DNA Binding and Dimerization Determinants for Thyroid Hormone Receptor α and Its Interaction with a Nuclear Protein

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The gel retardation assay was used to analyze the role of the thyroid hormone receptor α (TRα) ligand-binding domain (LBD) in controlling receptor interaction with a thyroid hormone responsive element (TRE). While wild type receptor TRα binds to the TRE mainly as monomer, deletion of 85 amino acids from its C-terminus results in a mutant receptor with enhanced DNA binding that forms several slow mobility complexes as revealed by gel retardation assay. Receptor deletion mutants that lack most of the LBD show significantly elevated DNA binding and are still able to bind to DNA as two complexes. Thus, the C-terminal end of TRα appears to interfere with the dimerization/oligomerization function and DNA binding of TRα. All C-terminal deletion mutants have lost their T_3-responsive activator function, but some show constitutive activity. Nuclear factor from several cell lines, including CV-1, F9, and GC cells, interacts with TRα receptor to form a larger molecular weight complex as determined by gel retardation assay. This factor could not be detected in HeLatk$^-$ cells, where TRα does not activate a TRE-containing reporter gene. The nuclear factor is heat sensitive and does not bind to TRE itself but can interact with TRα in the absence of DNA. Deletion analysis demonstrates that the leucine zipper-like sequence located in the LBD of TRα is involved in this interaction. Together, our data suggest that TRα contains a dimerization function outside the LBD which is inhibited by the carboxy-terminal region, while the leucine zipper-like sequence in the LBD is required for interaction with a nuclear factor. (Molecular Endocrinology 5: 1909–1920, 1991)

INTRODUCTION

The thyroid hormone T_3 regulates cellular growth, development, and metabolism predominantly through binding to specific nuclear receptors that modulate the transcription of responsive genes. These thyroid hormone receptors (TRs) belong to a large family of ligand-activated nuclear transcription factors that includes the steroid hormone, retinoic acid, and vitamin D_3 receptors (1–4). The members of this superfamily have a highly conserved DNA binding domain and a less conserved carboxy-terminal ligand binding domain (LBD) (reviewed in Refs. 5, 6). According to the DNA recognition sequences, the superfamily can be divided into subfamilies: the glucocorticoid (GR), progesterone (PR), mineralocorticoid, and androgen receptors bind to a similar DNA binding site (reviewed in 7), while the estrogen receptor (ER) and retinoic acid receptor (RAR) are able to interact functionally with T_3-responsive elements (TREs) (8–10). The thyroid hormone receptors are encoded by two separate genes, α and β (2, 11), both of which give rise to multiple receptor subtypes through alternative splicing events (3, 12–17). Binding of TRα and TRβ to TREs occurs in the absence of ligand (9, 18–19). This is of functional significance since, in the absence of T_3, TRα and TRβ are able to repress the activity of RAR (9, 20) and ER (10) from a common response element, presumably due to their high affinity for the response element. Thus, in the presence of T_3, TRs are transcriptional activators of responsive genes, while in the absence of T_3, they can function as negative regulators or transcriptional silencers (19, 21). The DNA binding activity of TRs is therefore likely to be essential for the bifunctional roles of TRs.

Recent results indicate that the DNA binding activity of several nuclear receptors including ER, RAR, and TR is not solely determined by their DNA binding domain but can be largely influenced by their LBD (19, 22). This region encompasses the carboxyl terminal half of the receptor and is responsible for multiple functions of the receptors, as shown by mutant and variant analyses. The v-erbA protein, that contains a nine-amino acid deletion and several amino acid exchanges in the LBD lacks ligand binding and *trans*-activation activity but has maintained repressor and DNA binding functions (18, 23, 24). The TRα isoforms that contain an altered carboxy-terminus as a result of alternative

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splicing events also lack ligand binding (13, 25) but in addition show largely reduced DNA binding activity (26). A mutation in the LBD of TRβ causes thyroid hormone resistance syndrome (27), while a deletion analysis of TRβ showed that the ligand binding/carboxy-terminal region is also involved in receptor oligomerization and modulates DNA binding (19). Here we report that although TRα contains a dimerization function outside the LBD and a second dimerization/oligomerization domain is present in the LBD, the LBD represses DNA binding and dimerization/oligomerization functions in the full-length receptor. In addition, we report that the LBD of TRα mediates the interaction of TRα with a cell type-specific nuclear protein that enhances DNA binding and may be essential for transcriptional activation.

RESULTS

Complex Role of the TRα Ligand Binding Domain in Controlling DNA Binding

To study DNA binding of TRα and how it is affected by its ligand binding domain, a number of TRα mutants were generated by sequential deletion of its C-terminal end (Fig. 1A). The gel retardation assay (9, 19) was used to compare the binding of equal amounts of *in vitro* translated TRα and TRα mutant proteins to a palindromic TRE (28). Figure 1B shows the binding pattern of mutant and wild type receptor proteins. TRα forms one major complex with the TRE under the conditions used, while a second, slower migrating complex (that overlaps with the nonspecific band) is seen at elevated TRα concentration (data not shown). Deletion of 8 amino acids from its C-terminus does not change the binding pattern of this receptor (ΔTR1). However, a deletion of additional 85 amino acids results in a mutant (ΔTR2) which binds to the TRE with enhanced intensity and forms additional complexes. These complexes migrated more slowly than the complex formed by wild type TRα, suggesting that this mutant may more efficiently dimerize and oligomerize. Deletion of 112 additional amino acids from the C-terminus of ΔTR2 significantly increased DNA binding of this mutant (ΔTR3). Although deleted for most of the ligand binding domain, the ΔTR3 protein still forms two major complexes with the TRE which are more prominent than the complexes formed by wild type TRα and the other mutants. ΔTR4, which lacks the entire LBD and most of the hinge region, also retains the ability to bind DNA and forms two complexes. However, a mutant, deleted up to amino acid 120 (ΔTR5), that lacks one amino acid from the DNA-binding domain, has lost all measurable DNA binding activity (Fig. 1B).

To demonstrate the specificity of the binding of these proteins to the TRE, an antigenic coding sequence (Flag) (29) was added to their 5′-ends. This Flag sequence, when connected to TRs, is recognized by a specific anti-Flag monoclonal antibody (26). When TRα and ΔTR3 were incubated with anti-Flag antibody and DNA, the protein-DNA complexes were upshifted to a slower migrating position, indicating that the complexes observed contained indeed the mutated proteins (Fig. 1C). Similar results were obtained with ΔTR3 and ΔTR4. The DNA binding activity of TRα and the mutants was not significantly affected by the presence of T_3. Thus this study strongly suggests that the TRα LBD influences (positively or negatively) DNA binding and oligomerization activity of the receptor.

