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Differential DNA Binding by Monomeric, Homodimeric, and Potentially Heteromeric Forms of the Thyroid Hormone Receptor

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Binding of the thyroid hormone receptor (TR) to thyroid hormone-responsive elements (TREs) is crucial for regulation of gene expression by thyroid hormone. The TR binds to each half-site of a palindromic TRE separately, as a monomer, or simultaneously, as a homodimer. In addition, the TR monomer interacts with a 42-kDa protein that may be responsible for an increase in the apparent size and stability of the TR-TRE complex after incubation with liver nuclear extract. The multiple DNA-binding forms of the TR contact the TRE differently but compete for binding in a dynamic equilibrium which is highly dependent on the relative concentrations of TR and nuclear protein. Thus, protein-protein interactions are likely to determine the context in which the TR binds to target genes and regulates the transcriptional response to thyroid hormone.

Thyroid hormone (T3) is required for normal growth, development, and adult function in a wide range of species, from *Xenopus laevis* to humans (44). T3 regulates gene transcription by binding to nuclear thyroid hormone receptors (TRs) (47). In mammals, three TRs (α1, β1, and β2) with different tissue-specific, hormonal, and pharmacological regulation have been described (34). The TRs are cellular homologs of the viral oncoprotein v-ErbA (48, 54) and are members of a superfamily of ligand-activated transcription factors (15, 24). The most highly conserved region of these receptors, located near their amino termini, is the DNA-binding domain, which contains basic amino acids arranged into two zinc fingers. The carboxyl termini, which are less well conserved, mediate binding of specific ligands. Although T3 is not required for interactions between TRs and T3-responsive elements (TREs) to occur in vitro (33, 35, 40), its presence can determine whether transfected TRs have repressive or stimulatory effects on gene transcription in cultured cells (6, 11, 23).

The palindromic sequence AGGTCATGACCT (TREp) has been shown to be a strong TRE (21), although some T3-responsive sequences contain direct repeats (7) while others consist of only a single half-site (28). It has been suggested that TRs bind as dimers to the two half-sites of TREp. This view has been supported by the observation of functional repression by non-T3-binding TR mutants which retain heptad repeats of hydrophobic amino acids in their carboxyl termini (18), which have been referred to as interaction domains (22). Such mutant TRs are similarly able to inhibit the action of retinoic acid receptors (RARs), also perhaps through formation of inactive heterodimers. TRs by themselves form multiple TRE-containing complexes, as revealed by the gel electrophoretic mobility shift assay (EMSA) (33), but biochemical identification of monomeric or dimeric TRs in these complexes has not previously been achieved. TR binding to TREs is also affected by nuclear proteins which enhance binding (8, 40) and retard the migration of the TR-TRE complexes in the EMSA (33).

Since TR-TRE interaction is a key step in T3 action, the existence of a nuclear protein which modifies the DNA-binding properties of the TR is of great interest. In this report, we provide evidence that the TR binds to TREs both as a monomer and as a homodimer. We also show that in the presence of liver nuclear extract, the TR forms an additional TRE-binding complex with a different pattern of DNA contact. The altered mobility of this complex, together with the finding that liver nuclei contain a 42-kDa protein which binds specifically to the TR, suggests that it may be due to a TR-nuclear protein heteromer, but determination of the actual mechanism by which nuclear proteins modulate DNA binding by the TR awaits their purification and identification. The potentially heteromeric TR-TRE complex is favored by its increased stability, although the TR monomer and homodimer are in dynamic equilibrium and can effectively compete for TRE binding when the TR concentration is sufficiently high.

MATERIALS AND METHODS

EMSA. In the standard assay, reticulocyte lysate-synthesized TRs or control lysates were incubated at room temperature for 20 min with DNA fragments in the presence of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 80 mM KCl, 1 mM dithiothreitol, 5% glycerol, 200 μg of poly(dI-dC) (Boehringer) per ml, and 25 μg denatured herring sperm DNA per ml. In some experiments, ^{35}S-labeled TRs were incubated with unlabeled DNA fragments, while in others the DNA fragments were ^{32}P labeled with the Klenow fragment of DNA polymerase I and 30,000 cpm (~0.5 to 1.0 ng) per binding reaction. For cold competition experiments, 5,000 cpm of labeled TRE was used with ~1,000-fold-molar-excess unlabeled competitor DNA, and binding reaction mixtures were loaded onto a running gel. Electrophoresis was performed on 5% polyacrylamide gels in 0.5× Tris-borate-EDTA at room temperature. Gels were dried and then subjected to autoradiography. When ^{35}S-labeled protein was used, gels were treated with Enhance (NEN) prior to drying and autoradiographed at room temperature. For studies of ^{32}P-labeled DNA, gels were autoradiographed with a Dupont Cronex intensifying screen at −70°C.

Preparation of DNA fragments. DNA-binding sites used were TREp (TCAGGTCATGACCTGA), TREx (TGGTGTC

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AAAGGTCAAACTT), vERE (AGGTCACAGTGACCT), and TREh (TGGTATGAAAAGGTCACATTTTAG). TREp was subcloned into the *Bam*HI site of Bluescript KS(−) and excised with *Xba*I and *Hin*dIII; the complete sequence of the DNA fragment used was CCTAGAACTAGTGGATCTCAG GTCATGACCTGAGATCCCCCGGGCTGCAGAATTCGA TATCAAGCT (the TREp insert is underlined). The other DNA fragments were subcloned into the *Bam*HI site of pUC18 and excised with *Hin*dIII and *Eco*RI for use in the EMSA. Fragments were either used unlabeled, labeled on both strands by fill-in with Klenow in the presence of the appropriate α-^{32}P-labeled deoxynucleotide after double digestion, or labeled on one strand for methylation interference assays by fill-in with Klenow after digestion with a single restriction enzyme followed by second enzyme digestion after heat inactivation of Klenow. In all cases, fragments were gel purified prior to use in the EMSA.

Synthesis of TRs in reticulocyte lysates. cRNA was transcribed from cDNA-containing plasmids (22, 26, 30, 54), using SP6, T3, or T7 RNA polymerase. The proteins were translated in reticulocyte lysates (Promega) in the presence of ^{35}S-labeled or unlabeled methionine. When translations were performed in the absence of label, parallel reactions were checked for full-length protein synthesis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were estimated to be >90% radiochemically pure in all cases.

Synthesis of TRα1 in *Escherichia coli*. An in-frame *Eco*RI site was substituted for the 5′ untranslated region of the rat TRα1 cDNA (35) by using the polymerase chain reaction, and this modified cDNA was subcloned into a modified version of the pET-3 expression vector (51) containing an *Eco*RI cloning site, called pAR (provided by M. Blanar). The construct containing the modified cDNA in the correct orientation (pAR-TRα1) was used to transform the BL21(pLysS) strain of *E. coli* (51), which was then grown to an optical density at 600 nm of 0.5 to 1.0, induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 to 3 h, and harvested by lysozyme and detergent treatment followed by sonication (2). SDS-PAGE-Coomassie blue analysis of induced and control bacterial proteins revealed a faint additional band of approximately 48 kDa in the induced cells, representing 0.5 to 1% of total cellular protein.

