Characterization of Thyroid Hormone Response Elements in the Gene for Chicken Malic Enzyme

FACTORS THAT INFLUENCE TRIIODOTHYRONINE RESPONSIVENESS*

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Triiodothyronine (T3) binds to the nuclear thyroid hormone receptor (TR) and regulates transcription of T3-responsive genes (1). TR binds to thyroid hormone response elements (T3REs) in the regulatory regions of T3-responsive genes. T3REs consist of pairs of hexameric repeats organized as palindromes, everted repeats or direct repeats, or as extended direct repeat T3REs with defined polarity; RXR and TR bind to the upstream and downstream half-sites, respectively (11, 12). RXR/TR dimers require a 4-bp spacer region between the half-sites (12, 13). The nucleotide sequence of the half-sites and flanking DNA and the spacing of the half-sites influence binding of the RXR/TR heterodimers and their interactions with other proteins. Consequently, the structure of the T3RE influences the transactivation potential of the bound heterodimer (4, 14–17). However, the mechanisms by which the transactivation signal is transmitted to the basal transcription machinery is not fully understood.

Several cDNAs have been cloned using strategies that rely on the ability of the encoded proteins to bind to TR and RXR; the proteins encoded by these proteins may be involved in transmission of the transactivation signal. In the absence of T3, co-repressors such as silencing mediator for retinoic acid and thyroid hormone receptors (18) and NCoR (19, 20) augment the silencing function of TR (21). In the presence of T3, transactivation is co-activated by steroid receptor co-activator 1 (22, 23) and inhibited by short heterodimer partner (24). CREB-binding protein and p300 bind to RXR and function as bridging proteins between components of this and other signal transduction pathways (25, 26). Similar bridging proteins (TAFII40 and TAFII110) are present in Drosophila tissues; this nonvertebrate also expresses transcription factors that belong to the steroid/thyroid superfamily of receptors (27, 28). These and other reports suggest that the binding of such proteins to RXR and TR influences transactivation.

Understanding of the binding of TR and RXR to T3REs is based primarily on studies that use characterized response elements and bacterially expressed or in vitro translated proteins. Functional analyses have used a variety of cell lines, many of which are normally unresponsive to T3. These experiments have profoundly increased our understanding of the mechanisms by which T3 regulates transcription. Nevertheless, results of functional analyses are sometimes consistent with results of in vitro binding experiments and sometimes not. Binding may not correlate with function, and functions deduced from experiments with one cell line may differ from those deduced from experiments using a different cell line. To determine physiologically relevant mechanisms, we must use T3REs of natural genes and test hypotheses about binding and function in physiologically relevant cell systems.
The gene for chicken malic enzyme is an appropriate model system for testing hypotheses about the relationship(s) between function of T3REs and binding of proteins directly to T3REs, to flanking DNA, or to T3RE-bound receptors. Malic enzyme is involved in the de novo synthesis of long chain fatty acids and responds to nutritional and hormonal signals in intact animals (29); these responses can be mimicked by manipulating the hormone and fatty acid milieu of chick-embryo hepatocytes in culture (30, 31). In hepatocytes transfected with chimeric genes containing 5′-flanking DNA of the malic enzyme gene linked to the gene for chloramphenicol acetyltransferase (CAT), a robust T3-induced increase in CAT activity is elicited without overexpressing TR or other protein (31, 32).

