. Here we demonstrate that hTRα directly contacts the TATA-binding protein (TBP) and that preincubation of hTRα with TBP completely alleviates TR-mediated repression in vitro. Using stepwise preassembled PICs, we show that hTRα targets either the TBP/TFIIA or the TBP/TFIIA/TFIIB steps of PIC assembly for repression. We also show that the repression domain of hTRα maps to the C-terminal ligand-binding region and that direct TR-TBP interactions can be inhibited by thyroid hormone. Together, these results suggest a model in which unliganded hTRα contacts promoter-bound TBP and interferes with later steps in the initiation of transcription.

Transcription initiation on protein-encoding genes involves the ordered assembly of RNA polymerase II (pol II) and other general initiation factors (TFIIA, -B, -D, -E, -F, and -H) to form a functional preinitiation complex (PIC) on core promoter elements (the TATA and initiator regions) (reviewed in references 10, 34, and 43). The binding of TFIID to the TATA element, in a step facilitated by TFIIA, initiates PIC formation and continues with the ordered assembly of TFIIB, pol II/TFIIF, TFIIE, and TFIIH. Recent studies revealed that TFIID is a multiprotein complex comprised of a TATA-binding protein (TBP) and several TBP-associated proteins (references 7, 14, 38, and 44 and references therein). Whereas TBP together with the other general initiation factors is sufficient for basal-level transcription, the intact TFIID complex as well as other soluble factors are required for activated transcription in response to gene-specific regulatory transcription factors (7, 14, 30, 38, 44; reviewed in reference 17). Activation or repression of transcription initiation by gene-specific regulatory factors involves facilitation (or inhibition) of limiting steps in PIC formation or function through interactions with one or more of the general factors.

Thyroid hormone receptors (TRs) are ligand-inducible transcription factors which belong to the nuclear hormone receptor superfamily (4, 15). A novel feature of TRs (which exist as α and β isoforms) is their dual ability to either activate or repress transcription from genes bearing TR-binding elements (TREs) (42). In general, TRs activate transcription when liganded to the thyroid hormone (triiodothyronine or T_3); in the absence of hormone, TRs can act as repressors (2, 6, 11, 18, 35). Although the precise mechanisms of activation and repression by TR are ill understood, it is generally held that, similar to other gene-specific regulatory factors, TRs regulate transcription by directly or indirectly affecting the assembly or function of the general initiation factors at the core promoter elements.

We recently demonstrated that unliganded human TRα (hTRα) can repress transcription in vitro from both natural and synthetic TRE-linked promoters by inhibiting the formation of a functional PIC (16). Two lines of evidence indicated that hTRα targets the basal transcription machinery for repression. First, hTRα repressed transcription more efficiently (and in some cases completely) from TRE-linked core promoter constructs than from TRE-linked promoters containing activator-binding sites. Second, hTRα repressed transcription efficiently from a TRE-linked core promoter in a fractionated transcription system consisting of only highly purified general initiation factors that included recombinant TBP in place of TFIID. Consistent with the notion that hTRα interacts with the basal transcription machinery, we and others have shown that TRs can directly contact TFIIB (1, 16, 19). Indeed, these observations have led to the hypothesis that TFIIB may be the target for TR-mediated repression, although an exclusive relationship to activation (19) was not eliminated. Here we show that hTRα can directly interact with TBP as well as TFIIB. Preincubation of hTRα with TBP alleviates TR-mediated repression in vitro, whereas preincubation with TFIIB has no effect. By staging PIC assembly in vitro, we show that hTRα can target either the TBP/TFIIA or the TBP/TFIIA/TFIIB steps of PIC formation for repression. Furthermore, we show that thyroid hormone can inhibit the interaction between TBP and the TR repression domain. Taken together, these results suggest that unliganded hTRα can contact TBP and disrupt transcription initiation.

MATERIALS AND METHODS

Plasmid construction. The plasmid encoding the glutathione *S*-transferase (GST)–TFIIB fusion protein was created by ligating the Klenow enzyme-blunted *Nde*I-*Bam*HI TFIIB cDNA into the *Sma*I site of pGEX-2T (Pharmacia). Plasmids encoding GST-TBP, GST-IIEα, and GST-RAP30 (the small subunit of TFIIF) were created by ligating the *Nde*I-*Bam*HI fragments containing each full-length cDNA into pGEX-2TL(+) (12). The GST-IIAα plasmid was described previously (12). Plasmids encoding the GST-TFIIB deletion mutants Δ4-11, Δ3-37, Δ3-103, and Δ244-316 were created by subcloning the *Nde*I-*Bam*HI fragment encompassing each mutant (20) into pGEX-2TL(+). An *Nde*I-*Ssp*I restriction fragment spanning TBP amino acids (aa) 1 to 163 was subcloned into the *Nde*I-*Sma*I sites of pGEX2TL(+), creating GST-TBP(1-163). An *Nde*I-*Bam*HI PCR fragment spanning TBP aa 154 to 335 was cloned into pGEX-2TL(+), creating GST-TBP(154-335). The bacterial expression vector for full-length hTRα was described previously (16). An *Nde*I-*Bam*HI PCR product spanning aa 1 to 123 of hTRα was cloned into pET11d6His (21), creating pET-TRα(1-123). Similarly, *Nco*I-*Bam*HI PCR products spanning hTRα aa 122 to 211 and aa 213 to 410 were cloned into pET11d (37), creating pET-TRα(122-211) and pET-TRα(213-410), respectively. pET-TRα-COOH (aa 122 to 410) was