Preparation of nuclear extracts. Rat liver nuclear extracts were prepared by the method of Surks et al. (52). JEG-3 and COS-7 nuclear extracts were prepared by the method of Dignam et al. (14). Five micrograms of nuclear extract protein was added per binding reaction unless otherwise noted. Protein determinations were made with a kit from Bio-Rad.

Methylation interference assays. DNA fragments of interest were labeled on one strand (see above) and methylated on approximately one G residue per molecule (2) prior to use in the EMSA, which was performed as described above except that the labeled DNA was electrophoretically transferred to DEAE-paper and then eluted, or the gel was autoradiographed while wet and labeled DNA was directly excised from the gel and eluted. The eluted DNA was cleaved with piperidine by using a standard protocol (2) and then electrophoresed on a sequencing gel adjacent to a sequencing reaction performed on a DNA fragment of known composition for standardization of size determinations. For all experiments (only some of which are shown in Results), results obtained for one labeled strand were confirmed for the other.

Preparation of antibodies recognizing TRβ. Peptides corresponding to amino acids 190 to 208 (NREKRRREELQKS IGNKPE) of human TRβ1 (54) and conserved in the rat (30, 41) were synthesized with carboxyl-terminal cysteines and then coupled to keyhole limpet hemocyanin by using maleimidobenzoyl succinimide. Rabbits were immunized with the coupled peptides and boosted approximately every 3 weeks (Berkeley Antibody Co.). The TRβ1 antisera immunoprecipitated reticulocyte lysate-synthesized TRβ1 but not TRα1 (25a).

Protein cross-linking. ^{35}S-labeled TRs were synthesized in reticulocyte lysates and then preincubated in the presence or absence of TREp (~4 ng/μl) and/or liver nuclear extract (0.4 mg/ml) under conditions identical to those described in the binding reactions for the EMSA in a volume of 30 μl for 20 min. Following preincubation, glutaraldehyde (0.01% final) or DSS (disuccinomide suberimidate) (0.12 mM final) was added, and the mixtures were incubated for an additional 35 min at 4°C. In the experiments shown, glutaraldehyde was used to cross-link TRα1 and DSS was used with TRβ1, but both agents gave similar results with either TR. The reactions were quenched with glycine (final concentration, 0.033 M) and run immediately on an SDS-PAGE gel, which was enhanced and dried prior to fluorography.

RESULTS

The TR binds to DNA as monomer and dimer. Figure 1A shows that TRβ1 synthesized in reticulocyte lysate formed two major complexes (arrowheads) with TREp. Formation of the TR-TRE complexes was independent of T3 (not shown). The presence of TR in each of the complexes was demonstrated by addition of anti-TRβ antibodies (which specifically recognize amino acids 190 to 208 of TRβ1), which supershifted both complexes. Interestingly, the anti-TRβ antiserum appeared to stabilize the TR-TRE complex, perhaps related to the bivalency of the antibodies. The hypothesis that the two TR-TRE complexes represented TR binding as monomer and homodimer was tested by mixing TRβ1 with an amino-terminally deleted form, TRβ1(ΔN), which lacks the first 100 amino acids but retains the interaction domain (22). TRβ1(ΔN) behaved similarly to TRβ1 in the EMSA, but the complexes that it formed migrated more rapidly than the corresponding TRβ1 complexes because of its lower molecular mass (~41 kDa compared with ~55 kDa). Figure 1B shows that as an increasing concentration of TRβ1(ΔN) was mixed with TRβ1, a novel complex which migrated between the upper TRβ1 and TRβ1(ΔN) complexes was observed. This complex most likely consisted of a heterodimer between TRβ1 and TRβ1(ΔN), indicating that the upper TRβ1 and TRβ1(ΔN) complexes were due to TR homodimerization. The lower TRβ1(ΔN) complex and the free probe were run off the gel in this experiment in order to increase the resolution of the multiple less rapidly migrating complexes.

The two major TRβ1 complexes thus appeared to represent TR monomer and homodimer binding to TREp. This conclusion was further substantiated by methylation interference experiments which demonstrated characteristically different DNA contact for each of the TRβ1-TRE complexes. Figure 1C shows that formation of the more retarded TR complex was prevented by methylation of any of the three G residues in TREp, indicating that both half-sites of the palindrome were contacted simultaneously. In contrast, methylation of none of the G's completely interfered with formation of a more rapidly migrating monomeric TRβ1-TRE complex, although the radioactivity in each of the three

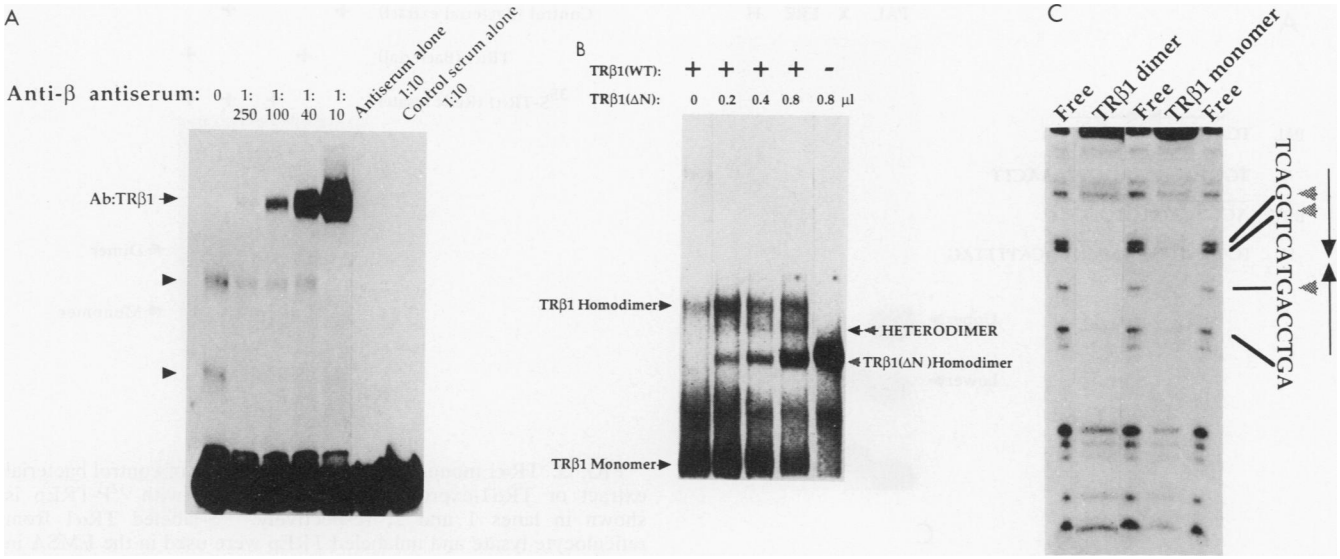

FIG. 1. TRβ1 monomers and dimers bind to TREp. (A) Formation of two TREp-binding complexes by TRβ1. EMSA of TRβ1 interactions with TREp was performed in the presence of increasing amounts of TRβ antiserum. Arrowheads indicate positions of monomeric and dimeric TRβ1-containing complexes, and the position of the antibody (Ab)-TRβ-TREp complex is labeled. (B) Evidence that mixture of TRβ1 and TRβ1(ΔN) results in formation of a heterodimer. Increasing amounts of TRβ1(ΔN) were added to wild-type (WT) TRβ1. Solid arrowheads indicate positions of the TRβ1 complexes; the shaded arrowhead indicates the TRβ1(ΔN) complex; double arrowheads point to the TRβ1-TRβ1(ΔN) complex. The rapidly migrating labeled probe and a smaller TRβ1(ΔN) has been run off the gel to increase separation of the slower-migrating bands. (C) Evidence that the TRβ1 homodimer contacts both half-sites of TREp. A methylation interference assay was performed on the TRβ1 monomer and dimer complexes (leftmost lane in panel A). The cleavage pattern of the unbound (Free) probe is shown for comparison. G residues in TREp which interfered with dimer formation are indicated by shaded arrowheads. Positions of the two half-sites and their orientations in TREp are also indicated.