The gene for chicken malic enzyme contains at least six putative T3REs; five are clustered in a 130-bp T3 response unit (T3RU) (32). The T3RU is centered about 3.8 kilobase pairs upstream of the start site for transcription and is complemented by an additional T3RE that is about 800 bp downstream of the T3RU. These T3REs resemble degenerate DR4-type elements; their contributions to T3 responsiveness of the T3RU vary considerably (32). We report here that, in addition to the nucleotides within the half-sites, nucleotides flanking each T3RE play critical roles in defining the differential function of each T3RE; they appear to do so by binding novel proteins.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were obtained from Life Technologies Inc., New England Biolabs, U.S. Biochemical Corp., and Boehringer Mannheim. Other enzymes were obtained from the indicated sources: T4 polynucleotide kinase and the Klenow fragment of Escherichia coli DNA polymerase I (Boehringer Mannheim) and Bet polymerase (Bio-Rad). Nucleotides were purchased from Sigma, Pharmacia Biotech, Inc., or Life Technologies. Radiolabeled nucleotides and Hybridfilm-HP were obtained from Amersham. D-Threo-[dichloroacetyl-1,2,14C]chloramphenicol was purchased from NEN Life Science Products. LipofectACE, Waymouth MD 705/1 medium, and competent E. coli cells of strain DH5α were obtained from Life Technologies. SeaKem LE agarose, NuSieve GTG agarose, and SpinBind DNA isolation columns were purchased from FMC Corp. Columns (BioSpin 6) for purifying oligonucleotides used in gel electrophoretic mobility shift assays were obtained from Bio-Rad. Hormones were from Sigma. Polyclonal antibody (IgG) against chicken TRα was from Santa Cruz Biotechnologies and recognizes both α and β forms. Monoclonal antibody to chicken RXR was provided by Pierre Chambon (Strasbourg, France) and recognizes α, β, and γ forms. Other chemicals were reagent grade or the best quality commercially available.

Plasmids—Construction of pBSV-LUC (luciferase) was described (32). Bruno Luckow and Gunter Schutz (Heidelberg, Germany) provided pBlColAT (pTKCAT; Ref. 33). Herbert H. Samuels (New York University) provided the cDNA for chicken TRα (6). Plasmid Bluescript KS+ was obtained from Stratagene Cloning Systems. Fragments of chicken malic enzyme DNA containing putative regulatory sequences were inserted into the multiple cloning site 5′ to the virus thymidine kinase (TK) promoter in pBlColAT.

A double-stranded herpes simplex polymerase chain reaction-generated DNA fragment (ME–3903/–3703) was subcloned into the SphI and BamHI sites of pTKCAT to generate pME–3903/–3703TKCAT (pT3RU-TKCAT) (32). Polymerase chain reaction-generated fragments of DNA were subcloned into the HindIII and BamHI sites of pTKCAT to make pME–3474/–2715TKCAT. Most of the TKCAT plasmids containing individual T3REs were constructed by inserting annealed complementary 36-bp oligonucleotides into the corresponding restriction sites of pTKCAT. T3RE 7 was subcloned into the HindIII and BamHI sites of pTKCAT, so that the insertion sites were the same as those in pME–3474/–2715TKCAT. Synthetic oligonucleotides were treated with T4 polynucleotide kinase prior to annealing and ligation. Structures of the resulting plasmid DNA were confirmed by restriction mapping and partial sequence analyses.

Cell Culture and Transient Transfection—Hepatocytes were isolated from livers of 19-day-old chick embryos; suspended in Waymouth medium MD 705/1 supplemented with penicillin (60 μg/ml), streptomycin (100 μg/ml), insulin (50 μg/ml), and corticosterone (1 μg/ml); and incubated in 35-mm tissue culture dishes at 40 °C in 5% CO2 in air (34). Cells were transfected 20 h after plating, using 40 μg of LipofectACE™/plate. Each plate contained 5 μg of plasmid DNA: pTKCAT (1 μg) or the molar equivalent of other test constructs, pBSV-LUC (0.5 μg), and pBluescript KS+ (balance). Transfection medium was removed at 24 h and replaced with the medium described above, with or without 1.6 μM T3.

Analysis of Cell Extracts and Statistics—For each transfection, cells from two plates were combined and harvested at 48 h after adding T3, lysed by three cycles of freezing and thawing, and analyzed for protein content (35), luciferase (36), and CAT (37) activities. Cell lysates to be used for CAT assays were heat-treated for 30 min at 60 °C; denatured protein was removed by centrifugation. CAT activity was normalized to protein in the unheated extracts (CAT activity μg of protein/15 h) and then adjusted for transfection efficiency. Statistical significances of differences were determined by the Wilcoxon signed rank test (38). S.E. values are provided to indicate the degree of variability.