The efficient oligomerization of the TRα2 mutant also could be demonstrated with a TRE dimer (Fig. 2A). In this case, wild type TRα formed three major complexes with the TRE dimer, which were not affected by the addition of T_3. ΔTR1 and ΔTR3 showed a similar binding pattern. ΔTR3 binds to DNA with highest intensity. In addition, it formed four major complexes, of which the highest molecular weight complex migrated at the same position as the highest molecular weight complex formed by TRα, although ΔTR3 is much smaller than TRα. This suggests that ΔTR3 can dimerize or oligomerize more efficiently than TRα. More surprisingly, when ΔTR2 was analyzed, it formed at least five complexes with the TRE dimer, three of which migrated much more slowly than the largest TRα complex (although this mutant protein is smaller than TRα). Thus, ΔTR2 displays a clearly enhanced dimerization and oligomerization activity. Taken together, these data suggest to us that the C-terminal region of TRα interferes with the dimerization function of TRα.

To determine whether such a highly dimerizing protein could efficiently interact with other receptors, ΔTR2 protein was mixed with TRα, or both proteins were cotranslated. In neither case could ΔTR2 dimerize with TRα to form a new complex (Fig. 2B). When TRα2 was mixed or cotranslated with ΔTR3, no new complexes were observed either. Negative results were also obtained when TRβ or RAR were used (data not shown). This indicates that although the dimerization and oligomerization functions of ΔTR2 are highly exposed, this mutant protein only, or at least primarily, oligomerizes with itself but not with other receptors.

TRα Binds to the TRE Mainly as a Monomer

TRα and the mutant proteins bind to the TRE in various patterns and complexes. To delineate the nature of these complexes, two additional oligonucleotides were designed and analyzed for their interaction with these receptor proteins. One sequence used was a TRE half-site (TRE/half); the other was an inverted perfect palindromic sequence, containing two TRE half-sites in the opposite orientation (TRE/op; Fig. 3A). Because of the palindromic nature of the TRE, TRs have been assumed to bind the TRE as a dimer. If the major complex (indicated in Fig. 3B) we observed here for TRα with the TRE however represents a TRα monomer, we expect a similar complex with the TRE/half and the TRE/op. As shown in Fig. 3B, TRα can efficiently bind to the TRE/half. It also binds to TRE/op forming one major complex. Thus the major TRα complex we ob-

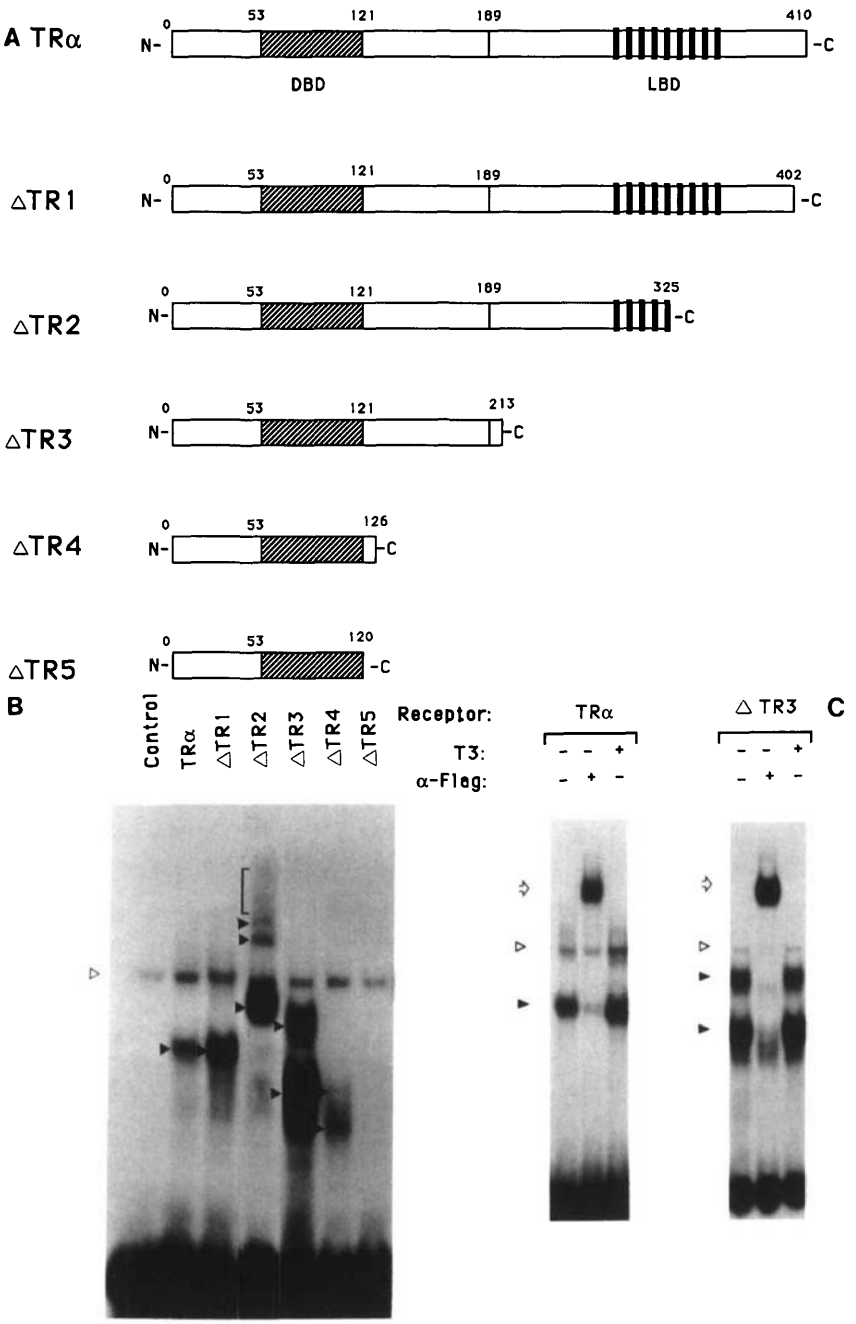

Fig. 1. DNA Binding Characteristics of TRα Deletion Mutants

A, Schematic representation of the TRα deletion mutants. *Numbers* above the bars indicate the amino acid positions. The DNA-binding domain (DBD) and the LBD are shown. A leucine zipper-like motif (41, 42) in the LBD containing 9-heptad repeats is indicated by the *black bars*. B, DNA binding of TRα deletion mutants. TRα deletion mutants were cloned into pBluescript, transcribed, and translated as described in *Materials and Methods*. Equal amounts of the *in vitro* synthesized TRα and mutant proteins were analyzed for their TRE binding activity by gel retardation assay. Control shows binding of unprogrammed reticulocyte lysate factor(s) to the TRE. *Open triangles* indicate the nonspecific TRE complex from unprogrammed reticulocyte lysate. *Solid triangles* indicate the specific complexes of TRα and the mutant proteins with TRE. *Brackets* indicate the minor high molecular weight TRE complexes of ΔTR2. C, Specific binding of TRα and mutant receptor. Binding of the TRα and ΔTR3 was analyzed by gel retardation assay in the presence or absence of 10^{-7} M T_3. To assure the specificity of binding of the *in vitro* synthesized proteins, a peptide (Flag)-encoding sequence was cloned 5′ to the TRα and ΔTR3 cDNAs as described in *Materials and Methods*. Anti-Flag antibody (α-Flag) generated against the peptide was incubated with the receptor protein before the DNA binding reaction. The specific receptor-DNA complexes (indicated by the *solid triangles*) but not the nonspecific protein-DNA complexes (indicated by the *open triangles*) were upshifted by the anti-Flag antibody to form higher molecular weight complexes indicated by the *open arrows*.