bands caused by cleavage at the methylated bases in the palindrome was nearly half of that in other bands in the same lane (a range of 39 to 65% in three experiments, using the Molecular Dynamics ImageQuant). These data are consistent with the lower TRβ1-TRE complex containing TR monomer bound to one or the other half-site in TREp but not to both simultaneously. This would explain the partial interference, since methylation of a G in one half-site would interfere with binding to that half-site but not to the adjacent one. The complete interference pattern of the more retarded band was therefore due to contact of both half-sites simultaneously by the TR homodimer.

We next studied an amino- and carboxyl-terminal truncation mutant, TRβ1(ΔNΔC), which contains amino acids 100 to 260 of TRβ1. Figure 2A shows that, like TRβ1, TRβ1(ΔNΔC) formed two major complexes with TREp. This suggested that two TRβ1(ΔNΔC) molecules could bind to one molecule of TREp (forming the more retarded complex) despite the fact that TRβ1(ΔNΔC) was lacking the carboxyl terminus, which has been implicated as an interaction and/or dimerization domain in transfection experiments (17, 18, 22, 27). To address this further, binding of TRβ1 to a variety of DNA fragments related to TREp was studied. The fragments used (Fig. 2A) were vERE, the vitellogenin estrogen response element, which is identical to TREp except for a 3-bp insertion between the two half-sites (21); TREx, the ovalbumin COUP response element (53), which contains two directly repeated half-sites with a single-base gap, in which one of the half-sites (GTGTCA; mutations underlined) is imperfect and has been shown not to cause T3 responsiveness (7); and TREh, which contains only a single half-site. Figure 2A shows that TRβ1(ΔNΔC) bound all of these DNA fragments but formed two complexes only with the DNA fragments which contained two perfect half-sites (TREp and vERE). Furthermore, the fast migration of the single complex formed with TREx and TREh suggested that only a single TRβ1(ΔNΔC) molecule bound per DNA molecule as a result of the presence of only a single copy of the AGGTCA motif in these fragments. Methylation interference experiments were carried out to test these hypotheses.

Figure 2B shows that formation of the more retarded (upper) complex was greatly interfered with by methylation of any of the three G residues in the palindrome, much as for the wild-type TRβ1 dimer complex. Thus, it is likely that one TRβ1(ΔNΔC) molecule was bound to each half-site in this complex, possibly without true interactive dimerization since TRβ1(ΔNΔC) lacks the putative interaction domain. The lower TRβ1(ΔNΔC)-TREp complex had an incomplete interference pattern similar to that of the TRβ1 monomer, presumably because this complex contained a lone TRβ1(ΔNΔC) molecule bound to one or the other half-site in TREp but not to both simultaneously. On the other hand, as suggested above, if TRβ1(ΔNΔC) formed only a single complex with TREx because it bound solely to the AGGTCA half-site, formation of this monomeric complex would be completely interfered with by methylation of either G in that half-site. Indeed, Fig. 2C shows that methylation of either G residue in the perfect half-site completely interfered with binding, while the imperfect half-site was not contacted at all, consistent with the hypothesis that the rapidly migrating TREx-binding complex was due to a single TRβ1(ΔNΔC) molecule bound exclusively to the perfect half-site. Thus, methylation of a G within a lone binding site completely prevented binding by the TR monomer, but when two half-sites were present (as in TREp), methylation within one of the half-sites had little effect on binding to the other.

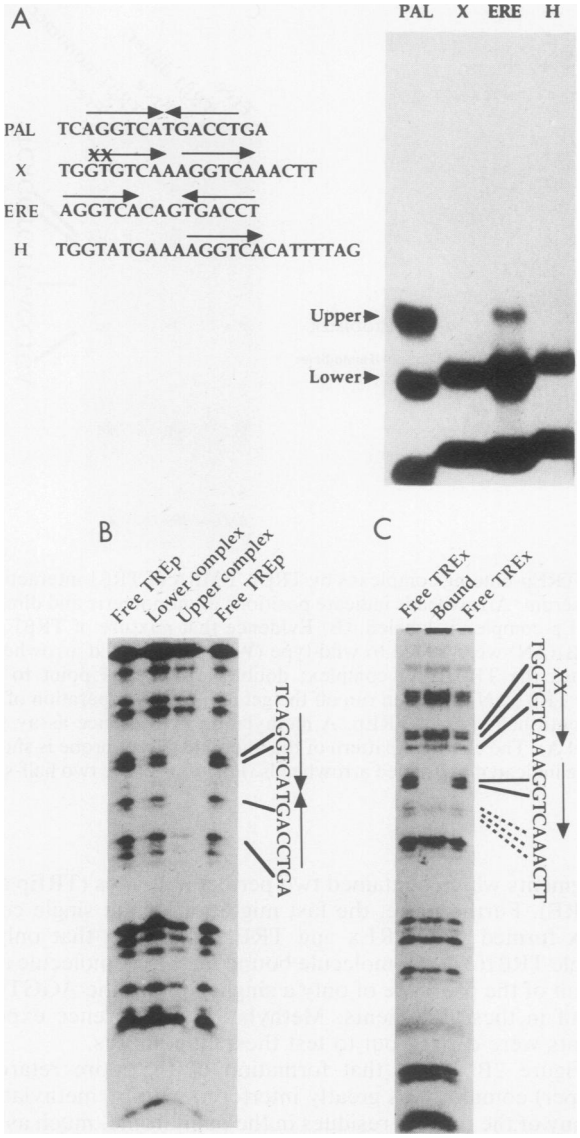

FIG. 2. Evidence that an amino- and carboxyl-terminal deletion mutant of TRβ1 (TRβ1ΔNΔC) can form two DNA-binding complexes in the presence of two half-sites. (A) EMSA of TRβ1 (ΔNΔC) binding to TREp-related DNA fragments. Half-sites and their orientations are indicated by arrows, and deviations from the AGGTCA motif are highlighted by X's. Surrounding polylinker sequences are described in Materials and Methods. DNA fragments are TREp (PAL), TREx (X), vERE (ERE), and TREh (H). (B and C) Methylation interference patterns of the TRβ(ΔNΔC)-TREp (B) and TRβ(ΔNΔC)-TREx (C) complexes. Upper and lower TRβ(ΔNΔC)-TREp complexes correspond to those indicated in panel A. Positions of DNA fragments corresponding to cleavage at specific G residues are indicated by solid lines. Some fragments corresponding to minor cleavage at adenosine residues are indicated by dashed lines.