RESULTS

T3REs of the Malic Enzyme Gene Are Functionally Distinct—Individual putative T3REs were subcloned into pTKCAT and transfected into hepatocytes to determine how much T3 responsiveness each T3RE would confer when it lacked the context of the entire T3RU (Fig. 1). T3RE 2 (32) and putative T3RE 5 bestowed large inductions by T3 and conferred basal levels that were less than 90% of the basal activity of the pBSV-LUC reporter gene linked to the gene for chloramphenicol acetyltransferase (CAT), a robust T3-induced increase in CAT activity is elicited without overexpressing TR or other protein (31, 32).
FIG. 1. T3 responsiveness conferred by putative T3REs of the chicken malic enzyme gene. A, T3REs of the T3RU. The T3RU spans bp −3903 to −3704 of the malic enzyme gene and is contained within pME−3903−3703/TKCAT; putative T3REs 2, 4, 5, and 6 contain 26 bp of malic enzyme DNA fused to pTKCAT. Chick embryo hepatocytes were transiently transfected using LipofectACE (40 μg/pllate) and pME−3903−3703/TKCAT (1 μg/pllate or an equimolar amount of the other constructs), pRSV-LUC (0.5 μg/pllate), and pBluescript DNA (sufficient to bring DNA to 5.0 μg/pllate) and treated with or without T3 for 48 h. Activities of the promoter constructs were expressed as CAT activity; CAT activity of cells transfected with pME−3903−3703/TKCAT treated with T3 was set equal to 100, and those transfected with other constructs were normalized thereto. Each point represents the mean ± S.E., of five or six independent sets of hepatocytes, using at least two independently prepared batches of each plasmid. Basal CAT activities were corrected for differences in transfection efficiency by dividing by luciferase activity of the same extract (light units/μg of protein). Relative basal CAT activities were calculated by setting the corrected CAT activities for T3-treated hepatocytes transfected with pME−3903−3703/TKCAT equal to 1 and adjusting all other activities proportionately. For each construct, fold response to T3 was calculated by dividing the relative CAT activity for hepatocytes treated with T3 (+T3) by that for hepatocytes not treated with T3 (−T3). Fold responses were calculated for individual experiments and then averaged; they are not the same as the quotients of the averaged relative CAT activities. Statistical significance between means within a column (p < 0.05) is as follows. a, versus pME−3903−3703/TKCAT. CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with pME−3903−3703/TKCAT equal to 1 and adjusting all other activities proportionately. Relative basal CAT activities were calculated by setting the corrected CAT activities for T3-treated hepatocytes transfected with pME−3903−3703/TKCAT DNA to 100 and adjusting all other activities proportionately. Basal CAT activities were corrected for differences in transfection efficiency by dividing by luciferase activity of the same extract (light units/μg protein). Relative basal CAT activities were calculated by setting the corrected CAT activities for T3-treated hepatocytes transfected with pME−3903−3703/TKCAT equal to 1 and adjusting all other activities proportionately. Statistical significance between means within a column (p < 0.05) is as follows. a, versus pME−3903−3703/TKCAT; b, versus pTKCAT. CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with pME−3903−3703/TKCAT were 0.9 ± 0.02 (mean ± S.E., n = 6) percentage conversion/15 h/μg of protein and 0.9 ± 1.8 × 10^6 (mean ± S.E., n = 6) light units/μg of protein, respectively. B, T3RE 7 of Dnase I-hypersensitive site III. Relative CAT activities were calculated by setting the CAT activities for T3-treated hepatocytes transfected with pME−3903−3703/TKCAT equal to 1 and adjusting all other activities proportionately. Statistical significance between means within a column (p < 0.05) is as follows. a, versus pME−3903−3703/TKCAT; b, versus pTKCAT. CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with pME−3903−3703/TKCAT were 0.9 ± 0.02 (mean ± S.E., n = 6) percentage conversion/15 h/μg of protein and 0.9 ± 1.8 × 10^6 (mean ± S.E., n = 6) light units/μg of protein, respectively. CAT and luciferase activities of extracts from non-T3-treated hepatocytes transfected with pME−3903−3703/TKCAT were 0.6 ± 0.1 (mean ± S.E., n = 6) percentage conversion/15 h/μg of protein and 4.3 ± 0.2 × 10^5 (mean ± S.E., n = 6) light units/μg of protein, respectively.