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created by cloning the *Nco*I-*Bam*HI fragment of the hTRα cDNA into pET11d. The TRE_1ML400, wtML200, and 2XSp1-HIV-CAT plasmids used as promoter templates for in vitro transcription were described previously (16). The template G5MLI containing five GAL4 binding sites preceding the adenovirus major late (AdML) core promoter region MLI (13) was obtained from Richard Bernstein. The GAL4-TRα-NH_2 expression plasmid was created by cloning a *Bam*HI-*Xba*I PCR product spanning hTRα aa 1 to 123 into pD10GAL4(1-94), obtained from Craig Rosen (24). The GAL4-TRα-COOH expression plasmid was created by cloning an *Sph*I-*Xba*I PCR product spanning GAL4 aa 1 to 98 together with an *Xba*I-*Bam*HI fragment from the hTRα cDNA (aa 126 to 410) into the *Sph*I-*Bam*HI sites of pD10GAL4(1-94). The FLAG epitope-tagged hTRα plasmid used for baculovirus expression was constructed by subcloning the full-length hTRα *Nde*I-*Bam*HI fragment (16) into pFLAG(s)-7 (8), creating pFLAG-TRα. The full-length FLAG-TRα fragment was then removed by using the *Nco*I-*Bam*HI restriction sites and subcloned into the baculovirus transfer vector pVL1392 (Pharmingen), creating pVL1392-FLAG-TRα. The promoter template used for DNase I footprinting was created by PCR amplification of the TRE_1ML400 promoter region between −95 and +10. The PCR fragment was then cloned into pCRII (Invitrogen), creating pCRII/TRE1ML400.

GST interaction studies. [^{35}S]methionine-labeled full-length hTRα and shorter derivatives were transcribed and translated in vitro, using a commercial kit (TNT; Promega). Glutathione-Sepharose-purified GST or GST fusion proteins (1 μg/20 μl of packed beads) were incubated with 1 μl of ^{35}S-labeled protein (from a 50-μl reaction) in 300 μl of BC100 (16) containing 0.05% Nonidet P-40 at 4°C for 30 min. The beads were then washed five times with BC300 (same as BC100 but containing 300 mM KCl) containing 0.1% Nonidet P-40. The beads were then suspended in sodium dodecyl sulfate (SDS) sample buffer, analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and autoradiographed. For experiments containing thyroid hormone (L-T_3 or TRIAC from Sigma), reactions were performed at room temperature, and hormone was added to both the reaction mix and the washes.

For experiments involving recombinant hTRα expressed in Sf9 cells (see below), 20 ng of purified hTRα1 protein was incubated with 1 μg of either GST-IIB or GST-TBP as described above and subsequently analyzed by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, blocked with TBS-T (10 mM Tris, 150 mM NaCl, 0.05% Tween 20) containing 5% milk, and incubated with an anti-hTRα rabbit polyclonal antiserum (FL-408; Santa Cruz Biotechnology, Inc.) in TBS-T (1:1,000 dilution). Immunoblots were developed as described by the manufacturer (ECL system; Amersham).

Purification of transcription factors. RNA pol II was immunopurified from calf thymus by using monoclonal antibody 8WG16 as previously described (39), with the following modification. RNA pol II was eluted from the immunoaffinity column with 40% propylene glycol–0.3 M ammonium sulfate. The fraction containing TFIIE and TFIIH was obtained by fractionating a HeLa nuclear extract on a phosphocellulose 11 column (30). The 0.5 M KCl elution was then applied to a DEAE-cellulose (DE-52) column and eluted with 0.3 M KCl. The natural TFIIA fraction was purified as described previously (30). The recombinant subunits of TFIIF, RAP30, and RAP74 were expressed in *Escherichia coli* and purified as described previously (41). The recombinant polypeptides TBP, TFIIB, TFIIEα, TFIIEβ, and TFIIAα(p55) were expressed in *E. coli* and purified as described previously (12, 16, 32).

Purification of the *E. coli*-expressed hTRα has been described elsewhere (16). Purification of the baculovirus-expressed hTRα was carried out as follows. The baculovirus transfer vector pVL1392-FLAG-TRα was transfected into Sf9 cells along with linearized baculovirus DNA (BaculoGold; Pharmingen), using cationic liposomes (Insectin; Invitrogen). The transfection supernatant harboring recombinant FLAG-TRα-expressing baculovirus was then amplified to increase the viral titer and used to infect Sf9 cells. Nuclear extract prepared from infected Sf9 cells was then applied to an anti-FLAG epitope immunoaffinity column (Anti-FLAG M2 Affinity Gel; Kodak). The FLAG-TRα protein was subsequently eluted by using a FLAG peptide (N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C). Human retinoid X receptor alpha chain (RXRα) was expressed as a GST fusion protein in *E. coli* as described previously (28); RXRα was liberated from GST by using thrombin (Sigma). GAL4(1-94) and GAL4-TRα fusion proteins were expressed in *E. coli* MC1061/PDMI and purified as described previously (24).