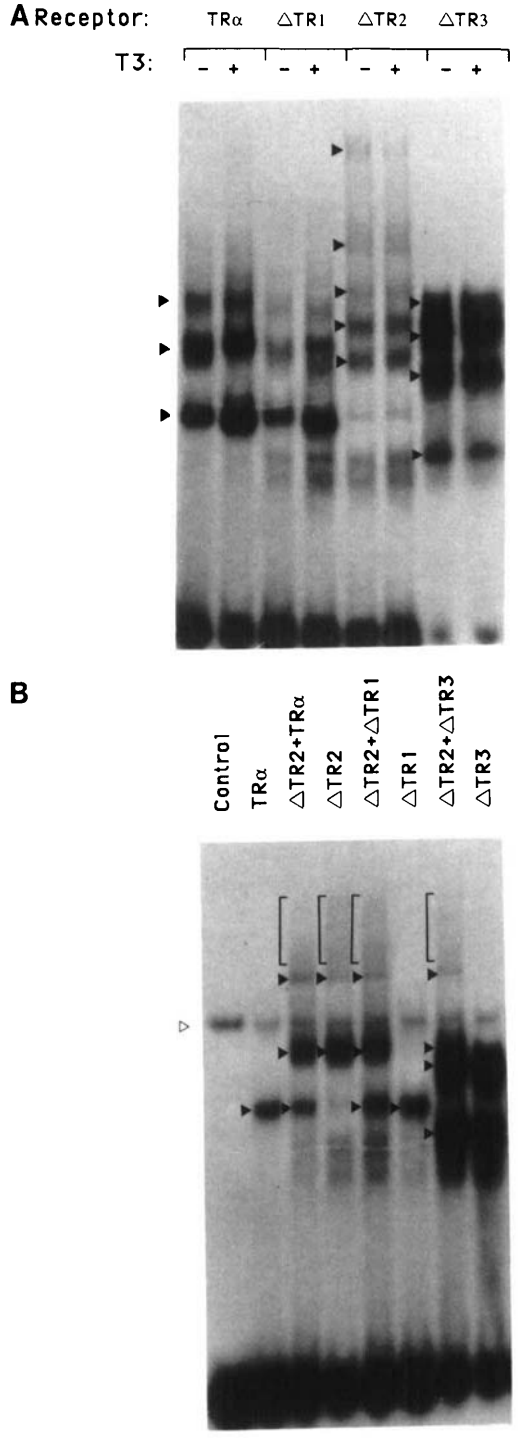

Fig. 2. DNA Binding Characteristics of ΔTR2

A, DNA binding of TRα and the mutant protein to TRE dimer. Equal amounts of *in vitro* synthesized TRα and the mutant proteins were analyzed for binding to the TRE dimer using the gel retardation assay in the presence or in the absence of T_3 (10^{-7} M). *Solid triangles* indicate the specific receptor-DNA complexes. Several faster migrating complexes not marked by triangles might represent the binding of truncated protein products that result from the *in vitro* translation process. B, ΔTR2 interaction with TRα and mutant receptors. To analyze whether the highly dimerizing/oligomerizing mutant receptor ΔTR2 can interact with TRα or other deletion mutants, *in vitro* synthesized ΔTR2 was incubated with equal amounts of *in vitro* synthesized TRα, ΔTR1, and ΔTR3 and subjected to the gel retardation assay using ^{32}P-labeled TRE oligonucleotide. Control represents the binding of the unprogrammed reticulocyte lysate to TRE. The complex formed with unprogrammed lysate is indicated by the *open triangle*. *Solid triangles* indicate the specific receptor-DNA complexes. *Brackets* indicate the minor higher molecular weight complexes formed by ΔTR2 with the TRE.

serve with the palindromic TRE, the TRE/half and TRE/op, most likely represents monomer TRα-DNA complexes, consistent with recent data reported by Forman and Samuels (30). That TRα binds mainly as a monomer is also supported by our studies on the mutants with TRE/half and TRE/op. TRα1 forms one prominent complex with the TRE, which also can be observed when TRE/half and TRE/op were used, indicating that this mutant also binds the TRE as a monomer. In contrast, ΔTR2 forms several higher molecular weight complexes with the TRE that could not be observed when TRE/half was used. Comparing the positions of the fastest migrating ΔTR2-TRE complexes with that of TRα and the TRE, we believe that the higher ΔTR2-TRE complexes represent dimer/oligomer binding. Thus, ΔTR2 binds to the TRE mainly as dimer and oligomer. Additional and strong support for the above conclusion comes from the study of ΔTR3. This mutant forms two major complexes with the TRE. However when TRE/half was used, the higher molecular weight complex disappeared, while the binding of the low molecular weight complex was retained. ΔTR3 can still form two complexes with TRE/op, although the higher molecular weight complex is slightly weaker than that observed with the TRE. Thus, the two complexes observed for ΔTR3 represent binding of monomer and dimer mutant protein.

Interaction of TRα with a Nuclear Protein

It has been shown that nuclear protein extracts can enhance the DNA binding activity of several members of the nuclear receptor family (31–36). Recent data suggest that nuclear protein(s) also can enhance TR DNA binding activity (37, 38, for recent review see 39). However, in several of these studies the ABCD assay was used where biotinylated DNA probes (and their interacting proteins) are precipitated with antibody against the DNA probe (28). In this assay system, the nature of the interaction or the complexes formed cannot be resolved. To characterize how nuclear protein(s) affect TRα binding, cell extracts from CV-1, HeLatk$^-$, F9, and GC cells were prepared and analyzed for their effect on the TRα-DNA interaction (Fig. 4A). When cell extract from CV-1 cells was mixed with *in vitro* synthesized TRα, we observed a significant change of the TRα binding pattern. The typical TRα-TRE complex was reduced when extract was added. The disappearance of TRα-TRE complex was dependent on the amount of extract added, and was accompanied by the appearance of a new and higher molecular weight complex which became more prominent with increasing amounts of extract added. Thus, the cell extract appears to contain a factor which interacts with the TRα-