FIG. 2. Binding of RXR/TR heterodimers to T3REs of the chicken malic enzyme gene. Gel electrophoretic mobility shift analyses were conducted using [α-32P]dATP-labeled double-stranded probes of 30 nucleotides as indicated below each gel. Nuclear extracts were prepared from hepatocytes incubated with T3 for 24 h as described under “Experimental Procedures.” Each reaction contained 7 μg of nuclear protein. Antibody to cTRα (1 μl) or cRXR (0.25 μl) was added to the binding reaction as indicated in the figure and allowed to incubate at room temperature for 15 min. DNA-protein complexes were visualized on 6% nondenaturing acrylamide gels. The position of the RXR/TR heterodimers is indicated by a bracket to the left of each set of gels.

Specific Binding of Proteins to T3REs—Each T3RE bound monomeric and dimeric TRα that had been partially purified from E. coli transformed with an expression vector for chicken TRα (results not shown). Nuclear proteins from T3-treated hepatocytes also bound to each T3RE (Fig. 2). Antibodies (IgG) to either cTRα or cRXR caused the same two closely spaced complexes to be supershifted, indicating that they contained both TR and RXR (bracketed in Fig. 2). Proteins that bind to TR or RXR also should be supershifted by TR or RXR antibodies. Thus, binding of TR accessory proteins, TR-interacting proteins, or integrator proteins to RXR/TR heterodimers may account for the two closely spaced bands (24–26, 39–41). Alternatively, the two complexes may contain RXR and/or TR of different sizes. These results suggest that binding of TR/RXR homodimers, as opposed to RXR/TR heterodimers, is not responsible for the weak functional activities of T3REs 3, 4, 6, and 7.

Unlabeled fragments containing each of the T3REs and DR4 competed for RXR/TR complexes when tested against each labeled T3RE (Fig. 3). When T3RE 3 was the probe, unlabeled T3REs 2, 4, and 5 competed less effectively for RXR/TR than unlabeled T3RE 3, suggesting that affinity of the weak T3RE 3 for RXR/TR may be higher than those of the strong T3REs 2 and 5. Thus, differing affinities of the T3REs for RXR/TR may not cause the differences in responsiveness to T3.
Antibodies to TR or RXR did not supershift or inhibit binding of all complexes bound to the T3REs; additional factors bound to each T3RE but did not form stable complexes with RXR/TR (Figs. 2 and 3, A–E). Migration patterns of the non-RXR/TR complexes formed with T3RE 2 were similar to those formed with T3RE 3 or 4 but different from those with T3REs 5 or 7, and that with T3RE 5 was different from that with T3RE 7. The non-RXR/TR complex(es) bound to each T3RE appeared to be specific for that T3RE. Competitions of DNA-protein complexes with a 100–500-fold molar excesses of the other T3REs failed to

**Fig. 3.** Non-RXR/TR proteins bind specifically to T3REs. Nuclear extracts were prepared from hepatocytes incubated with or without T3 for 24 h as described under "Experimental Procedures" and indicated for each panel. Gel electrophoretic mobility shift analyses were conducted using \( {\left( ^{32}P \right) dATP} \)-labeled double-stranded probes (30 nt). Each reaction contained 7 μg of nuclear protein. DNA-protein complexes were visualized on 6% nondenaturing acrylamide gels. The positions of RXR/TR heterodimers are denoted by a bracket to the left of each set of gels. A, T3RE 2 was the labeled probe. Double-stranded competitor T3REs were added to the binding reactions at either a 100- or 500-fold molar excess over probe as indicated. DR4 is an idealized T3RE with direct repeat orientation and a 4-bp spacer; half-sites match the consensus sequence AAGTCA. B, T3RE 4 was the probe. Double-stranded competitor T3REs were added to the binding reactions at 100-fold molar excess over probe. C, T3RE 3 was the probe. Other conditions were as described for T3RE 4. D, T3RE 5 was the probe. Other conditions were as described for T3RE 4. E, T3RE 7 was the probe. Other conditions were as described for T3RE 4. IC, insulin plus corticosterone; ICT3, insulin plus corticosterone plus T3.
displace, or displaced poorly, the non-RXR/TR complexes on nonidentical T3REs. These results suggest that different non-RXR/TR proteins bind to each of the T3REs.