In vitro transcription. Standard in vitro transcription reaction conditions involving the G-less cassette assay (Fig. 4 and 5) and the primer extension assay (Fig. 7) have been described elsewhere (16). The purified transcription system (Fig. 4) consisted of recombinant TBP (10 ng), recombinant TFIIB (10 ng), recombinant TFIIF (5 ng), a natural TFIIA fraction (0.5 μl; 0.5 mg/ml), a natural TFIIE/H fraction (1 μl; 0.85 mg/ml), and natural RNA pol II purified from calf thymus (1 μl; 0.55 mg/ml).

DNase I footprint assay. The TRE_1ML400 promoter region of pCRII/TRE_1ML400 was removed by digestion with *Hin*dIII-*Apa*I and ^{32}P end labeled by using Klenow enzyme. A typical 25-μl binding reaction mixture contained 2 fmol (25,000 cpm) of the ^{32}P-labeled DNA fragment, 50 ng of poly[d(G-C), 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 60 mM KCl, 5 mM β-mercaptoethanol, 0.2 mM EDTA, 4 mM $MgCl_2$, 10% glycerol, and 0.1 mg of bovine serum albumin (BSA) per ml. The reactions were incubated at 30°C along with the appropriate amounts of purified proteins as indicated in the legend to Fig. 6. Reaction mixtures were then treated with 2 μl of DNase I (0.4 ng/μl; diluted in 1 mM $CaCl_2$–0.1 mg of BSA per ml–10% glycerol just before use) for 2 min at room temperature. The reactions were terminated by addition of 25 μl of stop solution (10 mM EDTA, 0.8% SDS, 0.1 mg of sonicated salmon sperm DNA per ml, 0.6 M sodium acetate [pH 5.2]). After phenol-chloroform extraction and ethanol precipitation, the DNA pellet was resuspended in 5 μl of loading buffer (98% formamide, 10 mM EDTA, 0.02% bromophenol blue, 0.02% xylene cyanol) and loaded on an 8% polyacrylamide–7 M urea sequencing gel. The Maxam-Gilbert sequencing method (29) was used to prepare G/A and C/T footprinting markers.

RESULTS

hTRα can form a specific complex with both TFIIB and TBP. We previously demonstrated that hTRα can interact with TFIIB in an immunoprecipitation assay (16). To determine whether hTRα interacts with other components of the basal transcription apparatus, we screened hTRα against a panel of recombinant general transcription factors. In this experiment, interactions were monitored by the ability of corresponding GST fusion proteins to bind [^{35}S]methionine-labeled hTRα. After immobilization on glutathione-Sepharose beads, the specific complexes were subject to several stringent washes and analyzed by SDS-PAGE. As shown in Fig. 1A, hTRα bound not only to TFIIB but also to TBP. This result contrasts with our previous inability to detect a TRα-TBP interaction by immunoprecipitation and suggests that TR-TBP contacts mask critical epitopes recognized by the anti-TBP sera used in our earlier studies (16).

To more closely examine the specificity of the hTRα-TBP and hTRα-TFIIB interactions, we repeated the GST assay with purified, recombinant hTRα expressed via a baculovirus vector in Sf9 cells. Figure 1B shows that recombinant hTRα bound to both GST-IIB and GST-TBP in the presence of ethidium bromide, ruling out a nonspecific bridging effect by contaminating DNA. Similarly, specific hTRα-IIB and hTRα-TBP complexes could be eluted from the glutathione-Sepharose beads by using reduced glutathione, thus eliminating a nonspecific trapping effect (Fig. 1B). In addition, the inability of a luciferase protein control to bind any of the GST fusion proteins further substantiates the specificity hTRα-TFIIB and hTRα-TBP interactions (Fig. 1A).

To examine which domains of hTRα interact with TFIIB and TBP, different regions of hTRα were labeled and again screened for binding to either TFIIB or TBP. As shown in Fig. 1C, a C-terminal region of hTRα (aa 213 to 410) which includes the ligand-binding domain (LBD) efficiently bound to both TFIIB and TBP. In contrast, the N-terminal portion of hTRα (aa 1 to 123), which includes the DNA-binding domain, bound weakly to only TFIIB. A short hTRα segment which spans the hinge region of the receptor (aa 122 to 211) failed to interact with either general factor. These results indicate that hTRα can specifically interact with both TFIIB and TBP.