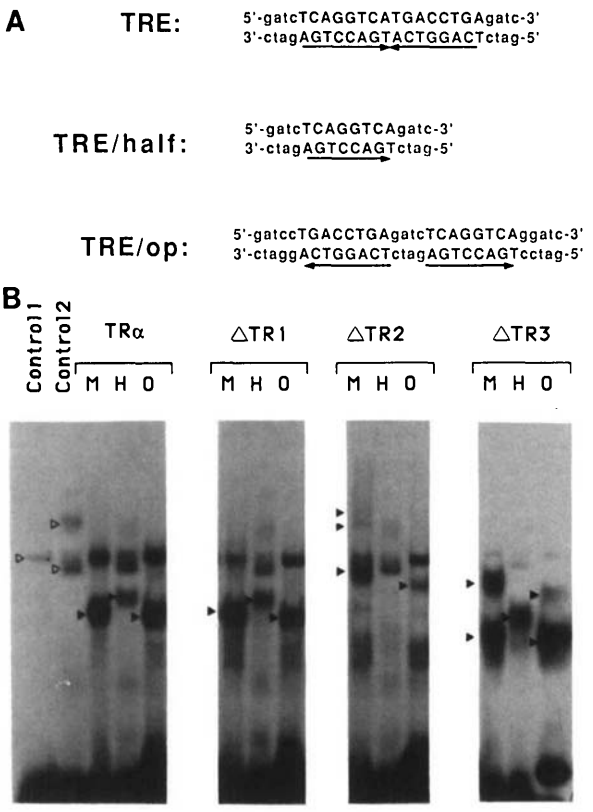

Fig. 3. TRα Binds the TRE Mainly as a Monomer

A, Sequences of three oligonucleotides were used for the gel retardation assay. TRE is the perfect palindromic T_3 response element. TRE/half is the half-site of the TRE. TRE/op is an oligonucleotide consisting of two TRE half-sites in the opposite orientation separated by 4 bp. B, DNA binding of TRα and TRα deletion mutants. *In vitro* synthesized TRα and TRα mutants were analyzed for their DNA binding characteristics to the palindromic TRE (M), TRE/half (H), and TRE/op (O) by gel retardation assay. Control 1 and control 2 represent the binding of unprogrammed reticulocyte lysate to TRE/op and TRE/half, respectively. *Open triangles* indicate the nonspecific lysate complexes. *Solid triangles* indicate the specific receptor complexes. Binding data of ΔTR2 represent a shorter exposure to obtain a clear picture. (Note: We have consistently observed that the unprogrammed reticulocyte lysate forms two complexes with TRE/half, of which the slower migrating complex was diminished or inhibited when the reticulocyte lysate had been used for translation. This is also seen in Fig. 5A).

TRE complex to form a higher molecular weight complex. The effect of cell extract is specific to TRα since it was not found when the same amount of cell extract was added to the unprogrammed reticulocyte lysate. When cell extracts were prepared from F9 and GC cells, similar upshifts were observed (Fig. 4A). In addition, endogenous TRE binding activity was observed when high amounts of extracts from these cells were used. The endogeneous TRE binding activity from F9 and GC cells however migrated slower than the upshifted complex. When cell extract was prepared from HeLatk$^-$, no upshift was seen (Fig. 4B). Thus, the factor which can interact with TRα is cell type specific or at least not present in all cell lines.

To confirm that the upshifted complexes resulted from the interaction between TRα and the nuclear factor, anti-Flag antibody was used. Incubation of this antibody with TRα and CV-1 cell extract resulted in upshifts of both the TRα-TRE complex and the nuclear factor complex. Because of the limited resolution of the polyacrylamide gel in the high molecular weight range, antibody upshifted TRα-TRE-nuclear factor complex migrated only slightly higher than the anti-Flag-TRα-TRE complex but was more smeared than the latter complex (Compare lane 3 and lane 5 or 7 of Fig. 4C, and lane 3 and lane 7 of Fig. 4D). Similar results were obtained when cell extract from F9 and GC cells were analyzed (Fig. 4, C and D). However, due to the strong endogenous TRE binding activity in GC cell extracts, the complexes observed in these studies were less clearly resolved. When HeLatk$^-$ cell extract was used, we again could not clearly observe a new upshifted complex, and the antibody-upshifted complex in the presence of HeLatk$^-$ cell extract was different from that in the presence of other cell extracts and was indistinguishable from the complex observed in the absence of HeLatk$^-$ cell extract (Fig. 4D, compare lanes 3 and 5). These data therefore suggest that CV-1, F9, and GC cells contain a nuclear factor that can specifically interact with TRα to form a higher molecular weight complex. This factor is not present or is only present in trace amounts in HeLatk$^-$ cells.

To investigate whether the interaction of TRα with the nuclear protein(s) was DNA sequence dependent, we analyzed the effect of CV-1 cell extract on TRα DNA interaction with different oligonucleotides. When cell extract was incubated with TRα and TRE/op, the TRα-TRE/op complex was upshifted to a position similar to that seen with the TRE but (Fig. 5A, *arrow*), in addition, a new and faster migrating complex was also observed. This new complex is specific since it was not found with CV-1 cell extract alone. Thus the TRE/op allows formation of different complexes between TRα and nuclear factor. When TRE/half was used, we found that CV-1 cell extract could also decrease the TRα-TRE/half complex as observed for the TRα-TRE complex; however, we could not detect an upshifted complex (Fig. 5A). Since the DNA sequence used contained only a TRα monomer binding site, we assume that the complex formed between TRα and the nuclear protein does not bind TRE/half efficiently. The data also suggest to us that the interaction between TRα and the nuclear factor identified here is not restricted to one TRE sequence and probably occurs in solution. Heating CV-1 cell extract for 10 min at 60 C before incubation with TRα and TRE abolished the interaction of this factor with TRα (Fig. 5B), suggesting that this factor is a protein or proteins.

Absence of Nuclear Factor Correlates with Lack of TRα Activity in HeLatk$^-$ Cells

To examine whether the absence of nuclear factor in HeLatk$^-$ cell extract correlated with altered TRα activity

