Identification of Nuclear Factors that Enhance Binding of the Thyroid Hormone Receptor to a Thyroid Hormone Response Element

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Using a gel shift assay, we analyzed the binding of *in vitro* translated α- and β-thyroid hormone (T_3) receptors to a T_3-response element (TRE) derived from the rat GH gene. No receptor-TRE complexes were observed when translated receptor alone was incubated with the TRE. However, addition of a nuclear extract from liver to the translational products resulted in the formation of two receptor-DNA complexes for both the α- and β-receptors. These complexes were shown to contain translated receptor by comigration of ^{32}P-labeled TRE and ^{35}S-labeled receptor in the gel shift assay. A competition experiment demonstrated that formation of the complexes was sequence specific. Preincubation of the liver nuclear extract at 60 C abolished formation of both complexes indicating that receptor-TRE binding required a heat-labile nuclear factor. Phosphocellulose chromatography of the nuclear extract resulted in separation of the activities required for formation of the two complexes. Analysis of nuclear extracts from different tissues revealed that one complex formed in the presence of all extracts, whereas the second complex appeared predominantly with a nuclear extract from liver. Addition of T_3 to the binding reaction had no effect on receptor-TRE complex formation. We suggest that nuclear factors interact with the T_3 receptor to enhance hormone-independent binding to a TRE. (Molecular Endocrinology 3: 1434–1442, 1989)

INTRODUCTION

Thyroid hormones regulate the expression of specific genes such as GH and hepatic spot 14 by interacting with a nuclear receptor in target tissues (1). The receptor has been characterized as a chromatin-associated protein that binds to T_3 and its analogs with a hierarchy of affinities that correlate with biological potency (1). The proposed mechanism of action is that the T_3 receptor binds with high affinity to specific DNA sequences adjacent to or within T_3-responsive genes and alters their rate of transcription. A specific DNA sequence upstream of the GH gene has been identified which confers T_3 regulation on a heterologous gene and is referred to as a T_3-response element (TRE) (2–5). Partially purified T_3 receptor from liver has been shown to bind to this sequence in a gel shift assay (6).

There is now strong evidence that the protooncogene, c-erbA, encodes the T_3 receptor (7, 8). This evidence includes the observation that the *in vitro* translated c-erbA protein binds with high affinity to T_3 and its active analogs (7–13). Specific binding of the c-erbA protein to TREs has also been observed (4, 14, 15). In addition, c-erbA has been shown to activate transcription of reporter genes linked to a TRE in a T_3-dependent manner after cotransfection into receptor-deficient cells in culture (4, 10, 16, 17). Based on sequence comparisons, c-erbA encodes a member of the gene family that includes the steroid hormone, retinoic acid, and vitamin D_3 receptors (18). Several laboratories have now isolated c-erbA cDNAs from a variety of sources and these cDNAs arise from two genes, designated α and β (7–15, 19). Alternatively spliced forms of c-erbA-α have been identified that are unable to bind to T_3 (14, 15, 19). We recently reported the isolation and characterization of α and β c-erbA cDNAs from rat liver (11). The *in vitro* translational products of these cDNAs bind with similar affinities to T_3 and its analogs, however, the α and β mRNAs differ markedly in their tissue distribution. To date, no functional differences between α and β have been identified.

Previous reports have indicated that both the α and β *in vitro* translated proteins bind to TREs in an avidin-biotin DNA binding assay (4, 14, 15). The objective of this study was to use a gel shift assay to analyze interactions between the α and β T_3 receptors and a TRE. We report that the α and β T_3 receptors each form two complexes with a TRE derived from the GH gene. Formation of each complex is dependent upon the presence of a nuclear accessory factor.

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RESULTS

Receptor-TRE Binding Requires the Presence of Nuclear Extract

We used a gel shift assay to analyze binding of the *in vitro* translational products encoded by rc-erbA-α and rc-erbA-β to a TRE derived from the rat GH (rGH) gene. An oligonucleotide was synthesized containing a modified TRE described by Koenig *et al.* (10). This sequence is located from −200 to −156 relative to the rGH gene and differs from the wild type TRE by two base substitutions (see *Materials and Methods*). By a cotransfection assay, Koenig *et al.* (10) have shown that this TRE confers c-erbA-β-dependent regulation of transcription; in the presence of T_3 they observed a 15-fold induction of transcription of a reporter gene linked to this sequence. Results from our laboratory indicate that c-erbA-α also activates transcription of a reporter gene linked to this TRE (Zilz, N. D., and H. C. Towle, unpublished results). Thus, this sequence is likely to contain a T_3 receptor binding site. When the α or β *in vitro* translated proteins were incubated with the ^{32}P-labeled TRE and analyzed on a nondenaturing polyacrylamide gel, no complexes were detected (Fig. 1A). To determine whether accessory nuclear factors might facilitate receptor-TRE binding, an extract from rat liver nuclei was added to the binding reaction. In the presence of nuclear extract, two distinct complexes were observed for both the α and β receptors (Fig. 1A). These complexes displayed a dose-dependent relationship with respect to the translated receptor and the nuclear extract and were not observed with a nonprogrammed lysate (data not shown) nor with the nuclear extract alone (Fig. 1A). The lack of receptor-TRE complexes with extract alone was likely due to a 40- to 90-fold lower level of T_3 binding activity in the nuclear extract compared to the translated receptor. The relative mobilities of the α- and β-complexes correlated with the mol masses of the receptors (α-45 K, β-52 K) suggesting that the complexes contained translated receptor. To confirm this possibility, a parallel assay was performed with ^{35}S-labeled receptor and unlabeled TRE. In the absence of nuclear extract and the TRE, the ^{35}S-labeled receptor did not migrate into the gel. However, in the presence of nuclear extract and the TRE, ^{35}S-labeled complexes were observed that migrated to the identical positions as the ^{32}P-labeled complexes (Fig. 1B). This result indicated that both the α and β T_3 receptors bound to the TRE in the presence of nuclear extract.