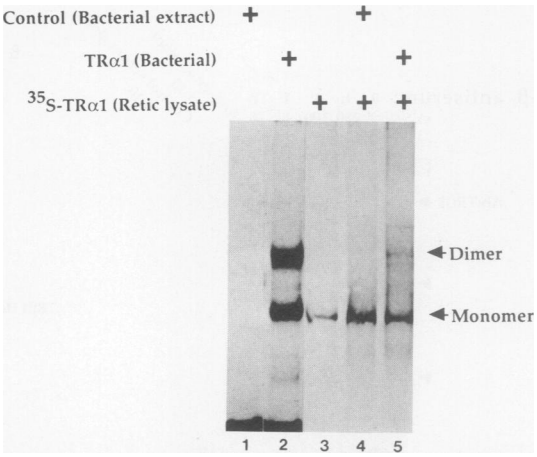

FIG. 3. TRα1 monomers and dimers. EMSA of control bacterial extract or TRα1-expressing bacterial extract with ^{32}P-TREp is shown in lanes 1 and 2, respectively. ^{35}S-labeled TRα1 from reticulocyte lysate and unlabeled TREp were used in the EMSA in lanes 3 to and 5, either alone (lane 3) or in the presence of control bacterial extract (lane 4) or TRα1-expressing bacterial extract (lane 5).

The DNA-binding of TRα1 was also investigated. We have previously noted that reticulocyte lysate-synthesized TRα1 formed a single complex with TREp (33). Figure 3 confirms that the reticulocyte lysate TRα1 formed a single complex with TREp (lane 3). The failure of TRα1 to bind to TREp as monomer and homodimer could be due to inadequate quantities of active protein generated by in vitro translation or to a fundamental difference between TRα1 and TRβ1. To distinguish between these two possibilities, TRα1 was synthesized in *E. coli* as described in Materials and Methods. Bacterially derived TRα1 formed two strong complexes with TREp (lane 2); these complexes were identified as TRα1 monomer and homodimer in methylation interference experiments (data not shown). The reticulocyte lysate TRα1-TREp complex comigrated with the monomer (lane 3). However, when labeled reticulocyte lysate TRα1 was incubated with the bacterially derived TRα1 (unlabeled) and with unlabeled TREp, a novel ^{35}S-containing complex was formed (lane 5). This complex most likely represented a heterodimer between the TRs from different sources, because it comigrated with the TRα1 homodimer and was not seen when ^{35}S-TRα1 was mixed with control bacterial extract (lane 4). This result confirmed the finding of TR homodimerization and showed that the reticulocyte lysate TRα1 was inherently able to dimerize at increased TR concentration.

Nuclear proteins heterodimerize with monomeric TR. The next set of experiments was designed to determine the mechanism by which nuclear proteins retard the electrophoretic mobility of the TR-TREp complex (33). Figure 4A shows that a rat liver nuclear protein, distinct from that in JEG-3 or COS-7 cells (33), decreased the electrophoretic mobility of the TR-TREp complex. In this experiment, ^{35}S-labeled TRα1 was allowed to interact with unlabeled TREp in the presence or absence of nuclear extracts from JEG-3 cells, liver, or both (Fig. 4A). By itself, TRα1 formed a single TRE-containing complex (as shown in Fig. 3). Nuclear proteins in liver and JEG-3 cells both caused a supershift of the TR-TRE complex; the TR was clearly present in these complexes, as neither the nuclear proteins nor the TRE was radiolabeled. The TR-TRE complex was retarded less by the liver protein than by that in JEG-3 extract, suggesting that the liver protein was of lower molecular mass. This was unlikely to be an artifact of extract preparation because proteolysis inhibitors were used and extracts from multiple sets of livers gave similar results. Note that the liver and JEG-3 proteins did not result in

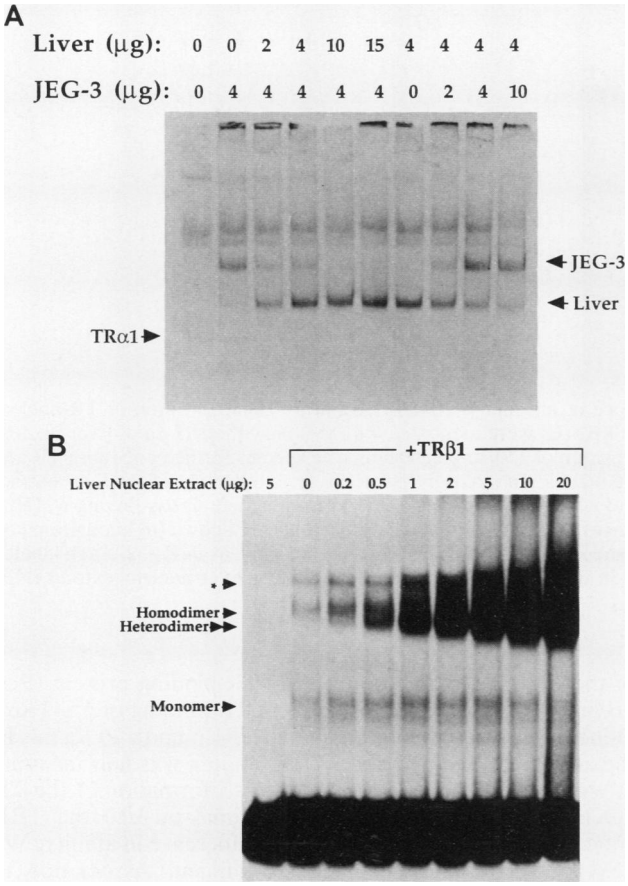

FIG. 4. Nuclear proteins form heteromers with TRs. (A) Interactions of liver and JEG-3 nuclear proteins with TRα1 in EMSA of ^{35}S-TRα1 and unlabeled TREp. The amount of liver or JEG-3 nuclear extract protein added to a 25-μl binding reaction is indicated above each lane. The arrow on the left indicates the position of the TRα1-TREp complex in the absence of added nuclear extract; arrows on the right show the positions of the TRβ1 heterodimers with liver and JEG-3 proteins. Nonspecific bands due to reticulocyte lysate alone are present in equal amounts in all lanes. (B) Enhancement of TR binding by liver nuclear protein. The liver nuclear protein was added to a 25-μl binding reaction. Positions of TRβ1 monomer, dimer, and heterodimer complexes are indicated by arrows. The asterisk indicates an additional TREp-binding complex seen in the absence of liver protein. This complex comigrated with the TRβ1-COS and TRβ1–JEG-3 heterodimer complexes and was also noted when bacterially expressed TRα1 was mixed with unprogrammed reticulocyte lysate (data not shown), indicating that it was due to minor contamination of this batch of lysate with nuclear proteins.

formation of an even larger complex when present in the same binding reaction, indicating that they did not simultaneously bind to the TR. Rather, saturating amounts of one protein reduced or eliminated the binding of the other, suggesting that the liver and the JEG-3 protein competed for the same region of the TR or that binding of one protein prevented binding of the other by a noncompetitive mechanism, e.g., allosterically or by steric hinderance.