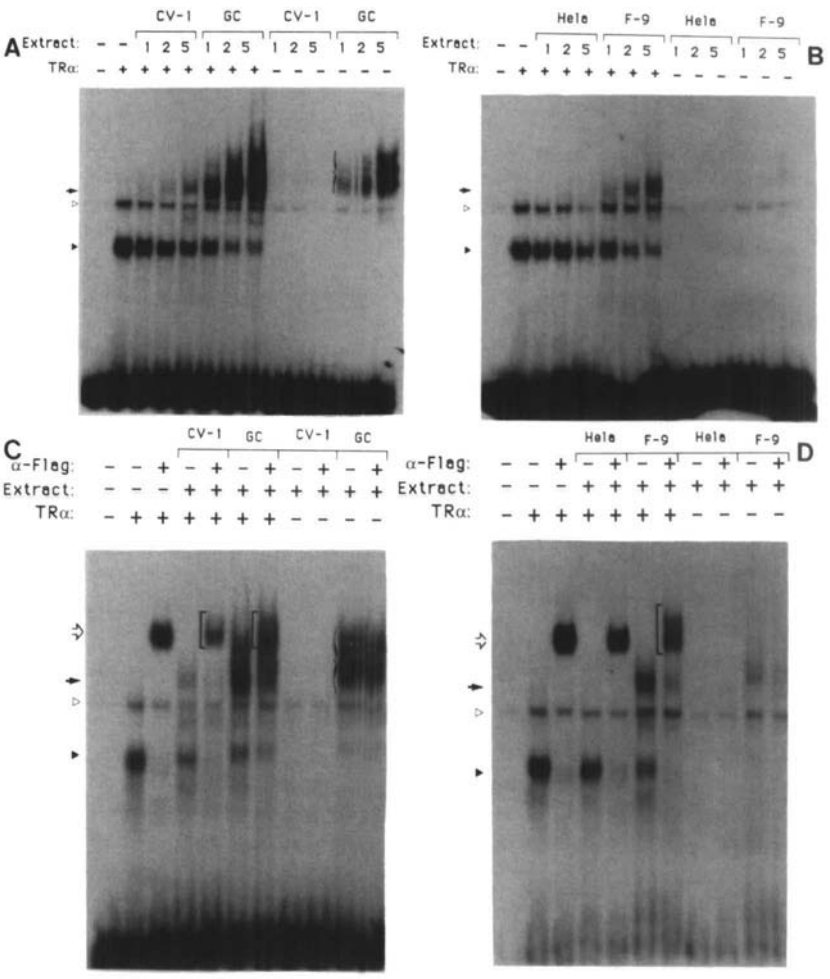

Fig. 4. Interaction of TRα with Nuclear Factor

A and B, Interaction of TRα with nuclear protein from CV-1, GC, HeLatk⁻, and F-9 cells. Cell extracts were prepared from CV-1, GC, HeLatk⁻, and F-9 cells as described in *Materials and Methods*. Indicated amounts of cell extract proteins (micrograms) were incubated with the *in vitro* synthesized TRα receptor protein (+) or equal amounts of unprogrammed reticulocyte lysate (−) at room temperature for 15 min before the DNA binding reaction. The mixtures were analyzed by gel retardation using ^{32}P-labeled TRE oligonucleotide. *Open triangles* indicate the binding of the unprogrammed reticulocyte lysate. *Solid triangles* indicate the specific TRα-TRE complexes, and the *arrows* indicate the complex formed between TRα and the nuclear protein. The *brace* (A) indicates binding of endogenous T_3 receptors from GC cell extract. C and D, Specificity of TRα-nuclear factor interaction. To demonstrate the specificity of the upshifted TRα complex by the nuclear protein, anti-Flag antibody (α-Flag) was incubated with TRα or a premixture of TRα and the cell extract (5 μg) from CV-1, GC, HeLatk⁻, and F-9 cells. After incubation for 15 min, the mixtures were analyzed by gel retardation assay using ^{32}P-labeled TRE oligonucleotide. *Open triangles* indicate the nonspecific binding of the unprogrammed reticulocyte lysate. *Solid triangles* indicate the specific TRα-DNA complex. *Solid arrows* indicate the complex of TRα upshifted by the nuclear protein. *Open arrows* indicate the complex of TRα upshifted by the anti-Flag antibody. *Brackets* indicate the TRα-nuclear protein complex upshifted by the anti-Flag antibody. The *brace* (C) indicates the binding of the endogenous T_3 receptors from GC cell extracts.

in these cells, we compared the transcriptional activation activity of transiently expressed TRα with that of other nuclear receptors in HeLatk⁻ cells. When a TRα expression vector was cotransfected with a TRE_2-tk-chloramphenicol aminotransferase (CAT) reporter, no reporter activity could be observed in the presence or absence of T_3 in HeLatk⁻ cells (Fig. 6). The inactivity of TRα in the HeLatk⁻ cells is not due to the reporter used, since it functions well in CV-1 cells, and in addition, ERE-tk-CAT, in which the ERE was cloned into the same position of the reporter vector as the TRE, functions well in HeLatk⁻ cells. Similar positive results were obtained with a GR expression vector/reporter plasmid cotransfection (data not shown). Thus, the inactivity of TRα in HeLatk⁻ cells is most likely due to the absence of a factor(s) which is required for TRα function, although the presence of a factor(s) which represses TRα activity also cannot be excluded.

Leucine Zipper-Like Domain Participates in the Interaction with Nuclear Protein

To delineate which domain of TRα is responsible for the interaction with the nuclear factor identified here,