To localize the regions of TFIIB which interact with hTRα, labeled N- and C-terminal hTRα domains were screened against a set of TFIIB deletion mutants (Fig. 2A). Figures 2B and C show that both the N- and C-terminal domains of hTRα can interact with the C-terminal domain of TFIIB (aa 244 to 316). This mapping was unexpected since it was previously reported that the N terminus of hTRβ interacts with the C terminus of TFIIB, while the C-terminal LBD of hTRβ interacts with the N terminus of TFIIB (1). One possible explanation for this discrepancy is that amino acid sequence differences between the TRα and TRβ isoforms (42) result in differential interactions with TFIIB. In support of this hypothesis, it has been observed that the C terminus of chicken TRα interacts exclusively with a C-terminal region of TFIIB (19). To localize the region of TBP recognized by hTRα, a labeled C-terminal hTRα domain (aa 213 to 410) was tested for bind-

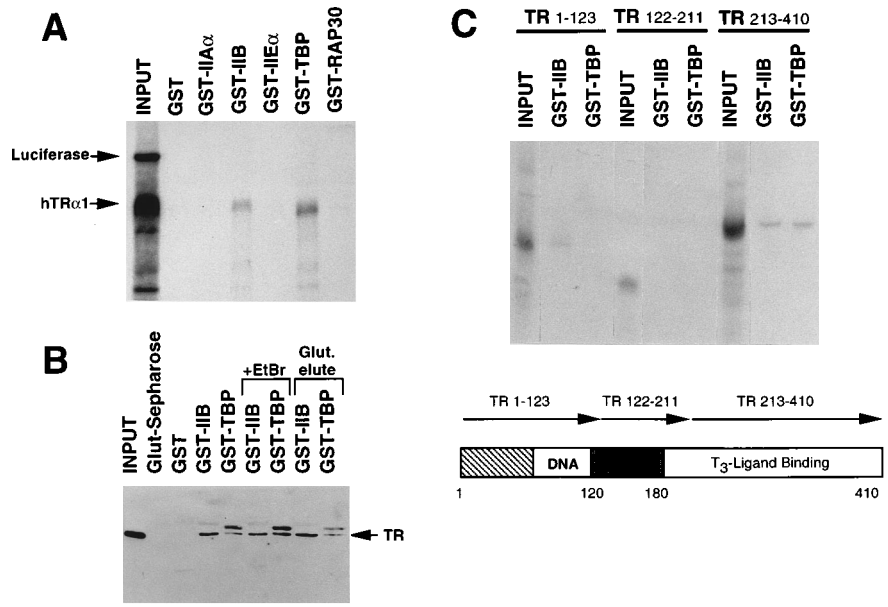

FIG. 1. hTRα specifically interacts with TFIIB and TBP. (A) Labeled hTRα interacts specifically with GST-IIB and GST-TBP fusion proteins. In vitro ^{35}S-labeled hTRα and luciferase were incubated with a panel of different GST fusion proteins (identified above the lanes). Specific complexes were immobilized on glutathione-Sepharose beads, and 50% of each reaction mixture was analyzed by SDS-PAGE. (B) Recombinant hTRα interacts specifically with TFIIB and TBP. Recombinant hTRα (expressed in Sf9 cells) was incubated with either GST-IIB or GST-TBP, and specific complexes were immobilized on glutathione-Sepharose beads; 50% of each reaction mixture was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Western blot (immunoblot) analysis was then carried out with a rabbit antiserum against hTRα. EtBr indicates that ethidium bromide was added to the binding reaction mixture at a concentration of 0.5 μg/ml. Glut. elute indicates that specific complexes were first eluted from the glutathione-Sepharose beads by using 20 mM reduced glutathione prior to SDS-PAGE analysis. (C) N- and C-terminal domains of hTRα interact differentially with TFIIB and TBP. Various in vitro ^{35}S-labeled segments of hTRα (diagramed schematically at the bottom) were incubated with either GST-IIB or GST-TBP. Specific complexes were immobilized, and 50% of each reaction mixture was analyzed by SDS-PAGE. Input indicates 100% of either the ^{35}S-labeled hTRα (A and C) or recombinant hTRα (B) added to the reaction mixtures. Estimated binding efficiency ranged between 10 and 20%.

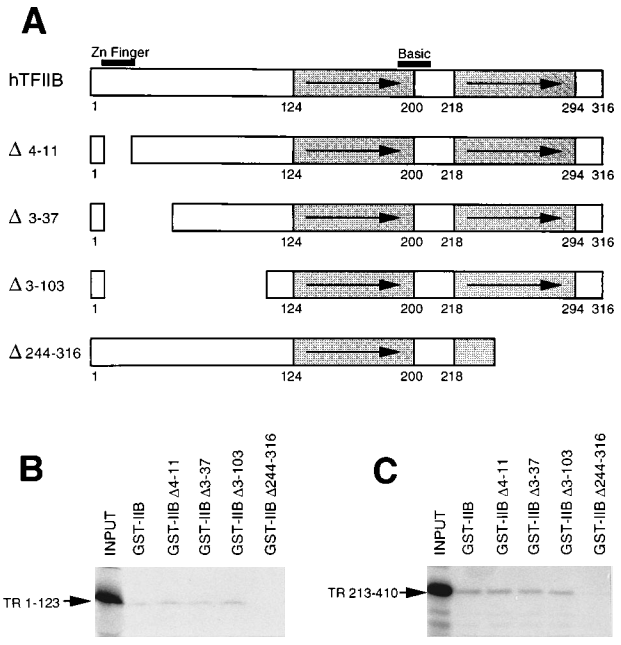

FIG. 2. Both N- and C-terminal domains of hTRα interact with the C terminus of TFIIB. (A) Schematic representation of the TFIIB mutants used in this study. (B) Interaction of the N-terminal hTRα domain (aa 1 to 123) with GST-TFIIB deletion mutants. Specific complexes were immobilized and analyzed by SDS-PAGE. (C) Interaction of the C-terminal hTRα domain (aa 213 to 410) with GST deletion mutants.