To demonstrate that the receptor-TRE complexes were sequence specific, a competition experiment was performed. Increasing concentrations of unlabeled TRE or control oligonucleotide were incubated with the ^{32}P-labeled TRE in the binding reaction. A 10-fold molar excess of unlabeled TRE competed for binding to the α- and β-receptors, whereas an equivalent amount of unlabeled control oligonucleotide did not compete (Fig. 2). Other minor bands which varied in intensity between experiments were not specifically competed by unlabeled TRE. We conclude that formation of the receptor-TRE complexes is sequence specific.

Identification of Nuclear Accessory Factors

The above results suggested that binding of the *in vitro* translated α- and β-receptors to the TRE required nuclear accessory factor(s). To initially explore the nature of the nuclear component(s) involved in TRE binding, we analyzed the heat sensitivity of the activity present in the nuclear extract. The α- and β-receptors were incubated with aliquots of nuclear extract that had been preincubated at various temperatures and TRE binding was analyzed in the gel shift assay. Preincubation of the nuclear extract at 50 C significantly reduced complex formation, while preincubation at 60 C eliminated binding completely (Fig. 3). Both complexes formed by the α- and β-receptors showed similar sensitivity indicating that all required a heat-labile factor. To address whether each α- or β-complex required a different nuclear factor, we fractionated the nuclear extract by phosphocellulose chromatography. Fractions were collected after elution with a step gradient of increasing NaCl concentrations and analyzed in the gel shift assay in the presence of the α-receptor (Fig. 4A). The factor involved in formation of the upper complex eluted earlier (≈200 mM NaCl) than the factor required for formation of the lower complex (≈300 mM NaCl). Thus, two separable factors are involved in formation of the two α-receptor-TRE complexes. To determine whether the same factors also interacted with the β-receptor, fractions containing the two activities were analyzed in the gel shift assay in the presence of the β-receptor. The factors also stimulated formation of the corresponding β-complexes suggesting that both receptors interact with the same factors (Fig. 4B). The fractions were analyzed for TRE binding activity in the absence of *in vitro* translated receptor and no binding was observed (Fig. 4B). This result indicated that fractionation did not unmask any endogenous TRE-binding activity. These results suggest that two nuclear accessory factors enhance T_3 receptor binding to a TRE.

To further address whether there were two nuclear factors involved in binding of the T_3 receptor to a TRE, we analyzed the tissue specificity of the nuclear factors. Nuclear extracts were isolated from liver, brain, heart, kidney, lung, pituitary, spleen, and testis of adult rats and incubated with the α- or β-receptors in the binding reaction. The pattern of complexes observed was the same for α and β (Fig. 5). A complex which migrated similarly to the upper complex observed with the liver extract was formed to some extent with nuclear extracts from all tissues. In parallel experiments using ^{35}S-labeled receptor, ^{35}S-labeled complexes were observed with all tissue extracts at the same relative intensity as the ^{32}P-labeled complexes (data not shown). The similarity in migration of the upper complex in the presence of the various tissue extracts suggested that the same nuclear factor was present in all tissues examined.