Figure 4B demonstrates that in addition to its monomer and dimer complexes, TRβ1 formed a complex in the presence of as little as 0.5 μg of liver nuclear protein. Increasing amounts of the liver protein resulted in a tremendous enhancement of TREp binding. The binding enhancement was especially pronounced in this experiment (and others in which labeled TREp was used) because of the low concentration of TREp (0.5 ng per binding reaction) relative to that (250 ng per reaction) used when the TR itself was labeled. The dependence of the enhancement of binding upon TRE concentration will be examined in greater detail later (see Fig. 7A). However, little TRE binding by the liver extract was detected in the absence of added TR, suggesting that the protein which dramatically enhanced TR binding had much lower affinity for TREp than did any of the TR-containing complexes. Weak TREp binding was noted upon much (5- to 10-fold) longer exposure of the autoradiogram, perhaps because of low levels of TR, RAR, or other TREp-binding proteins in the amount of liver extract used. Of note, the complex resulting from the TRβ1-liver protein interaction migrated more rapidly than the TRβ1 homodimer-TRE complex. Since no additional complex larger than the TR homodimer was detected, we hypothesized that the liver nuclear protein heterodimerized with the TR monomer but did not interact with the TR homodimer.

The TR-binding nuclear protein in liver is ~42 kDa and binds to the TR monomer in the absence of DNA. The degree of retardation of the TR-TRE complex strongly suggested that a liver protein heterodimerized with the TR. A protein-protein cross-linking strategy was used to prove that a nuclear protein in the liver extract indeed bound specifically to the TR, as well as to estimate the size of this TR-binding protein and to determine whether DNA binding was necessary for TR-nuclear protein heterodimerization. ^{35}S-labeled TRα1 and TRβ1 were incubated with or without liver nuclear extract prior to cross-linking and analysis by SDS-PAGE. Figure 5A shows that a novel ~89-kDa complex containing ^{35}S-TRα1 (~47 kDa) was detected only when the cross-linking was performed in the presence of liver protein. TRβ1 was similarly cross-linked to a liver nuclear protein whose TR-binding activity was heat labile (Fig. 5B). The TRβ1 translation product is a doublet of 55 and 52 kDa (54), and the product of cross-linking was a poorly resolved doublet of which the largest species was ~97 kDa. The molecular mass of the TR-binding protein, estimated by subtracting the size of the TR (α or β) from that of the heterodimer in four separate experiments, was 42 ± 2 (standard error of the mean) kDa, which, in agreement with the EMSA data, is lower than that of either TR. Interestingly, the TR heterodimers formed in the absence of added TREp, indicating that the association occurs in solution without DNA binding. Cross-linking of the heterodimer was similar in the presence or absence of TREp-containing DNA. The cross-linking was specific, since labeled standard proteins did not cross-link to one another or to liver proteins under identical conditions (not shown). Of note, cross-linking of TR homodimers was not observed in these experiments, presumably because of the weakness of the TR-TR interaction.

The finding that the TR heterodimer formed in solution in the absence of DNA suggested that its formation could be detected by EMSA by using DNA fragments other than TREp, including those containing half-sites recognized by the TR. To test this, TRα1 was incubated with labeled TREx, vERE, or TREh in the presence or absence of liver nuclear extract and then subjected to EMSA. As indicated in Fig. 5C, TR heterodimers bound to all of the TRE-related DNA fragments, and binding was considerably enhanced in each case (in the case of the TREh, binding in the absence of nuclear extract was detectable only after long exposure times). Similar results were obtained with TRβ1 (not

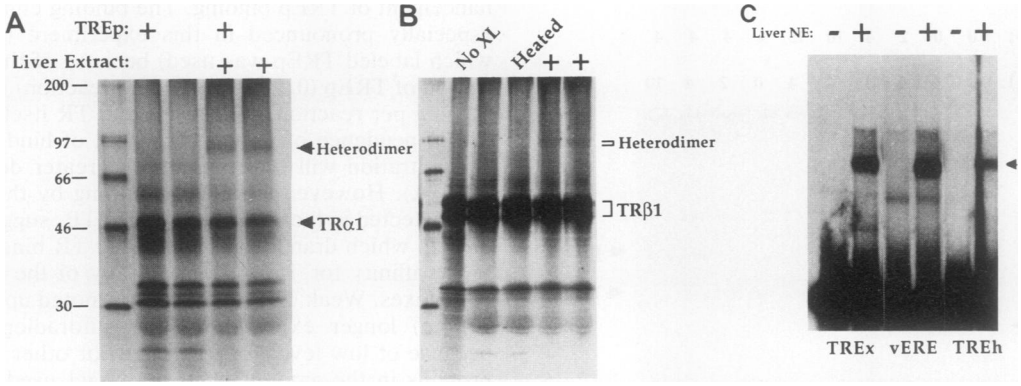

FIG. 5. Binding of TRs to a 42-kDa liver nuclear protein in the presence or absence of DNA. (A and B) Demonstration of TR-nuclear protein heterodimers by protein cross-linking. ^{35}S-labeled TRα1 (A) and TRβ1 (B) were incubated with (+) or without 5 μg of liver nuclear extract and then subjected to cross-linking prior to SDS-PAGE analysis. Position of TRs and heterodimeric species formed only with TR and native liver extract are indicated. When indicated, TREp was added to the incubation reaction. Heated refers to heating of the liver extract to 100°C for 2 min prior to incubation with TR; No XL indicates a sample to which cross-linking agent was not added. Cross-linking of TRβ1 to native liver protein was performed in duplicate as shown. Molecular masses of ^{14}C-labeled standard proteins are shown (in kilodaltons) on the left. (C) Binding of TR heterodimers to multiple TRE-related DNA fragments. The DNA fragments are the same as in Fig. 2. Each labeled DNA fragment was incubated with reticulocyte lysate-synthesized TRα1 in the presence or absence of 5 μg of liver nuclear extract (NE) protein. The arrowhead points to the heterodimer complexes.

shown). Thus, the TR heterodimer bound to a variety of half-site-containing fragments.

The TR heterodimer binds asymmetrically to TREp. We next used the methylation interference assay to compare TREp contact by the TR heterodimer with that of the monomer and homodimer. Figure 6 contrasts the methylation interference patterns of TR monomer, homodimer, and heterodimer, using both TRα and -β as well as liver and COS nuclear extracts. The TR heterodimer contacted the TRE differently than did either the monomer or homodimer; remarkably, methylation of only the single, most central G in TREp completely interfered with binding (Fig. 6A to C), indicating that the heterodimer preferentially contacted a specific, single half-site in the shifted TREp-containing fragment. TRα and TRβ heterodimers had similar binding preferences. In contrast, confirming our earlier finding, the TR homodimer contacted both half-sites (Fig. 6A) and the monomer interacted with either half-site at approximately equal frequencies (Fig. 6B). Furthermore, the methylation interference pattern of the heterodimer formed with the TR-binding protein in COS-7 cells was indistinguishable from that of the TR-liver protein heterodimer (Fig. 6C). Figure 6D shows that when the opposite strand of the TREp-containing fragment was labeled, methylation of either of two adjacent G residues (base paired with cytosines on the ^{32}P-labeled strand in Fig. 6A to C) interfered with heterodimer binding. These G residues are part of the same half-site that the TR heterodimer was shown to contact on the other strand (which contained a single G), confirming that the heterodimer bound preferentially to one of the TREp half-sites, whereas the monomer and homodimer bound similarly to either and both half-sites, respectively. This difference was not observed when a only single perfect half-site was present, such as in TREx (data not shown). It is likely that heterodimer binding to the specific half-site in TREp is favored by interactions with adjoining sequences. However, these interactions must be weaker than the contact with the half-site because methylation of G residues in this regions did not detectably interfere with binding.