ing to TBP deletion mutants (Fig. 3A). Figure 3B shows that the C-terminal LBD of hTRα interacts specifically with the highly conserved C-terminal domain of TBP (aa 154 to 335). In sum, these results show that the C-terminal LBD of hTRα is capable of interacting with distinct C-terminal domains of both TFIIB and TBP, whereas the hTRα N-terminal domain can weakly interact with only the C terminus of TFIIB.

hTRα inhibits an early step in PIC assembly. By using a fractionated transcription system containing highly purified general initiation factors, we previously showed that unliganded hTRα interferes with the formation or function of a PIC (16). In the course of these studies, we defined the minimal basal factor components necessary for repression in vitro and found that only TBP, not the entire TFIID complex, was absolutely required. Since a preformed PIC was resistant to the inhibitory effects of hTRα, we reasoned that TRα might inhibit or block one or more of the discernible ordered steps of PIC assembly. To examine this possibility, the ordered steps were staged by preincubating subsets of purified general initiation factors and pol II with a TRE-linked AdML promoter (Fig. 4A). The preformed PICs were then challenged with unliganded hTRα (expressed in *E. coli*) and, with concomitant addition of other general transcription factors, tested for transcriptional inhibition in vitro. Figure 4B shows that promoter templates containing preformed TBP/IIA or TBP/IIA/IIB complexes were efficiently repressed upon the addition of hTRα and remaining general factors (lanes 3 and 4). Conversely, the subsequent steps of PIC formation, generating TBP/IIA/IIB/polII/IIF and TBP/IIA/IIB/polII/IIF/IIE/IIH complexes, were resistant to hTRα repression (Fig. 4B, lanes 5 and 6). Identical experiments using unrelated recombinant proteins (*E. coli*-expressed human USF and GAL4-AH [17])

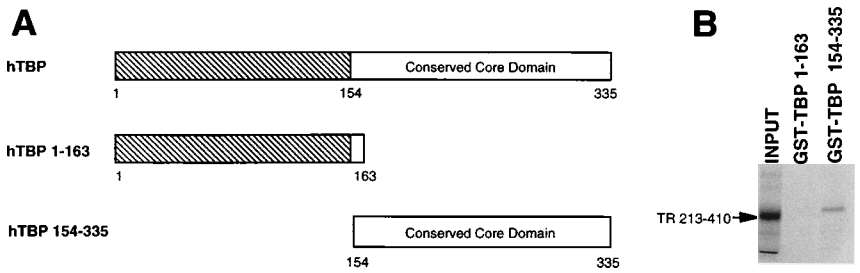

FIG. 3. The C-terminal domain of hTRα interacts with the core domain of TBP. (A) Schematic representation of the TBP deletion mutants used in this study. (B) Interaction of the C-terminal hTRα domain (aa 213 to 410) with GST-TBP deletion mutants. Specific complexes were immobilized and analyzed by SDS-PAGE.

had no effect on transcription (data not shown). These results suggest that hTRα can target TBP, and possibly TFIIB, for repression during PIC assembly. The role of TFIIA as a potential target for repression is unlikely since hTRα can efficiently repress transcription in fractionated assays lacking TFIIA (data not shown). The inability of hTRα to repress the more completely formed PICs suggests that assembly of the additional factors renders TBP and/or TFIIB inaccessible to the inhibitory effects of hTRα.

Preincubation of hTRα with TBP alleviates repression. If hTRα directly targets TBP and/or TFIIB for repression, it is conceivable that preincubation of unliganded hTRα with either general initiation factor might block or mask the hTRα repression domain and alleviate hTRα-mediated repression. To test this hypothesis, unliganded hTRα (expressed via baculovirus in Sf9 cells) was first preincubated with a series of different general initiation factors together with template before addition to complete in vitro transcription reactions. As shown in Fig. 5 (lane 3), preincubation of hTRα with TBP resulted in complete alleviation of repression. Conversely, preincubation of hTRα with either TFIIB (lane 4) or the large subunit of TFIIA (lane 7) had no effect. Similar results were obtained when preincubation was performed in the absence of template (data not shown). As expected, preincubation with thyroid hormone (TRIAC; lane 10) also alleviated TRα repression, again suggesting that ligand either blocks the hTRα repression domain or induces a conformational change which alleviates repression (16). Addition of human RXRα (lane 11) had no effect on hTRα-mediated repression, indicating that unliganded hTRα can inhibit transcription as either a homodimer or heterodimer. These findings strengthen our supposition that unliganded hTRα can target TBP for transcriptional repression.

hTRα does not prevent binding of TBP to the core promoter. The results of Fig. 4 and 5 suggest that one mechanism by which hTRα might act to repress transcription is to prevent TBP and/or TBP-containing complexes (TBP/IIA or TBP/IIA/IIB) from binding at the TATA region of the core promoter. To address this question, DNase I footprinting assays were performed on the promoter region of the TRE_1ML400 template (−95 to +10). Figure 6 shows that preincubation of the TRE_1ML400 promoter with hTRα, under conditions where TRE sites are fully occupied, did not alter the ability of a TBP/IIA/IIB complex to assemble over the TATA region. Thus, these results indicate that hTRα does not act to displace or compete with TBP for binding at the core promoter; rather, these results suggest hTRα might actively impinge on or mask promoter-bound TBP and interfere with subsequent steps in PIC formation or function.