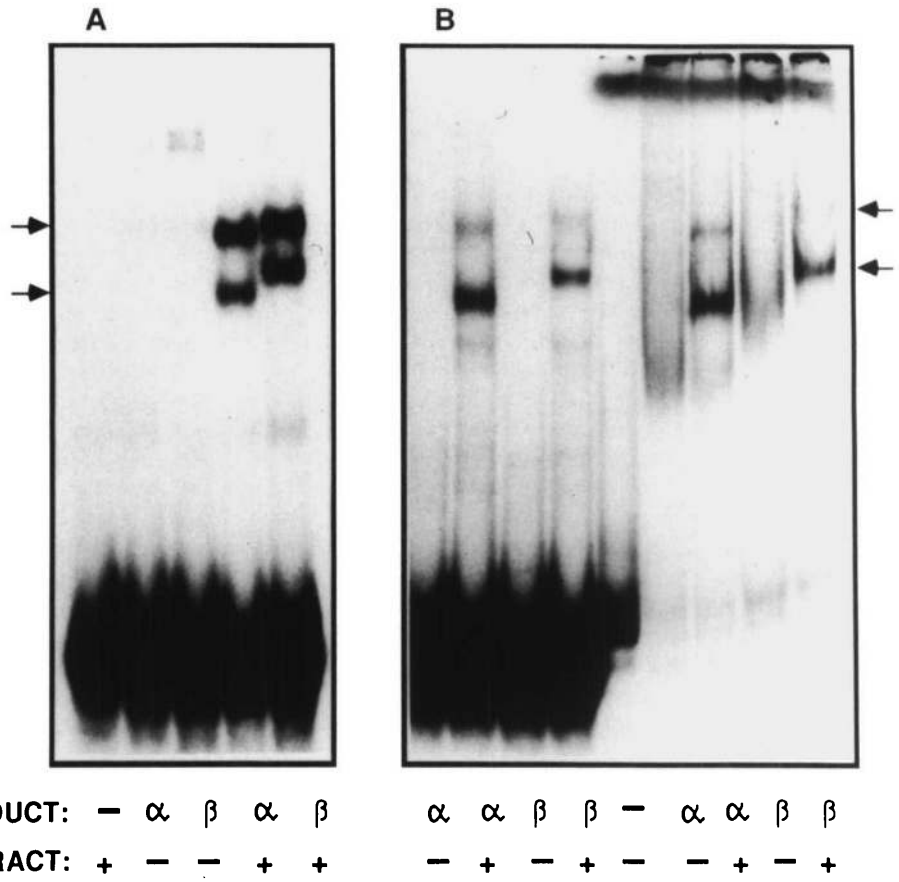

Fig. 1. Binding of the α and β T_3 Receptors to a TRE Requires the Presence of Liver Nuclear Extract

A, Formation of complexes requires both *in vitro* translated c-erbA protein and liver nuclear extract. α- or β-translational products and liver nuclear extract (1.5 μg) were incubated alone or together in the binding reaction and analyzed in the gel shift assay as described in *Materials and Methods*. B, Complexes contain *in vitro* translated receptor. Parallel binding reactions were performed with ^{32}P-labeled TRE and unlabeled α- or β-translational products (lanes 1–4) or ^{35}S-labeled translational product and unlabeled TRE (lanes 5–9) in the absence (−) or presence (+) of liver nuclear extract.

However, the level of the accessory factor in the different tissues appeared to vary significantly. Whether this is due to differences in extraction efficiency or reflects inherent differences in activity of the factor is unknown. The lower complex formed predominantly with the liver nuclear extract and to a lesser extent with extracts from heart and kidney. This activity may represent a tissue-specific factor involved in receptor-TRE binding. None of the additional complexes observed with tissue extracts were seen in a parallel gel shift assay with ^{35}S-labeled receptor (data not shown). We concluded that these complexes did not contain translated receptor and were due to binding of other extract proteins to the TRE. The difference in the tissue distribution of the factors provides further evidence for the involvement of two nuclear accessory factors in receptor-TRE interactions.

Effect of T_3 on Receptor-TRE Binding

To examine the role that hormone plays in the interaction of the T_3 receptor with specific DNA sequences, we analyzed the effect of T_3 on receptor-TRE binding. In the presence of nuclear factors and absence of T_3, both receptors bound to the TRE (Fig. 6). A similar level of binding was also observed in the presence of nuclear factors from hypothyroid rat liver suggesting that binding was independent of any T_3 present in the euthyroid nuclear extract (data not shown). Incubation of the receptors with increasing concentrations of T_3 did not alter the intensity of the complexes (Fig. 6). We conclude that T_3 is not required for binding of the receptor to the TRE.

DISCUSSION

Using a gel shift assay to detect protein-DNA interactions, we have found that binding of the *in vitro* translated α or β T_3 receptors to a modified TRE of the GH gene requires the presence of nuclear factors. Using the avidin-biotin DNA binding (ABCD) assay, other groups have demonstrated that the *in vitro* translated α- and β-receptors bind to TREs in the absence of accessory factors (4, 14, 15, 19). The most reasonable