The pattern of migration of the non-RXR/TR proteins and competition for binding of non-RXR/TR proteins by the various T3REs were similar for T3REs 5 and 6. Furthermore, T3REs 5 and 6 contain 21 bp of overlap. T3RE 5, however, bestowed a much higher T3 responsiveness to transfected cells than T3RE 6. A series of binding and transfection experiments suggested that only one of these two DR4 elements is functional in the natural gene (results not shown). No additional analyses of T3RE 6 are reported in this study.

Medium containing T3 affected binding and migration of the non-RXR/TR complexes with some of the T3REs. For T3REs 2 (results not shown) and 4 (Fig. 3B), the migration patterns and extents of binding of non-RXR/TR complexes to each T3RE were similar in extracts prepared from cells incubated with or without T3. In contrast, for T3REs 3, 5, and 7 (Fig. 3, C–E), major non-RXR/TR binding complexes were formed when the nuclear extracts were from hepatocytes not treated with T3; these complexes were absent in extracts prepared from T3-treated cells. Antibodies to TR and RXR did not supershift non-RXR/TR complexes in extracts prepared from cells that were not treated with T3, confirming that these complexes did not bind to TR or RXR under these conditions. T3RE 7 also bound non-RXR/TR complexes that formed in extracts prepared from T3-treated cells, but not, or less so, in those from cells not treated with T3. In sum, the non-RXR/TR complexes appear to contain factors that are specific for the elements to which they bind; binding of these factors may contribute to the differences in T3 responsiveness and basal activities of cells transfected with TKCAT linked to the different T3REs.

T3 in the medium caused a slight increase in the mobility of complexes formed between most of the T3REs and RXR/TR (Fig. 3). A similar phenomenon has been reported previously (42). For most of the T3REs, the extent of binding of RXR/TR was unaffected by T3. For T3RE 7, however, increased binding of RXR/TR in extracts from T3-treated cells was correlated with decreased binding of one of the non-RXR/TR complexes. At this T3RE, RXR/TR and one of the non-RXR/TR proteins may bind to the same site and compete with one another for binding to the site in a T3-dependent manner.

**T3RE 5**—A gel electrophoretic mobility shift assay that used a high ionic strength buffer permitted resolution of the slowly migrating complex containing T3RE 5 into two distinct bands (Fig. 4C). The mutations in mD reduced binding of RXR/TR to T3RE 5 because they destroyed the downstream half-site of T3RE 5 (Fig. 4B). In addition, however, they reduced binding of protein (NP5) in the more slowly migrating of the two non-RXR/TR complexes. Formation of this complex may be dependent on binding of RXR/TR to the T3RE. The mutations in m3' also reduced binding of NP5 (Fig. 4C), but they had no effect on binding of RXR/TR (Fig. 4B).

Mutant m3' of T3RE 5 was introduced into the TKCAT vector (p5 m3'[ME-3794/-3769]TKCAT) to characterize function of NP5 (Fig. 4D). This mutation caused a significant decrease in T3 responsiveness. The decrease was due entirely to elevated basal activity. The NP5 binding activity was present only in extracts made from hepatocytes that were not treated with T3. This suggests that the binding of NP5 to T3RE 5 may contribute to the silencing function of TR.

**T3RE 2**—Mutant forms of T3RE 2 were linked to TKCAT (Fig. 5A), and their promoter activities were tested. Mutation of the 5'-flanking region did not reduce T3 responsiveness significantly compared with that of wild-type T3RE 2 (Fig. 5B). Mutation of the spacer region (2mS) reduced (by 93%) but did not completely...
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A. Mutant Identifier | Sequence |
|----------------------|----------|
| T3RE 2 | -3883 GGTATGCGGCGCCGGACCTC CGTG |
| 2mS | -3883 GGTATGCGGCGCCGGACCTC CGTG |
| 2mD | -3883 GGTATGCGGCGCCGGACCTC CGTG |
| 2m5 | -3883 GGTATGCGGCGCCGGACCTC CGTG |
| 2mU | -3883 GGTATGCGGCGCCGGACCTC CGTG |