The hTRα repression domain maps to the C terminus. In an effort to localize the domain of hTRα which is responsible for

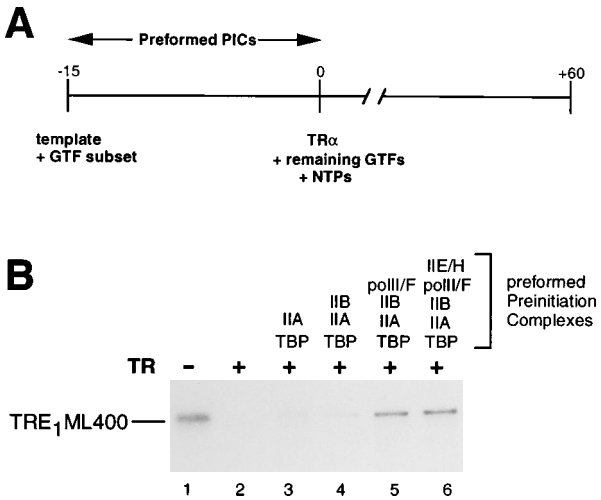

FIG. 4. hTRα inhibits an early step in PIC assembly. (A) Experimental scheme for preformed PIC assay. (B) The TRE_1ML400 promoter template was preincubated with different subsets of general initiation factors (indicated above the lanes) for 15 min, allowing different steps of PIC formation to assemble. No factors were preincubated in lanes 1 and 2. *E. coli*-expressed hTRα (100 ng) was then added as indicated together with ribonucleotide triphosphates (NTPs) and the remainder of the general transcription factors (GTFs). The reaction was allowed to proceed for 60 min, and transcripts were analyzed by using the G-less cassette assay.

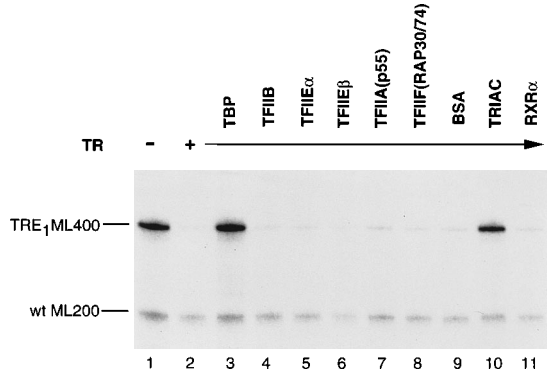

FIG. 5. Preincubation of hTRα with TBP alleviates repression. hTRα (90 ng, baculovirus expressed) was preincubated with the TRE_1ML400 template (50 ng) along with 150 fmol of each general initiation factor indicated above lanes 3 to 8. In lane 9, BSA was used as a negative control. In lane 10, hTRα was preincubated with 20 μM TRIAC. In lane 11, hTRα was preincubated with *E. coli*-expressed RXRα (100 ng). The preincubated factors (plus templates) were then added to a reaction mixture containing 25 ng of wtML200 (as an internal control) together with 50 μg of HeLa nuclear extract under standard conditions. Transcripts were analyzed by using the G-less cassette assay.

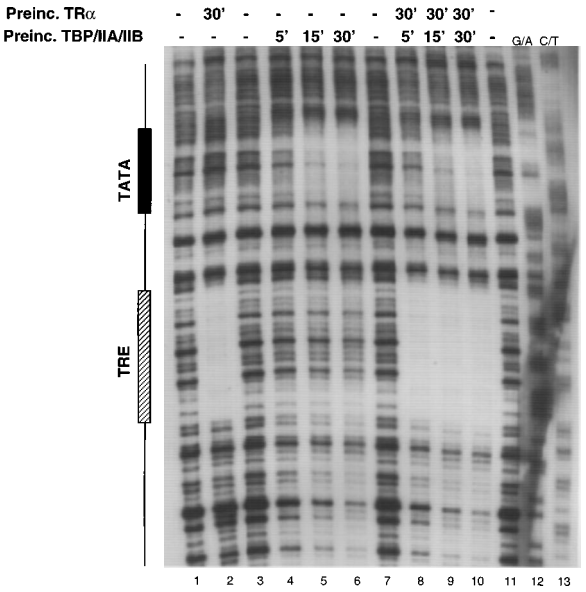

FIG. 6. hTRα does not prevent TBP binding to the promoter. DNase I footprinting analysis of a TBP/IIA/IIB complex over the TATA region of the TRE_1ML400 promoter (−95 to +10) in the presence or absence of unliganded hTRα (*E. coli*). In lanes 4 to 6 and 8 to 10, TBP (10 ng), TFIIA (0.5 μl; 0.5 mg/ml), and TFIIB (10 ng) were incubated with the TRE_1ML400 promoter for 5, 15, or 30 min as indicated. In lanes 8 to 10, the TRE_1ML400 promoter was preincubated with *E. coli*-expressed hTRα (50 ng) for 30 min prior to the addition of TBP/IIA/IIB. In lane 2, the TRE_1ML400 template was incubated with hTRα alone. Lanes 12 and 13 contain G/A and C/T footprinting markers, respectively (see Materials and Methods).