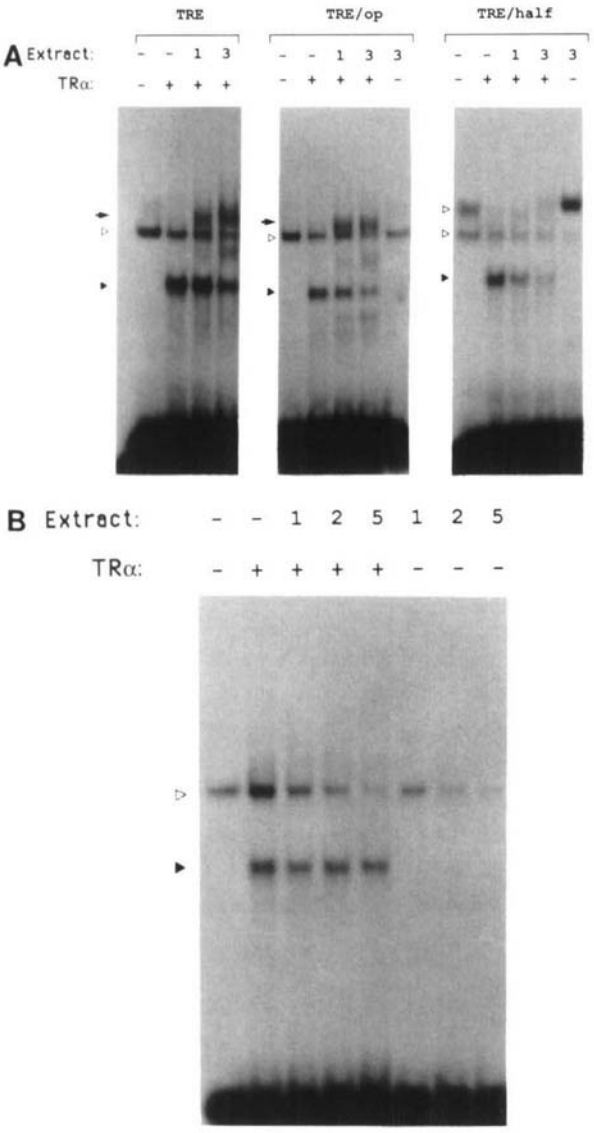

Fig. 5. Characteristics of the Nuclear Factor-TRα Interactions

A, Interaction of the nuclear factor and TRα is DNA independent. Indicated amounts of protein extract (micrograms) from CV-1 cells were incubated with *in vitro* synthesized TRα (+) or the equal amount of the unprogrammed reticulocyte lysate (−) at room temperature for 15 min. The mixtures were then analyzed by gel retardation using ^{32}P-labeled TRE, TRE/op, and TRE/half oligonucleotides. *Open triangles* indicate nonspecific binding of the unprogrammed reticulocyte lysate. *Solid triangles* indicate the specific binding of TRα. *Arrows* indicate the complex of TRα upshifted by the nuclear protein. B, The nuclear factor is heat sensitive. The indicated amounts of the protein extract (micrograms) from CV-1 cells were heated at 60 C for 10 min before incubation with *in vitro* synthesized TRα protein (+) or the equal amount of unprogrammed reticulocyte lysate (−). The mixtures were then analyzed by gel retardation assay using ^{32}P-labeled TRE oligonucleotide.

the TRα deletion mutants were analyzed for their ability to interact with protein from CV-1 cell extract. CV-1 cell extract was able to upshift the complexes formed by TRα and ΔTR1 with TRE to a similar position (Fig. 7), indicating that the C-terminal eight amino acids of the TRα C-terminus are not essential for the interaction. When ΔTR2 was analyzed we were unable to distinguish an upshifted complex in the presence of CV-1 cell extract, but we observed repeatedly inhibition of formation of the high molecular weight complexes seen with ΔTR2 alone (Fig. 7). This result suggests that the nuclear protein may still interact with ΔTR2 and interferes with the formation of the higher ΔTR2 oligomers. Thus ΔTR2 appears to retain the ability to interact with the nuclear factor. When ΔTR3 (deleted for most of the ligand binding domain) was analyzed, we found that addition of CV-1 cell extract neither decreased ΔTR3 binding to the TRE nor resulted in the appearance of a new complex. The CV-1 cell extract similarly did not affect DNA binding of ΔTR4 either. Thus, a domain responsible for the interaction with the nuclear protein is located between amino acid 213 and amino acid 325, a region that contains a leucine zipper-type motif (Fig. 1A), previously suggested to mediate receptor dimerization (40, 41).

Transcriptional Activation Activities of the TRα Mutants

To assess the transcriptional activation activities of the mutant receptors, expression vectors of the mutant proteins were cotransfected together with a TRE-tk-CAT reporter into CV-1 cells (Fig. 8). While cotransfection of wild type TRα with TRE-tk CAT resulted in a ligand-dependent reporter activity, mutant receptors showed little T_3-dependent activity. However, ΔTRα2, ΔTRα3, and ΔTRα4 exhibited a significant constitutive activity. The constitutive activity increased when higher amounts of mutant receptor expression vectors were used. The constitutive activity appears to require DNA binding, since ΔTRα5, a mutant that lacks DNA binding activity, did not exhibit the constitutive activity. Thus, deletion of the TRα C-terminal sequences impairs receptor ligand-induced transcriptional activity but exposes a constitutive activation activity, observed so far mostly for GR (42, 43).

It has been shown that v-erbA inhibits TRα activity due to its strong DNA binding. To investigate whether the receptor mutants that elicited strong DNA binding can also repress wild type receptor activity, TRα was cotransfected with the mutant receptors into CV-1 cells. Surprisingly, none of the mutant proteins showed a significant repressor activity (data not shown). Thus although the TRα mutants bind the TRE, they are inefficient inhibitors of TRα.

DISCUSSION

The ligand-binding carboxy-terminal domain of nuclear receptors encompasses multiple functions. It determines the ligand specificity and mediates receptor dimerization and transactivation functions of the receptor.

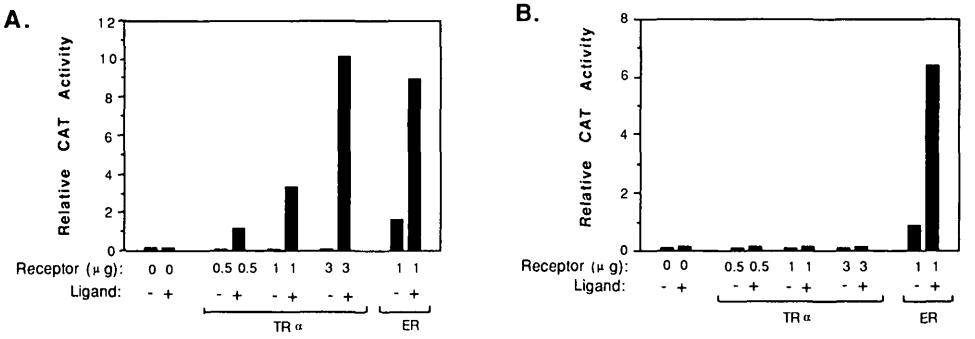

Fig. 6. Transcriptional Activity of TRα in HeLatk$^-$ and CV-1 Cells

CV-1 (A) and HeLatk$^-$ (B) cells were transiently transfected or cotransfected as described in *Materials and Methods* with 2.0 μg TRE_2-tk-CAT reporter plasmid and the indicated amounts of the receptor expression plasmids. The reporter cotransfected with ER is ERE-tk-CAT. Cells were grown in the absence (−) or presence (+) of ligands ($T_3 = 10^{-7}$ M, $E_2 = 10^{-7}$ M). The data represent means of two separate experiments.

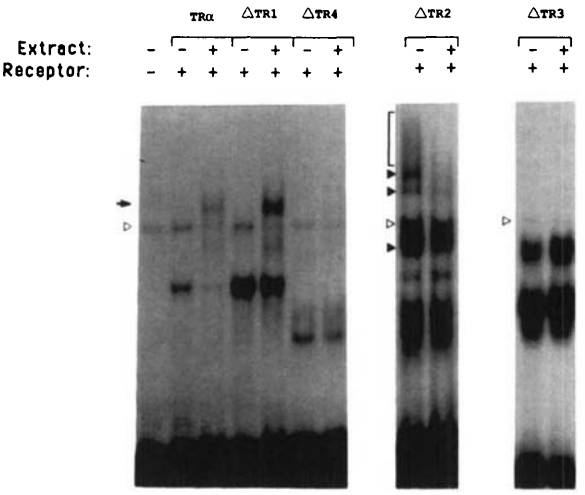

Fig. 7. LBD of TRα Is Responsible for the Interaction with Nuclear Factor

Five micrograms of protein extract from CV-1 cells were incubated with TRα and TRα deletion mutants before the DNA binding reaction. The mixtures were then analyzed by gel retardation assay using ^{32}P-labeled TRE oligonucleotide. *Open triangles* indicate the binding of unprogrammed reticulocyte lysate. The *arrow* indicates the complex of TRα or ΔTR1 upshifted by the nuclear protein. *Solid triangles* and the *bracket* indicate the complexes of ΔTR2 which are inhibited by the nuclear protein.