B. Relative CAT activity and fold change in CAT activity (\(+\)T3/\(-\)T3) for the various T3REs and their mutants.

C. Nucleotide sequences of functional T3REs. The 5’- and 3’-end points are listed for each T3RE; half-sites are boxed. Unfavorable nucleotides are indicated by a shaded circle, favorable nucleotides by a shaded square.
T3RE 3 contains unfavorable nucleotides in positions IV of the spacer and VI of the downstream half-site. Favorable nucleotides were inserted at these positions either simultaneously (3mS-mD) or separately (3mS and 3mD) (Fig. 7A). Compared with cells transfected with wild-type T3RE 3, T3 responsiveness of hepatocytes transfected with p3mS-mD[ME-3833/-3808]TKCAT or p3mD[ME-3833/-3808]TKCAT were increased only about 2-fold (Fig. 7B). Mutation of the spacer had no effect on T3 responsiveness (3mS). Basal activity conferred by the double mutant was reduced compared with that for the wild type and was significantly different from that conferred by either pT3RE 2[ME-3833/-3858]TKCAT or pTKCAT. Thus, a large increase in T3 responsiveness of T3RE 3 may require more than high affinity binding of RXR/TR.

Competition with wild-type or mutated versions of T3RE 3 was used to localize binding of the high molecular weight complex (Fig. 7C) found in cells incubated without T3. Only 3mD DNA failed to compete with wild-type T3RE 3 for binding of HNP3 (high molecular weight novel protein of T3RE 3). This result suggests that in extracts from cells not treated with T3, the unfavorable nucleotide in the downstream half-site may contact HNP3. Decreased binding of HNP3 to 3mD correlated with increased T3 responsiveness of cells transfected with p3mD[ME-3833/-3808]TKCAT. Extracts from cells incubated with or without T3 also contain a low molecular weight protein that binds to T3RE 3 and potentially could inhibit function of T3RE 3.

T3RE 4 contains one unfavorable nucleotide in position II of the downstream half-site; this substitution should exert a strong negative effect on function. This nucleotide was changed to a more favorable one in p4mD[ME-3809/-3784]TKCAT and in a double mutant, p4 mS' mD[ME-3809/-3784]TKCAT (Fig. 8A). The latter construct contains a second mutation in the 5'-flanking region that also should improve function. The constructs that contained the 4mD substitutions conferred increased responses to T3 (Fig. 8B). Although basal activities were increased significantly relative to that bestowed by wild-type T3RE 4, relative CAT activities in the presence of T3 were increased much more, resulting in T3 responsiveness similar to that conferred by T3RE 2. Insertion of a more favorable nucleotide in the 5'-flanking region increased basal but not T3-induced activity and reduced the "fold" T3 response to that for the vector alone. These results suggest that the unfavorable nucleotide in the downstream half-site of T3RE 4 suppresses T3 responsiveness of a potentially strong T3RE 3.

Except for the single substitution at position II of the downstream half-site, all mutations of T3RE 4 decreased the ability of competitor oligonucleotides to bind to NP4 (novel protein of T3RE 4) (Fig. 8C). Mutant 4mD competed better than the wild-type T3RE 4 for binding of both RXR/TR and NP4 to the...
FIG. 8. T3RE 2, T3RE 4, and mutant forms of T3RE 4: T3 responsiveness of transfected cells and binding and relative affinity of nuclear proteins. A, sequences of wild-type T3RE 2 and wild-type and mutant forms of T3RE 4. Changed nucleotides are shaded; boxed regions denote T3RE half-sites. B, hepatocytes were transfected as described in the legend to Fig. 1. The results are expressed as described in the legend to Fig. 1 and represent the means ± S.E. of 6–10 independent experiments using at least two independently prepared batches of each plasmid. Relative CAT activities were calculated by setting the corrected CAT activities for T3-treated hepatocytes transfected with pT3RE2TKCAT equal to 100 and adjusting all other activities proportionately. Basal CAT activities were corrected for differences in transfection efficiency by dividing by luciferase activity of the same extract (light units/mg of protein). Relative basal CAT activities were calculated by setting the corrected CAT activities for T3-treated hepatocytes transfected with pTKCAT equal to 1 and adjusting all other activities proportionately. Statistical significance between means within a column is as follows. a, versus pT3RE2TKCAT (p < 0.05); b, versus pTKCAT (p < 0.01); c, versus pT3RE4TKCAT (p < 0.05). CAT and luciferase activities of extracts from T3-treated hepatocytes transfected with pT3RE2TKCAT were 2.9 ± 0.6 (mean ± S.E., n = 10) percentage of conversion/15 h/mg of protein and 13 ± 2 × 10^3 (mean ± S.E., n = 10) light units/mg of protein, respectively. CAT and luciferase activities of extracts from non-T3-treated hepatocytes transfected with pTKCAT were 0.09 ± 0.01 (mean ± S.E., n = 10) percentage of conversion/15 h/mg of protein and 5.8 ± 0.6 × 10^3 (mean ± S.E., n = 10) light units/mg of protein, respectively. C, double-stranded competitor DNAs were added to the binding reactions at a 100-fold molar excess over probe as indicated. Gel electrophoretic mobility shift analyses were conducted using [α-32P]dATP-labeled double-stranded probe (30 nt). Nuclear extracts were prepared from hepatocytes in culture incubated with or without T3 for 24 h; each reaction contained 7 μg of nuclear protein. DNA-protein complexes were visualized on a 6% nondenaturing acrylamide gel. The
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wild-type probe (Fig. 8D). The increased binding affinity of 4mD is correlated with a dramatic increase in T3 responsiveness of cells transfected with p4mD[ME-3809/−3784]TKCAT. Thus, high affinity RXR/TR binding and the ability to bind NP4 may be characteristic of a very strong T3RE.