transcriptional repression, we fused either the N terminus (aa 2 to 123) or the C terminus (aa 126 to 410) of hTRα to the DNA-binding domain of the yeast transcription factor GAL4 (Fig. 7A). The GAL4-TR fusions were then tested for the

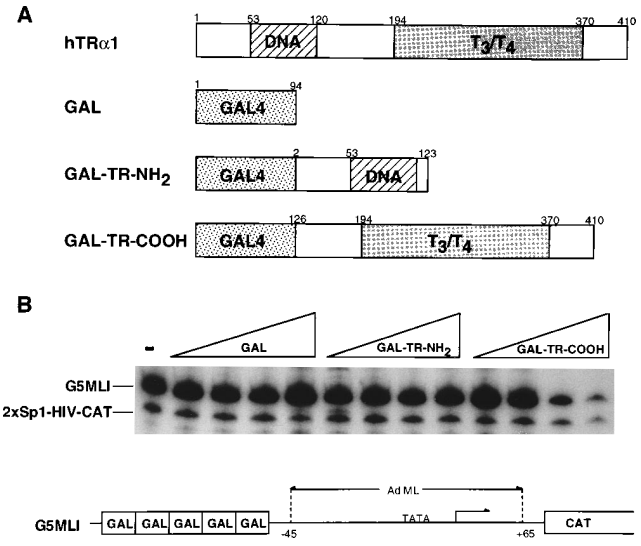

FIG. 7. hTRα repression domain maps to the C terminus. (A) Schematic diagram of GAL4-TRα fusion proteins. (B) Increasingly equimolar amounts of GAL4, GAL4-TR-NH_2 and GAL4-TR-COOH (0.1, 0.25, 0.5, and 1 pM) were added to transcription reactions containing 75 μg of HeLa nuclear extract and 50 ng of the G5MLI template under standard conditions; 10 ng of the template 2XSp1-HIV-CAT (lacking GAL4 binding sites) was added as an internal control. Transcription was analyzed by using a primer extension assay.

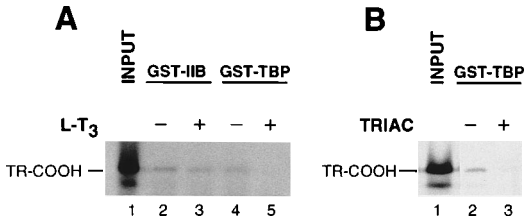

FIG. 8. Thyroid hormone reduces the efficiency of the C-terminal hTRα domain binding to TBP. (A) Interaction of a ^{35}S-labeled hTRα C-terminal domain (aa 122 to 410) with GST-TFIIB or GST-TBP in the presence or absence of 5 μM natural thyroid hormone (L-T_3). (B) Interaction of the hTRα C-terminal domain (aa 122 to 410) with GST-TBP in the presence or absence of 5 μM synthetic thyroid hormone (TRIAC).

ability to repress transcription in vitro from an AdML core promoter containing five GAL4 binding sites (G5MLI). The ability of the GAL-TR-COOH fusion protein to specifically inhibit transcription from the G5MLI template (Fig. 7B) demonstrates that the repression domain of hTRα maps to the C terminus. This finding confirms previous in vivo data linking the rat TRα silencing domain with the C terminus of the receptor (2). The small decrease in transcription from the reference promoter (2XSp1-HIV-CAT) in reactions containing high concentrations of GAL-TR-COOH may be the result of nonspecific sequestration of TBP. In sum, these results are consistent with the observation that hormone blocks TRα repression, since the LBD also maps to the C terminus.

Thyroid hormone reduces the efficiency of the C-terminal hTRα domain binding to TBP. Given our observations that (i) TBP interacts with the C-terminal domain of hTRα and (ii) both the hormone-binding domain and the repression domain of hTRα map to the C terminus, it was of interest to determine what effect thyroid hormone would have on the interaction between TBP and the C-terminal end of hTRα. To address this question, a labeled hTRα fragment spanning the entire C-terminal domain (aa 122 to 410) was tested for interactions with TBP in the presence or absence of thyroid hormone. Addition of either natural thyroid hormone (L-T_3; Fig. 8A, lane 5) or synthetic thyroid hormone (TRIAC; Fig. 8B, lane 3) to the binding reaction significantly reduced the interaction between the hTRα C terminus and TBP. Conversely, addition of thyroid hormone (Fig. 8A, lane 3) did not affect the interaction between the hTRα C terminus and TFIIB. Given the fact that thyroid hormone masks or alleviates hTRα-mediated repression, these findings further support our contention that unliganded hTRα can target TBP for repression and that addition of hormone blocks this negative interaction.