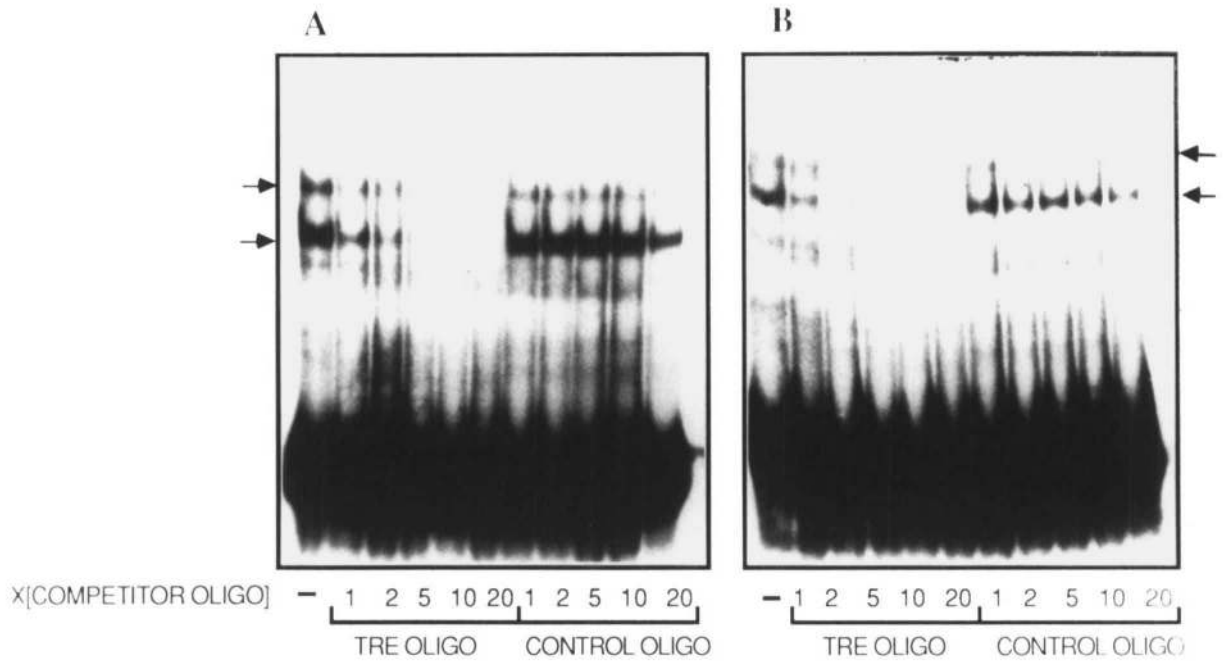

Fig. 2. Competition for Binding of the TRE to the α and β T_3 Receptors

α (A) or β (B) translational products were incubated with ^{32}P-labeled TRE in the absence (–) or presence of increasing molar amounts (1–20×) of unlabeled TRE or a control oligonucleotide.

explanation for these contradictory results is that the ABCD assay may be able to detect lower affinity protein-DNA interactions than the gel shift assay. This distinction might be expected based on the differences in time necessary for separation of bound and free complexes by the two techniques. Thus, the translated α- and β-receptors may be capable of binding to a TRE to form a weakly bound complex, but one which is stable under the conditions of the ABCD assay. Interaction with accessory factors may increase the affinity of the receptor for the TRE and lead to formation of a higher affinity complex which is stable under conditions of the gel shift assay. These observations would argue that the T_3 receptor can exist in at least two forms with differing affinities for the TRE and that accessory nuclear factors are necessary for formation of the higher affinity complexes.

We have found that the α- and β-receptors did not bind in the gel shift assay to an oligonucleotide containing a wild type GH TRE located from −193 to −161 relative to the transcription start site. A sequence comparable to this TRE has been shown to confer transcriptional activation in a cotransfection assay, but to a lesser extent than the modified TRE (16). Thus, the T_3 receptor appears to bind more weakly to the wild type TRE than to the modified TRE and this weaker interaction may not be stable in the gel shift assay even in the presence of accessory factors. We also found that no complexes were formed between the alternatively spliced form of c-erbA-α, designated α2 (14), and the modified TRE under the conditions of the gel shift assay. Lazar *et al.* (15) reported that a splicing variant of c-erbA-α bound to a TRE in the ABCD assay, but at a level consistently lower than that measured for either α or β. These data suggest that the proposed zinc finger, DNA binding domain of the receptor, which is identical for α and α2, is not sufficient to confer high affinity binding to a TRE and that sequences at the C-terminus of the receptor, which differ for α and α2, may be important.

The involvement of accessory factors in binding of a receptor to its target site has also been suggested for certain steroid hormone receptors. Feavers *et al.* (20) found that binding of the estrogen receptor to an estrogen response element of the vitellogenin gene was increased by interaction with two nonhistone proteins. In this case, the nonhistone proteins were DNA-binding proteins which interacted with DNA sequences adjacent to the estrogen response element. Likewise, Edwards *et al.* (21) found that binding of the progesterone receptor to its response element in mouse mammary tumor virus DNA was increased in the presence of nuclear extract, suggesting that accessory factors were increasing the affinity of the receptor for the response element. In both of these studies, the stimulatory activities were found in all tissues examined, similar to the accessory factor that gives rise to the upper complex with the α or β T_3 receptor.