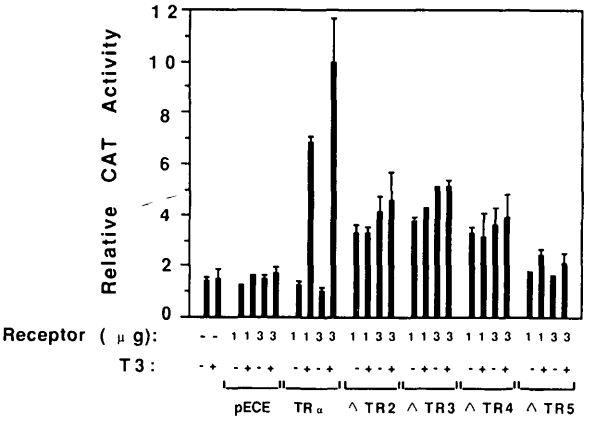

Fig. 8. Constitutive Transcriptional Activity of TRα Deletion Mutants

CV-1 cells were transiently cotransfected as described in *Materials and Methods* with 2.0 μg TRE_2-tk-CAT reporter plasmid and the indicated amounts of the empty vector, TRα and TRα mutant expression plasmids. Cells were grown in the absence or presence of 10^{-7} M T_3.

Here we report that the LBD of TRα has a prominent role in regulating its DNA-binding activity, dimerization/oligomerization, constitutive activity, and interaction with a nuclear factor. Wild type TRα binds to the palindromic TRE sequence mainly as a monomer. However, deletion of its C-terminal region exposes dimerization/oligomerization functions of the receptor. Deletion of 85 amino acids yields a receptor which binds the TRE mainly as dimer and oligomers. The efficient dimerization/oligomerization of this mutant may be due to the exposure of the hydrophobic leucine zipper-like sequence in the ligand binding domain (40, 41), a sequence which consists of several heptad repeats (see Fig. 1A). Thus one can propose that the C-terminal end of TRα inhibits or interferes with wild type receptor dimerization on the perfect palindromic TRE, *i.e.* the wild type TRα binds to TRE in such a conformation that it cannot effectively dimerize itself. (However, wild type TRα does interact with nuclear factor to form a larger complex as discussed below). The C-terminal region of TRα may interfere with TRα dimerization by either directly inhibiting the dimerization motif or forming such a conformation that the dimerization domain (leucine zipper-like region) is not exposed. This is consistent with a previous study in which the extreme C-terminal end of TRβ was shown to inhibit the oligomerization of TRβ in the presence of T_3 (19).

Surprisingly, ΔTR3, a mutant deleted for almost the entire ligand binding domain, still forms two complexes with the TRE and the TRE/op, which we interpret as monomer and dimer complexes. The binding of ΔTR3 dimer may be due to the binding of dimerized receptor

subunits or the independent binding of receptor subunits. However, the ΔTR3 mutant no longer contains the leucine zipper-like sequences, and we therefore expect that this mutant dimerizes through another region. This dimerization function is likely to be contained in the DNA-binding domain since two complexes were also observed with ΔTR4. In addition, three-dimensional structure analyses on the GR and ER DNA-binding domains have demonstrated a dimerization function for this domain in both receptors (44, 45). Thus, while ΔTR4 contains only the dimerization function of the DNA binding region, ΔTR2 contains in addition the leucine zipper-type dimerization/oligomerization function and therefore formed oligomers efficiently. The carboxy-terminal region that interferes with TRα dimerization also represses a ligand-independent transcriptional activation function of TRα. Such ligand-independent transcriptional activation activities have been reported for other steroid hormone receptors (42, 43, 46) and in one case have been subscribed to the DNA-binding domain alone (47).

We have demonstrated that protein from some cell lines, including CV-1, F9, and GC cells, interacts with TRα on the palindromic TRE. The TRα-TRE complex is upshifted by protein from these cells to form a slower migrating complex. The complex formed in the presence of the nuclear extracts is TRα dependent, as this new complex does not appear when the nuclear factor is incubated with the unprogrammed reticulocyte lysate. Moreover, the complex can be further shifted by an anti-TRα-specific antibody. Experiments with cell extracts from different cell lines indicate that the nuclear protein is present in CV-1, F9, and GC cells, and it is not present in HeLatk$^-$ cells. The nature of this factor is not known, although its heat sensitivity suggests that it is a protein. The nuclear factor upshifted the TRα-TRE complex to a position higher than the TRα dimer. These results suggest that the mol wt of the factor we identified here is about 55K. Based on its molecular weight and the suggested interaction between nuclear receptors (26, 39, 40, 48), the possibility that this factor may be a member of steroid thyroid receptor family is under investigation.

The function of this TRα-associating factor is not yet clear. Besides its ability to stabilize the TRα-TRE interaction, it could directly mediate the transcriptional activity of TRα by serving as a link between TRα and the transcriptional machinery. This hypothesis is supported by our observation that TRα does not activate the palindromic TRE in HeLatk$^-$ cells, which also lack any measurable activity of this protein. Interestingly, the related ER and GR show high activity in HeLatk$^-$ cells, supporting our hypothesis that this protein is TR specific. It will be interesting to investigate whether this protein interacts with RARs, which are members of TR/RAR subfamily. The ER binds to a single palindromic ERE very efficiently *in vitro* and forms several complexes (22). Importantly, TRα-ER hybrid receptors that contain the ER LBD also bind efficiently to the TRE (19, 22). These data therefore suggest to us that the TRα LBD may inhibit TRα DNA binding, dimerization/oligomerization, and transcriptional activation activity on the TRE. Interaction with the nuclear factor would counteract the inhibitory function of the LBD and allow efficient DNA binding, dimerization, and transcriptional activation in the presence of T_3. In contrast, ER appears to bind a perfect palindromic ERE and dimerize efficiently in the absence of a nuclear factor (22).

Deletion analysis suggested that amino acids 213–325 are critical for TRα interaction with the nuclear factor, since the absence of this region abolished it, as observed by Lazar and Berrodin (35). Amino acids 213–325 encompass a stretch of hydrophobic amino acids that could represent such a domain. This domain consists of several heptad repeats of hydrophobic amino acids (40, 41) and enables receptor dimerization/oligomerization as revealed by our deletion analysis. Our data indicate that, in addition, interaction of TRα with the nuclear factor may be mediated through these hydrophobic sequences. The interaction of TRα with the nuclear factor also could be detected when the TRE/op was used, indicating that the complex containing TRα and the nuclear protein is able to bind to several DNA sequences. However, when TRE/half was used, we only observed the disappearance of the TRα-TRE complex, and the TRα-nuclear protein-DNA complex was not detected. This suggests that TRα-nuclear factor complex does not interact significantly with TRE/half and, importantly, that TRα and the nuclear factor can interact in solution. Thus, the nuclear protein does not bind the TRE alone effectively, but mediates high affinity binding when it is complexed with TRα. This could occur through the direct formation of a TRα-nuclear protein heterodimer. Our finding that several deletion mutants showed strong DNA binding *in vitro* but little repressor activity may be due in part to the constitutive activity of these mutants but also may suggest that complexing of the receptor with the nuclear protein is required for optimal repressor activity of TRα (10, 19).