DISCUSSION

We demonstrated previously that unliganded hTRα can target the basal transcription apparatus for active repression (16). In this study, we examined more precisely the molecular mechanism of hTRα-mediated repression and found that hTRα directly contacts TBP. Several lines of evidence suggest that TBP is a basal factor target for TRα-mediated repression. First, by using stepwise, preassembled PICs (Fig. 4), we found that hTRα targets either the TBP/IIA or the TBP/IIA/IIB steps of PIC formation for repression. Second, preincubation of unliganded hTRα with TBP completely alleviates repression (Fig. 5). Third, thyroid hormone decreases the affinity of the hTRα C-terminal domain for TBP but not for TFIIB (Fig. 8). This last finding is especially significant since both repression

and ligand-binding domains of hTRα map to the C terminus and since thyroid hormone blocks TRα-mediated repression.

The question arises as to how unliganded hTRα might physically target TBP for repression. For instance, the ability of unliganded hTRα to interact with TBP in solution might suggest that hTRα nonspecifically sequesters TBP from pol II promoters in a process termed squelching (33). However, it is unlikely that such a squelching mechanism accounts for hTRα-mediated repression, since promoter templates lacking TREs are not significantly affected by unliganded hTRα (Fig. 5) (16). Alternatively, hTRα might compete with or displace TBP binding at the TATA element as previously described for other repressors (22, 31). We feel that this mechanism also is unlikely since unliganded hTRα does not qualitatively change the DNA-binding pattern of TBP/IIA/IIB over the TATA region as assayed by DNase I footprinting on a TRE-linked AdML promoter (Fig. 6). Instead, the data presented here suggest that unliganded hTRα can contact TBP during the early steps of PIC assembly and, with both components remaining promoter bound, inhibit the subsequent formation or function of a complete PIC. In this model, inhibitory contacts between DNA-bound hTRα and TBP prevent TBP from making proper contacts with the remainder of the basal transcription machinery. Addition of thyroid hormone presumably blocks the inhibitory interaction between unliganded hTRα and TBP and, instead, reveals (or potentiates) an activator function. In support of this model and our past (16) and present data, Tong et al. (40) recently demonstrated that unliganded hTRβ can both repress transcription in vitro from a TRE-linked minimal promoter and directly contact a TBP/IIB complex on a core promoter; addition of thyroid hormone both alleviated the repression and blocked the interaction with the TBP/IIB promoter complex. Similarly, TBP is believed to be a target for p53-mediated repression by an analogous mechanism (27, 36). Although we demonstrated earlier that TBP-associated factors (coactivators) are not required for TRα-mediated repression (Fig. 4) (16), unliganded hTRα can presumably target the entire TFIID complex for repression, as evidenced by its ability to efficiently repress transcription in a nuclear extract (Fig. 5) (16).

By virtue of the ability of hTRα to both contact TFIIB and target a TBP/IIA/IIB complex for repression, it could be argued that TFIIB is also a basal factor target for TRα-mediated repression. Although we cannot rule out this possibility, we have no additional functional data that would support this hypothesis. Given the fact that TFIIB is a basal factor target for transcriptional activators (9, 23, 25), the ability of hTRα to contact TFIIB might be more relevant to a role in activation rather than repression (19). Indeed, Blanco et al. (5) have suggested that TFIIB-nuclear hormone interactions may play a critical role in ligand-dependent transcriptional activation. Along these lines, the ability of hTRα to target a TBP/IIA/IIB complex might suggest only that TBP is still accessible for repression at this step, whereas formation of a more complete PIC (TBP/IIA/IIB/polII/IIF) renders TBP inaccessible to hTRα. In support of the primary role of the TRα-TBP interaction in repression, ligands which alleviate repression decreased the TRα-TBP interaction but not the TRα-TFIIB interaction (Fig. 8). It has been recently suggested that an additional corepressor protein(s) may be required for efficient transcriptional repression by hTRβ (3). The possibility of an accessory factor which might serve to stabilize or enhance TRα-mediated repression is not inconsistent with the model proposed here. Thus, just as specific activation domains in DNA-bound activators and interacting coactivators may act synergistically (26), possibly by targeting different general factors, so might repression domains in DNA-bound repressors and interacting corepressors act synergistically to repress transcription. In sum, our results suggest that one way unliganded hTRα can directly target the basal transcription apparatus for repression is through inhibitory interactions with TBP. Studies examining alternative modes of repression by hTRα and hTRβ involving other basal factor targets and/or accessory factors are under investigation.

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ADDENDUM IN PROOF

Recent studies by J. D. Chen and R. Evans (Nature [London] **377:**454–457, 1995) and by Hörlein et al. (A. J. Hörlein, A. M. Näär, T. Heinzel, J. Torchia, B. Gloss, R. Kurokawa, A. Ryan, Y. Kamei, M. Söderström, C. G. Glass, and M. G. Rosenfeld, Nature [London] **377:**397–404, 1995) have provided direct evidence for corepressors that interact with TR.

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