The nature of the accessory factors is unknown. The observation that the two activities can be separated by phosphocellulose chromatography suggests that each factor is a distinct protein. However, it is also possible that a single protein is involved in formation of both complexes and the difference in chromatographic properties reflects different modifications of the protein. This latter possibility is suggested by the similar heat sensi-

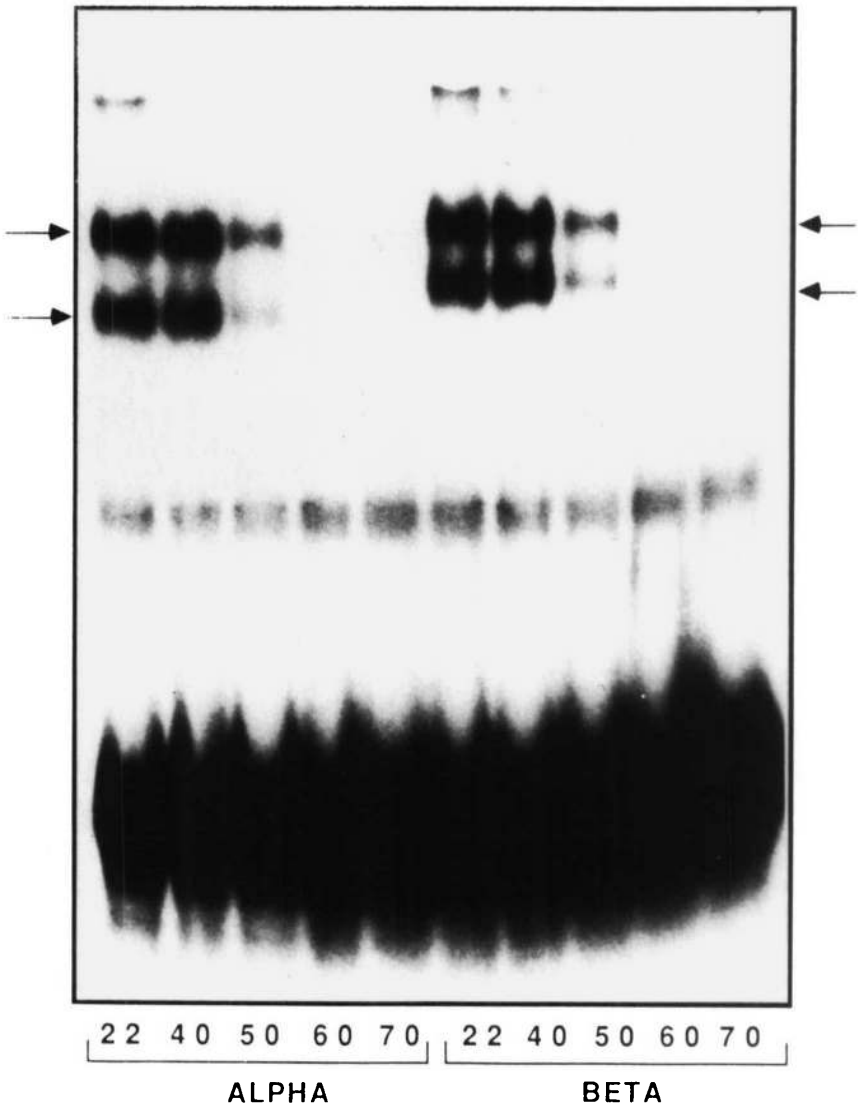

Fig. 3. Heat Sensitivity of Liver Nuclear Extract

Aliquots of liver nuclear extract (1.5 μg) were preincubated for 10 min at 22, 40, 50, 60, or 70 C before addition to the α- or β-translational products in the binding reaction.

tivities of the two activities. The proposed physiological role of the accessory factors is to promote high affinity binding of the receptor to the TRE. There are several possible mechanisms by which the accessory factors could increase the affinity of the receptor for the TRE. The action could be indirect by promoting the dissociation of an inhibitor of TRE binding from the receptor or by promoting the formation of an active oligomer of the receptor, a form which has been reported for steroid hormone receptors (22, 23). A second possibility is that the factors may act by covalently modifying the receptor. Third, it is possible that the accessory factors stabilize receptor-TRE binding by interacting directly with the TRE, as has been suggested for the estrogen receptor (20). In our studies, no protein-TRE complexes were observed in the presence of nuclear extract alone, however, there may be a cooperative interaction between the receptor and the factors which allows the factors to bind to adjacent DNA sites in the presence of the receptor. Finally, by analogy to other well characterized transcription factors (24–26), the accessory factors may bind directly to the receptor forming a complex with increased affinity for the TRE. The fact that two complexes with different migration properties are formed with either receptor suggests that other factors may be bound in the complex.

Our results suggest that specific binding of the T_3 receptor to a TRE is independent of T_3 binding to the receptor. This is consistent with the fact that the receptor is chromatin-bound even in the hypothyroid state. Lavin *et al.* (6) have reported similar results with a partially purified T_3 receptor from liver. Additional evidence suggesting that T_3 is not essential for TRE binding comes from the observation that one of the nonhormone-binding forms of c-erbA-α was capable of TRE binding in the ABCD assay (15). T_3-independent binding of the receptor to TREs suggests a role of hormone at a step subsequent to receptor-DNA inter-