The TR-binding protein stabilizes the TR-TRE complex. We next studied the mechanism of the marked enhancement of the TR-TRE interaction by the TR-binding protein (Fig. 4B and 5C). Figure 7A shows the TRE binding of ^{35}S-TRα1 at increasing amounts of unlabeled TREp up to 20 μg/ml. In the absence of liver extract, TRE binding was half-maximal at ~4 μg of TREp per ml. In contrast, formation of the TR heterodimer complex was half-maximal at 1/10 the TRE concentration, indicative of a 10-fold increase in affinity. We next studied the rates of association and dissociation to determine the mechanism of the increased affinity of the heterodimer. To estimate the off rates of the TR-TRE complexes, TRα1 was incubated with ^{32}P-TREp (0.1 ng) for 20 min in the presence or absence of liver extract, after which 1,000-fold molar excess of unlabeled TREp was added for various periods of time (30 s to 10 min) prior to EMSA. Figure 7B shows that the labeled TR heterodimer complex had a half-life of ~1.5 min under these conditions, while the half-lives of the monomeric and homodimeric TR-TRE complexes were clearly much shorter since these complexes were reduced by ~98 and ~99%, respectively, at 30 s, which was the earliest feasible time point. Thus, the increased stability of the TR heterodimer-TRE complex accounted for much and perhaps all of its ~10-fold increased affinity.

To estimate the association rate of the TR-TRE interaction, the time course of TRα1 binding to TREp in the absence or presence of liver nuclear extract was studied. Figure 7C shows that in the absence of liver extract, TR binding was so rapid that steady-state levels of complex formation were observed in the time necessary to mix the reaction components and load the gel, which was under 30 s. This result indicated that TRE binding of the TR was nearly instantaneous in the absence of the TR-binding protein and confirmed the rapid dissociation rate of the nonheterodimeric TR-TRE complex, since the time to steady state is dependent on the half-life. In contrast, formation of the supershifted TR-nuclear protein heterodimer complex was not at equilibrium until about 10 min of preincubation and was less abundant than the nonheterodimeric TR-TRE complexes at the earliest time points. Interestingly, the amount of TR-TRE complex formed in the absence of liver extract increased four- to fivefold when the EMSA was performed at 4°C (data not shown), presumably because TR-TRE disso-

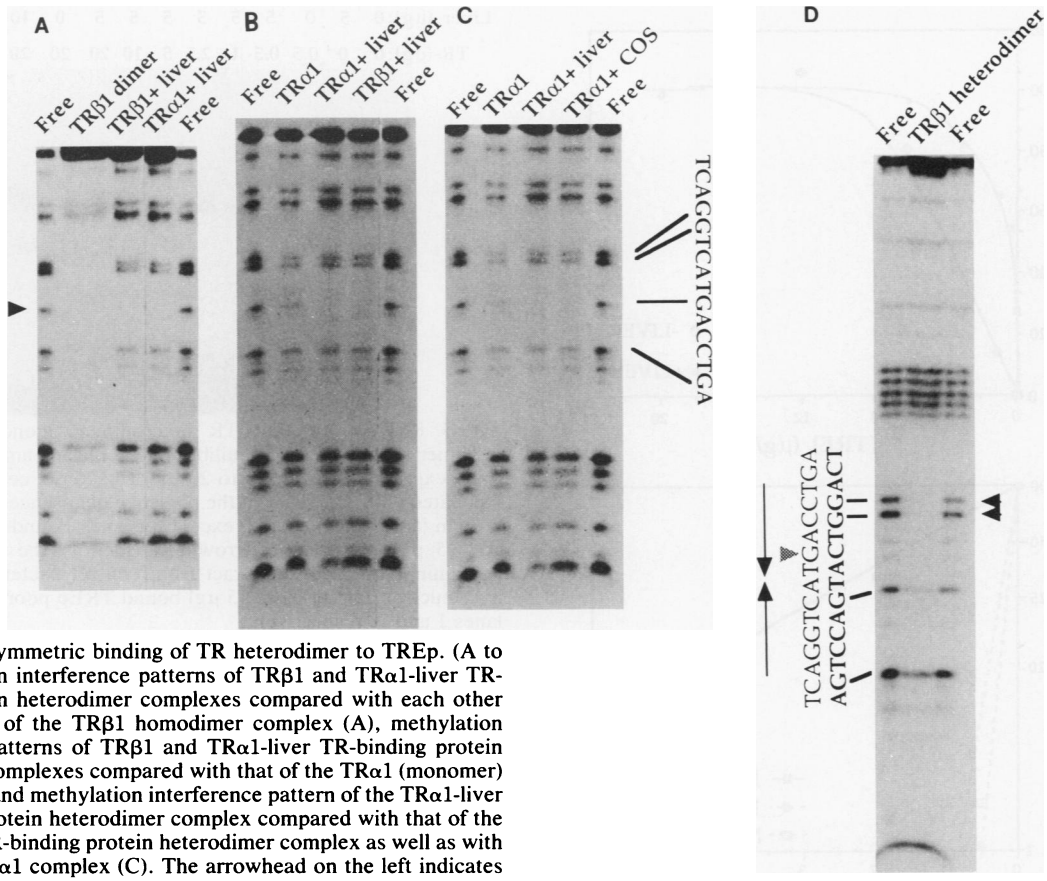

FIG. 6. Asymmetric binding of TR heterodimer to TREp. (A to C) Methylation interference patterns of TRβ1 and TRα1-liver TR-binding protein heterodimer complexes compared with each other and with that of the TRβ1 homodimer complex (A), methylation interference patterns of TRβ1 and TRα1-liver TR-binding protein heterodimer complexes compared with that of the TRα1 (monomer) complex (B), and methylation interference pattern of the TRα1-liver TR-binding protein heterodimer complex compared with that of the TRα1-COS TR-binding protein heterodimer complex as well as with that of the TRα1 complex (C). The arrowhead on the left indicates the G residue in a single half-site which interferes with TR heterodimer-TREp complex formation. (D) Methylation interference pattern of TRβ1-liver nuclear protein heterodimer, with ^{32}P labeling of the strand opposite that labeled in panels A to C; the G residue whose methylation inhibited heterodimer complex formation in panels A to C is indicated by the shaded arrowhead. Solid arrowheads indicate the two G's in the opposite strand of the same half-site which are seen to be contacted by the heterodimer in this experiment.

ciation is temperature dependent. Taken together, the data strongly suggest that the mechanism of binding enhancement by the TR-binding protein is stabilization of TRE binding.