Taken together, we have shown here that TRα may contain two dimerization domains, one most likely located in the DNA-binding domain, while the other is part of the LBD. This second dimerization/oligomerization domain—probably the previously described leucine zipper-type motif—is also important for interaction with a nuclear factor which in fact may be its major function. While the analysis of the characteristics of this protein awaits molecular cloning of its gene, it appears to have a fundamental role in controlling TRα function. Finally, the very carboxy-terminal region of TRα appears to inhibit TRα dimerization activities as well as DNA binding. This region is replaced through an extended carboxy-terminal region in TRα2, a protein that reveals very little DNA-binding activity (26). It will be of interest to investigate whether the carboxy-terminal regions of TRα isoforms and related receptors directly interact with the DNA-binding domain and the leucine zipper region of the receptor and thereby inhibit their activities.

MATERIALS AND METHODS

Construction of Expression and Reporter Plasmids

The coding sequence of TRα was inserted into the multiple cloning sites of the eukaryotic vector pECE (49) or pBluescript (Stratagene, La Jolla, CA). The construction of these plasmids has been described previously (9). To obtain TRα deletion mutants, existing restriction enzyme sites on TRα were used to digest TRα cDNA. The resulting TRα cDNA fragments were purified and cloned into pECE and pBluescript. ΔTR1, ΔTR2, ΔTR3, ΔTR4, and ΔTR5 were generated by digesting TRα cDNA with *Xho*1 (1530), *Hin*cII (1310), *Stu*1 (964), *Xba*1 (698), and *Bal*1 (685), respectively. Numbers within the brackets indicate the DNA sequence positions of the restriction enzyme sites (4).

The construction of reporter plasmids, TRE-tk-CAT, TRE_2-tk-CAT, and ERE-tk-CAT also has been described previously (19). Briefly, one copy or two copies of the synthetic palindromic TRE sequence (TCAGGTCATGACCTGA) or one copy of the synthetic ERE oligonucleotide (TCAGGTCACTGTGACCTGA) was inserted into the *Bam*HI site of pBL-CAT_2 (50) by using *Bgl*II linkers to generate TRE-tk-CAT, TRE_2-tk-CAT, or ERE-tk-CAT, respectively. The construction of Flag-TRα has been described (26).

Tissue Culture, Transient Transfection, and CAT Assay

CV-1 cells were grown in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum (FCS). HeLatk$^-$ cells were maintained in DME medium with 10% calf serum (CS). CV-1 cells were plated at 1.5×10^6-2.0×10^6 per dish 16–24 h before transfection. A modified calcium phosphate precipitation procedure was used for transient transfection (51) and is described elsewhere (52). In general, 2 μg reporter plasmid, 3 μg β-galactosidase (β-gal) expression vector (pCH 110, Pharmacia, Piscataway, NY), and variable amounts of receptor expression vector were mixed with carrier DNA (Bluescript) to 20 μg total DNA per plate. β-Gal was determined as described (52) with a modification to allow the assay to be run in microtiter plates (53). CAT activity was determined by using [^{3}H]acetyl coenzyme A as substrate (54). To normalize for transfection efficiency, we corrected the CAT activities for β-gal activity. For CAT assays, cells were also seeded in 24-well dishes, using approximately 1/20 of the cells and 1/20 of the DNA mixtures that were used for transfection in large dishes (53).

Preparation of Receptor Proteins

cDNAs for TRα and the deletion mutants cloned into pBluescript (Stratagene) were transcribed by using T7 or T3 RNA polymerases, and the transcripts were translated in the rabbit reticulocyte lysate system (Promega, Madison, WI) as described (19, 52). The relative amount of the translated proteins was determined by separating the [^{35}S]methionine-labeled proteins on sodium dodecyl sulfate-polyacrylamide gels, quantitating the amount of incorporated radioactivity and normalizing it relative to the content of methionine residues in each protein.

Preparation of Specific DNA Fragments

The TRE used in the experiments was a 16-base pair (bp) perfect palindromic TRE (TCAGGTCATGACCTGA) (28). An oligonucleotide flanked by a *Bgl*II adaptor sequence was synthesized (Applied Biosystems DNA Synthesizer, Foster City, CA) and purified by polyacrylamide gel electrophoresis. Oligonucleotides were annealed and were radioactively labeled using the Klenow fragment of DNA polymerase. To obtain TRE dimer, the synthetic TRE monomer was ligated, and double-stranded dimer TRE was purified by polyacrylamide gel electrophoresis. In these dimers, the two TREs are separated by 4 bp of the *Bgl*II restriction site. TRE/op is an oligonucleotide consisting of two TRE half-sites with a 4-bp spacer (GATCCTGACCTGAGATCTCAGGTCAG). TRE/half is an oligonucleotide consisting of one TRE half-site (GATCTCAGGTCA). Both oligonucleotides are synthesized with a *Bgl*II adaptor sequence. Labeled TRE probes were purified by gel electrophoresis and used for the gel retardation assay.

Preparation of Cell Extracts

F-9 cells were maintained in α-Medium supplemented with 10% FCS, and GC cells were grown in DME medium supplemented with 5% horse serum and 2.5% FCS. Cell extracts were prepared from HeLatk$^-$, CV-1, F-9, and GC cells in a buffer containing 20 mM HEPES, pH 7.9, 0.4 M KCl, 2 mM dithiothreitol, and 20% glycerol as described (9).

Gel Retardation Assay

In vitro translated receptor protein (1–5 μl) was incubated with the ^{32}P-labeled oligonucleotides in a 20-μl reaction mixture containing 10 mM HEPES buffer, pH 7.9, 50 mM KCl, 1 mM dithiothreitol, 2.5 mM MgCl, 10% glycerol, and 1 μg poly(dI-dC) at 25 C for 20 min. The reaction mixture was then loaded on a 5% nondenaturing polyacrylamide gel containing 0.5 × TBE (1 × TBE = 0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA). To analyze the effect of the nuclear proteins on receptor DNA binding activity, the cell extracts were preincubated with receptor protein at room temperature for 15 min before performing the DNA binding assay. When anti-Flag antibody was used, 0.5 μl of the antiserum was incubated with the receptor protein or the preincubated receptor-nuclear protein mixture at room temperature for 30 min before performing the DNA binding assay described above.

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