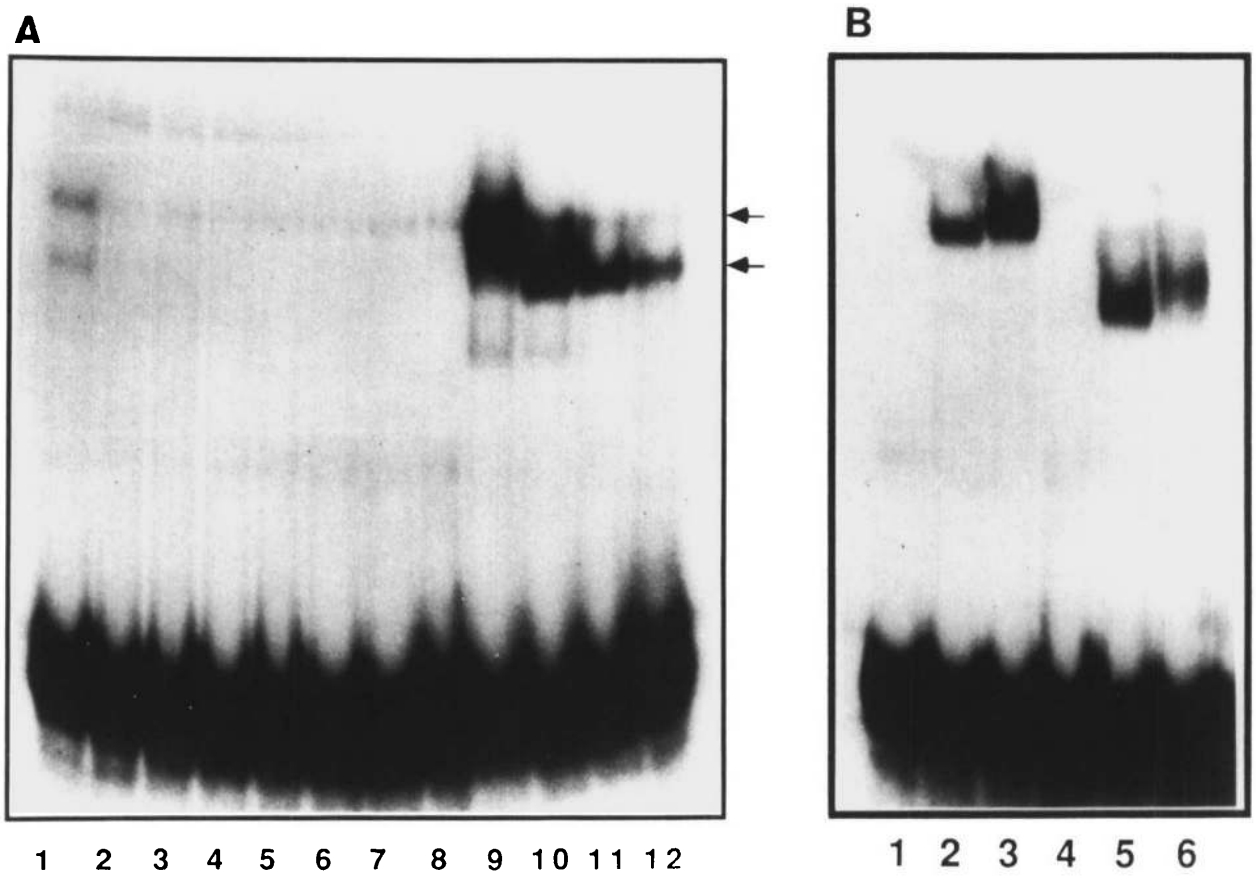

Fig. 4. Fractionation of Nuclear Accessory Factors

A (*left panel*), Phosphocellulose fractionation of liver nuclear extract. Nuclear protein was loaded onto a phosphocellulose column and eluted with DNA binding buffer containing NaCl concentrations of 50 mM to 450 mM. Liver nuclear extract (1.5 μg, lane 1) or 20 μl aliquots of different phosphocellulose fractions (lanes 2–12) were analyzed in the gel shift assay in the presence of the α-translational product. B (*right panel*), Nuclear accessory factors enhance TRE binding of both α- and β-receptors. Aliquots (20 μl) of the fractions giving rise to the upper complex (fraction 8, lane 9, A) or the lower complex (fraction 10, lane 11, A) were analyzed in the gel shift assay in the absence or presence of the α- or β-translational products. Lane 1, Fraction 8 alone; lane 2, fraction 8 + α; lane 3, fraction 8 + β; lane 4, fraction 10 alone; lane 5, fraction 10 + α; lane 6, fraction 10 + β.

action. For example, the hormone may activate transcription by inducing interactions between the receptor and other transcription factors required for T_3 regulation of gene expression.

Analysis of receptor-TRE binding using the gel shift assay has allowed us to identify accessory factors involved in DNA binding. The major question which arises is whether these factors play a physiological role in mediating hormone action. Future studies to explore the nature of the accessory factors will hopefully shed light on this question.