The TR heterodimer is in dynamic equilibrium with TR monomers and heterodimers. The data thus far show that formation of the TR heterodimer-TRE complex is favored over formation of the TR-TRE complex because of its increased stability. Since TR concentrations were limiting in these experiments, we next used the bacterially overexpressed TRα1 to determine whether high concentrations of TR could drive the formation of nonheterodimeric TR complexes even in the presence of the TR-binding protein. As shown above (Fig. 7B), this preparation of TRα1 retained the ability to heterodimerize with the TR-binding protein, and just as with TR synthesized in reticulocyte lysate, formation of the TR heterodimer considerably enhanced TRE binding at low TR concentrations (Fig. 8; compare lanes 3 and 4). Binding of the TR monomer and homodimer to the TRE increased as the TRα1 concentration was increased despite the presence of a constant amount of liver protein. Under some conditions, such as when 5 μg of bacterial TRα1 extract and 5 μg of liver protein were coincubated, the monomer, homodimer, and heterodimer forms of TR were all distinguishable. The TRα1 heterodimer migrated faster than the homodimer because of its smaller size, as was earlier shown to be the case for TRβ1. Interestingly, at very high concentrations of TR, heterodimer binding was not observed even in the presence of liver protein (lanes 4 to 9). However, at the same high concentrations of TR, inclusion of additional liver protein in the binding reaction once again allowed the heterodimer-TRE complex to form (lanes 11 and 12). Thus, the extent of TR binding to the TRE as monomer, homodimer, and heterodimer is determined by the relative concentrations of TR and TR-binding protein. In the absence of TR-binding protein, low TR concentration favors the binding of the TR monomer whereas TR homodimers predominate at high TR concentration. In the presence of the TR-binding protein, the heterodimer is the main TR-TRE complex formed except when the TR concentration is high enough to drive homodimerization despite the increased stability of the TR heterodimer-TRE complex.

DISCUSSION

Although the cloning of multiple TRs has provided insight into the mechanism of T3 action, the mechanisms regulating TR-TRE interaction are not well understood. Here we have presented evidence that TRs bind to half-sites and palindromes as monomers and dimers which compete in a dynamic equilibrium with a more stable nuclear protein-dependent complex with unique properties.

DNA binding by TR homodimers. The ability of TRs to dimerize has been predicted from studies of other transcrip-

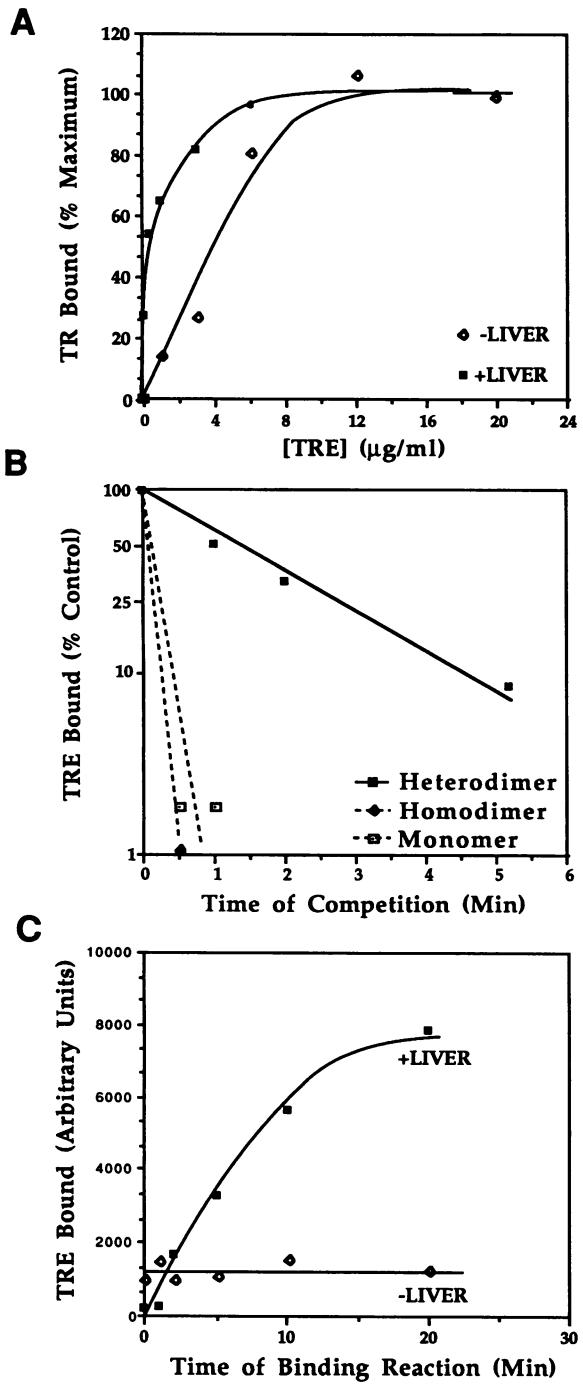

FIG. 7. Increased stability of heteromeric TR-TREp complexes. (A) Increased affinity of the TR heterodimer-TRE complex. ^{35}S-labeled TRα1 was incubated with various amounts of unlabeled TREp in the absence or presence of liver extract (5 μg/25-μl binding reaction) at room temperature for 20 min. (B) Decreased off rate of the TR-heterodimer-TRE complex. Bacterially derived TRα1 was incubated with ^{32}P-labeled TREp for 20 min prior to addition of a 1,000-fold molar excess of unlabeled TREp for various times before gel loading. (C) Time dependence of TR-TRE complex formation. Unlabeled TRα1 was incubated with ^{32}P-labeled TREp for various times in the absence or presence of 5 μg of liver nuclear extract per 25-μl binding reaction. In all cases, levels of complex formation were determined densitometrically.

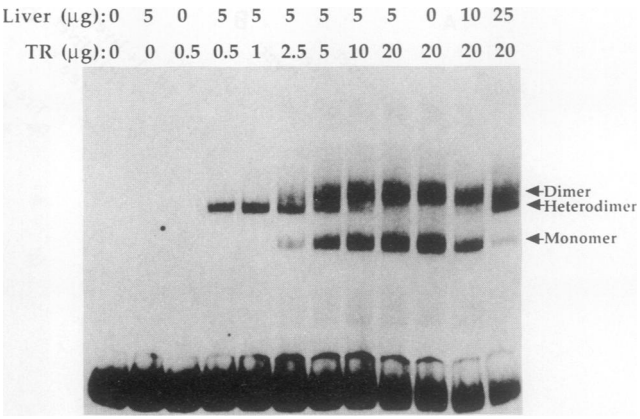

FIG. 8. Evidence that TR heterodimer, monomer, and homodimer are in dynamic equilibrium. Increasing amounts of bacterially expressed TRα1 (0.5 to 20 μg of bacterial cell extract) were incubated with ^{32}P-TREp in the presence of absence of liver nuclear protein (5 μg/30-μl reaction except for lanes 11 and 12, in which 10 and 25 μg were used). Arrowheads identify the various TREp-containing complexes. Extract from control bacteria (10 μg) and liver nuclear extract alone (5 μg) bound TREp poorly, as shown in lanes 1 and 2, respectively.

tion factors, including the highly related estrogen receptor (ER) (16, 31), as well as from functional studies suggesting that TRs can interact with themselves and with RARs (18, 22). TRβ has been previously cross-linked to RAR (22) and to itself (27) in the context of binding to adjacent half-sites on precipitated, biotinylated TREs. In that assay, cross-linking was also observed between TRβ mutants lacking the interaction domain (27). This can now be explained by our finding that the carboxyl terminus is not required for binding of two TR molecules to TREs containing two perfect half-sites. However, it is therefore unclear whether the dimerization of wild-type TRs which was observed is indicative of a facilitative TR-TR interaction or merely due to independent binding of two TRs to adjacent half-sites. TR homodimerization is favored at higher TR concentrations (Fig. 3 and 8 and data not shown), which probably explains our failure to detect homodimerization of reticulocyte lysate-synthesized TRα1. However, the concentration dependence of dimerization may be due not to cooperativity but rather to the second-order kinetics inherent in the binding of two TRs to a single DNA molecule. Formation of the TR homodimer in solution is reversible, since dimerization was demonstrated between TRs synthesized in separate translation reactions (Fig. 1B) or in different systems (reticulocyte and bacterial; Fig. 3) altogether. This property of the TR is shared with the orphan receptor ARP-1, in which dimerization of separately translated proteins is readily observed (32). It suggests that TR homodimer is relatively unstable, which is confirmed by the ready detection of DNA binding by TR monomers as well as by the failure to cross-link the TR homodimer in solution.

DNA binding by TR monomers. The relative ease with which wild-type and truncated TRs bind to TREs as monomers contrasts with the strong tendency of related transcription factors, including the ER (16, 31), to dimerize. Indeed, unlike the TR, demonstration of ER dimerization with truncation mutants requires cotranslation, and detection of specific DNA binding as well as dimerization by the ER requires a 22-amino-acid domain located in its carboxyl terminus (amino acids 501 to 522 of the mouse ER) (36). It has been

pointed out that this region of the ER has some similarity to a region of the TRs (amino acids 407 to 428 in TRβ1) included in the carboxyl-terminal domain which appears to be involved in TR-TR and TR-RAR interactions (16, 18). However, TRβ1(ΔNΔC) lacks this domain yet can still bind to TREs. Indeed, the finding that two TRβ1(ΔNΔC) molecules can bind to the two half-sites in TREp means that facilitation of TR-TR interactions by their carboxyl termini is not absolutely required for simultaneous binding of two TR molecules. Furthermore, the ability of the TRs to bind as monomers indicates that dimerization is not necessary for TR binding to TREs.

DNA binding by potential TR heteromers. Previous work had indicated that nuclear proteins can enhance TR binding (8, 12, 33, 40) and alter the electrophoretic mobility of TR-TRE complexes (33). We hypothesize that nuclear protein-TR heterodimers are present in the novel TR-TRE complexes. A TR-containing TRE-binding complex which is liver protein dependent migrated between the TR monomer and homodimer complexes, though closer to the homodimer. If this complex were due to a TR-nuclear protein heteromer, then its relative migration suggests that the TR-binding protein is of lower molecular mass than the TR. Indeed, cross-linking experiments indicated that even in the absence of DNA, the TR interacts in solution with an ~42-kDa liver nuclear protein. Also, our finding that the liver protein-dependent TR-TRE complex is more mobile than the JEG-3 protein-dependent TR-TRE complex suggests that if the novel complexes are due to TR heterodimers, then the TR-binding protein in JEG-3 cells should be larger. This is consistent with the recent report by O'Donnell et al. (43) that JEG-3 cells contain an ~63-kDa protein which could be cross-linked to the TR. Thus, there may be a family of TR-binding proteins. However, it should be noted that while the relative migration of the various TRE-binding complexes is consistent with the hypothesis that a TR-nuclear protein heterodimer binds DNA directly, the data do not rule out the possibility that additional protein(s) are present in the nuclear extract-dependent complexes. Furthermore, the proteins that can be cross-linked to the TR are not necessarily the ones that enhance its DNA binding. A detailed molecular analysis of the nuclear protein-dependent TR-TRE complexes will require the purification and identification of the proteins involved.

Interestingly, DNA binding by the RAR (20) and vitamin D receptor (38) has also been shown to be enhanced by nuclear proteins, indicative of another property shared by these TR-related receptors. Although RAR-TR interactions have been documented (18, 22), the TR-binding protein that we are studying is probably not the RAR because it does not bind TREp. The TR-binding protein could, however, be another member of the thyroid/steroid hormone receptor family. Other recently described proteins which enhance ER binding (39) or stimulate binding of bacterially synthesized Jun and Fos (1) are found in reticulocyte lysates and thus are also unlikely to be the TR-binding protein. We have also considered whether Jun and Fos, which have recently been shown to interact with glucocorticoid (13, 29, 50, 55) and estrogen (19) receptors, could be TR-binding proteins. However, both Jun and Fos failed to supershift the TR-TRE complexes (data not shown). TFIID was likewise unable to substitute for the TR-binding protein, which could nonetheless be another transcription factor previously described in another context. Alternatively, the TR-binding protein could be an adaptor which does not itself interact with DNA but signals the productive interaction of a TR, T3, and a TRE to the cellular transcriptional machinery (37, 45), although the failure of the TR-binding protein to bind to the TR homodimer makes this model less attractive.

Unlike the TR alone, which interacts with both half-sites equally, the potential TR heterodimer preferentially binds to a specific half-site in TREp. This asymmetrical binding to a symmetrical TRE appears to be the converse of the situation recently described for the helix-loop-helix protein E47, homodimers of which surprisingly prefer to interact with asymmetrical DNA-binding sites (5). The molecular mechanism by which the TR-binding protein alters the DNA binding of the TR is unclear. A TR heterodimer may selectively recognize the asymmetric sequences adjacent to the preferred half-site in a relatively weak interaction that is beyond the sensitivity of the methylation interference assay. Indeed, some sequence specificity for TR-binding enhancement by nuclear proteins has recently been reported (3), and we have found that although the liver protein binds poorly to TREp, it binds to heparin agarose as is characteristic of many DNA-binding proteins (4a).

The increased stability of the potential TR-heterodimer-TRE complex is reminiscent of the situation of Jun and Fos, where the heterodimer binds more stably to the AP-1 site than does the Jun homodimer and little, if any, binding of the Fos homodimer is detectable (9, 10, 25, 42, 46, 56). In this analogy, the TR would be more like Jun since it forms homodimers which bind DNA less stably than the TR heterodimer, while the TR-binding protein would be more like Fos since it binds poorly in the absence of TR. However, the ability of the TR to bind as a monomer adds another level of complexity since these other transcription factors, which utilize different DNA-binding motifs, have strict dimerization requirements for DNA binding. In addition, it remains to be established whether, as is the case of Jun and Fos, the heterodimer is a more potent transactivator than the homodimer (9, 49). It is also possible that, as for MyoD and the Id protein, TR heterodimer formation leads to a transcriptionally inactive complex (4).

The present data show that the TR dimerizes rather weakly and prefers to bind to DNA in association with other nuclear proteins. The different properties of the multiple DNA-binding forms of the TR make it likely that their dynamic equilibrium is important, and they must be taken into account by models of transcriptional regulation by the TR. Indeed, given the low abundance of TRs and the presence of potentially distinct TR-binding proteins in many mammalian cell types, it is possible that a TR-nuclear protein heteromer is the T3-dependent regulator of gene expression by T3 in vivo. This hypothesis can be further tested when the proteins that interact with the TR have been purified and identified.

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