MATERIALS AND METHODS

Materials

All enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Poly(dI-dC) was obtained from Pharmacia (Piscataway, NJ). The rabbit reticulocyte lysate was from Promega (Madison, WI). [^{35}S]Methionine (>800 Ci/mmol) was obtained from Amersham (Arlington Heights, IL) and [α-^{32}P]dCTP (3000 Ci/mmol) was from New England Nuclear (Boston, MA). L-T_3 was purchased from Sigma (St. Louis, MO). Normal adult male Sprague-Dawley rats and rat tissues were obtained from Bio-Lab Corp. (St. Paul, MN). The TRE oligonucleotide was synthesized by the Microchemical Facility, Institute of Human Genetics (University of Minnesota), and the control oligonucleotide was kindly provided by Dr. David Bernlohr (University of Minnesota).

In Vitro Transcription and Translation

To achieve efficient expression of the β-receptor, a large portion of the 5′-untranslated region of rc-erbA-β was removed yielding the plasmid, rc-erbA-β(-37), which retained 37 base pairs (bp) of 5′-untranslated region. The α-receptor was expressed from the plasmid, rc-erbA-α(-29), which was described previously (11). The α and β cDNAs were linearized and RNA was transcribed in the presence of 40 U T7 RNA polymerase, 0.5 mM NTPs, and 20 U human placental RNase inhibitor in 40 mM Tris-HCl, pH 8.0, 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, and 5 mM dithiothreitol for 1–2 h at 37 C. RNA was treated with RNase-free DNase, extracted with phenol-chloroform, and precipitated. Receptor RNA was translated in a rabbit reticulocyte lysate in the presence or absence of 10 μCi [^{35}S]methionine for 1 h at 30 C.

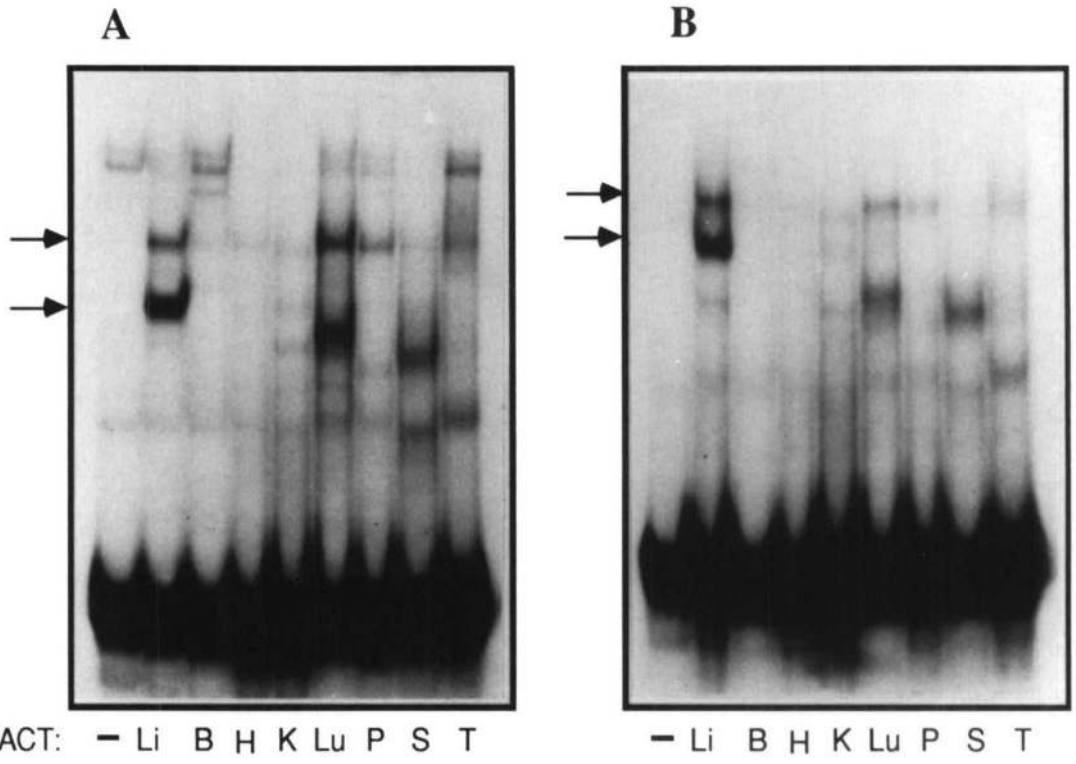

Fig. 5. Tissue Specificity of Nuclear Accessory Factors

Binding reactions contained α (A) or β (B) translational products incubated in the absence (–) or presence of 1.5 μg nuclear extract from rat liver (Li), brain (B), heart (H), kidney (K), lung (Lu), pituitary (P), spleen (S) or testis (T).

Nuclear Extract Isolation and Fractionation

Nuclear extracts were isolated by the method of Oppenheimer *et al.* (27). All procedures were carried out at 4 C. Liver nuclei were prepared by homogenization of tissue in 0.32 M sucrose, 3 mM $MgCl_2$, and centrifugation at 2500 rpm for 10 min. The crude nuclear pellet was resuspended in 2.4 M sucrose, 3 mM $MgCl_2$, and pelleted by centrifugation at 22,000 rpm for 1 h. The nuclei were washed with 0.14 M NaCl, 3 mM $MgCl_2$, centrifuged at 3000 rpm for 10 min, and resuspended in 0.2 ml/g tissue of 0.4 M NaCl, 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 5 mM $MgCl_2$, 5 mM dithiothreitol, and 10% glycerol. Nuclear proteins were extracted on ice for 45 min. The nuclear pellet was removed by centrifugation at 10,000 rpm for 20 min. Nuclear extract from lung was isolated by the above method and nuclear extracts from other tissues were prepared by minor modifications of this procedure as described by Oppenheimer *et al.* (27). The nuclear extracts were stored at −70 C. Protein concentrations were determined by the Bradford assay using BSA as the standard (28).

Phosphocellulose was prepared according to the manufacturer (Whatman, Clifton, NJ) and equilibrated in DNA binding buffer (see below). Nuclear protein (1–2 mg) was dialyzed against DNA binding buffer, loaded onto a 2.5 ml column, washed with DNA binding buffer, and eluted with a step gradient of DNA binding buffer containing NaCl concentrations of 50–450 mM.

DNA Binding Assay

Oligonucleotides were annealed and the 5′-overhanging ends were labeled with Klenow fragment and [α^{32}P]dCTP to specific activities of 3–8 × 10^7 cpm/μg. The TRE oligonucleotide contained the sequence from −200 to −156 of the rGH gene flanked by linker sequences: 5′-AGCTTGGCGGTGGAAAGGTAAGATCAGG**T**A**A**GTGACCGCAGGAGAG CAGTCTAG-3′. The two nucleotides in bold type were mutated compared to the wild type GH gene to yield a more efficient TRE (10). The control oligonucleotide used in the competition experiment contained the sequence: 5′-TCTAGAAAGGAGATATACATATGTGTGATGCCTTTGTGGGAACC TGGAAGCTT-3′.

Aliquots of *in vitro* translated products (5 μl; unlabeled or ^{35}S-labeled) were incubated with 40 μg/ml Polydl-dC, 1.5 μg nuclear extract and 10,000–30,000 cpm ^{32}P-labeled TRE oligonucleotide (or 15–18 ng unlabeled TRE oligonucleotide) in DNA binding buffer (50 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, and 5 mM 2-mercaptoethanol) for 20 min at 22 C. Bromophenol blue and xylene cyanol were added and the reactions were loaded immediately onto a 6% polyacrylamide gel in 89 mM Tris, 89 mM borate, 2 mM EDTA at 4 C. Gels were run at 200 V for 3 h, dried, and exposed for autoradiography.

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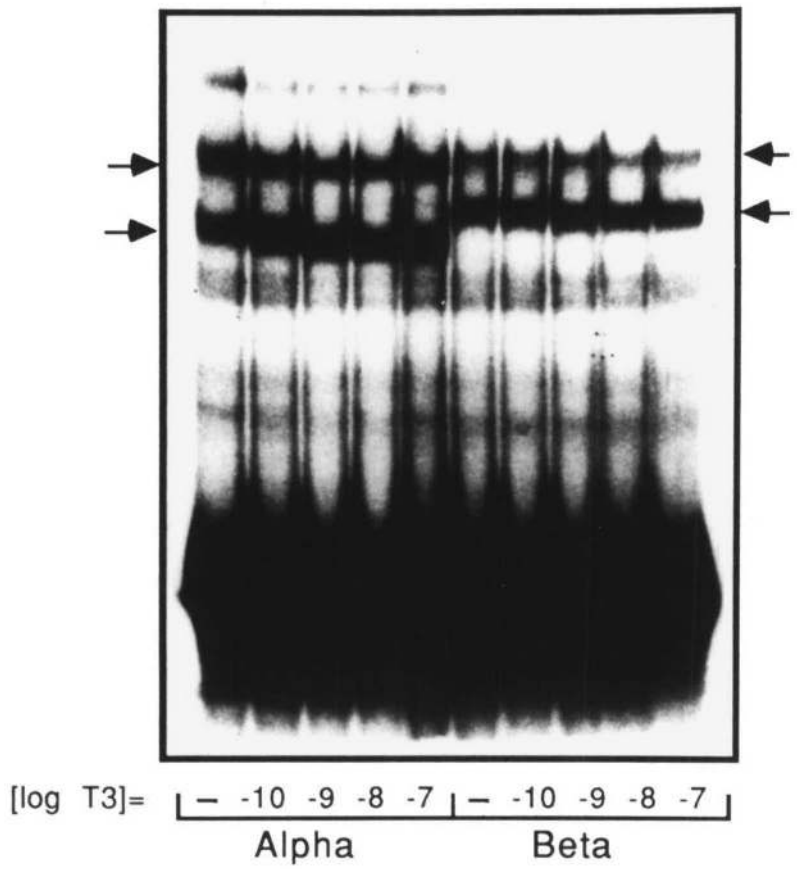

Fig. 6. Effect of T_3 on Binding of the Receptor to the TRE

α- or β-translational products were incubated in the absence (—) or presence of increasing molar concentrations (10^{-10} to 10^{-7} M) of T_3 in the binding reaction.

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