T3RE 7 contains five unfavorable nucleotide contacts. One construct was designed with favorable substitutions at all sites (p7 m5'-mS-mD[ME−3081/−3056]TKCAT) to determine if T3 responsiveness could be improved. Unexpectedly, these favorable mutations resulted in a loss of T3 responsiveness (results not shown). Basal activity of the mutated T3RE 7 was not significantly different from that for wild-type, so that decreased activation by T3 was the affected step. This confirms the unusual nature of T3RE 7.

DISCUSSION

These studies were designed to examine T3RE function and modulation of TR action using physiologically relevant natural T3REs. The natural genes for human and rat growth hormone were used in earlier transfection studies, but rat liver TR that was partially purified and devoid of other DNA-binding proteins was used with radiolabeled T3 in the corresponding T3RE binding analyses. Thus, only TR and proteins that interacted directly with TR were detected (14, 43). Our binding analyses used endogenous proteins rather than purified or in vitro translated receptors. We wanted to know if non-RXR/TR proteins bound to these T3REs. Differential functional activity of T3REs could be due to the binding of different proteins and/or to differences in T3RE structure. We report here that novel proteins bind to and alter the function of T3REs; for at least one T3RE, they do so without interacting directly with TR or RXR/TR. For some T3REs, an alternative explanation is that nucleotide sequence influences conformation of RXR/TR and alters interactions with coactivators and/or corepressors.

Some hormone response elements are functional when present in tandem or in the context of other elements but fail to bestow large responses when utilized individually (43–46). Plasmid T3RE2TKCAT bestowed T3 responsiveness equivalent to that of the idealized T3REs, pTREpalTKCAT and pDR4TKCAT, each of which contain two or more copies of the T3RE. A version of T3RE 4, p4mD[ME−3809/−3784]TKCAT, that contains only favorable nucleotides conferred T3 responsiveness equivalent to those of the strong idealized T3REs and T3RE2. Thus, weak T3REs can become strong T3REs if appropriate bases are changed.

High-affinity RXR/TR binding is a critical factor in the T3 responsiveness conferred by strong T3REs (5, 14, 16, 47). Given their variable responsiveness, we were surprised that all of the chicken malic enzyme T3REs bound RXR/TR; binding of TR/TR was not detected using nuclear extracts from hepatocytes. Binding of TR/TR dimers or TR monomers was observed only with partially purified cTRs. RXR/TR bound to 4mD with higher affinity than it bound to T3RE 4, suggesting that differences in RXR/TR binding affinity may account for the observed functional differences. However, the affinity of a non RXR/TR protein also was increased.

Binding sites for the novel proteins that bound to T3RE 3 and 5 were localized to specific sequences in each T3RE. Decreased binding of HNP3 was correlated with a small but significant increase in T3 responsiveness. In contrast, decreased binding of NP5 resulted in a large decrease in T3 responsiveness. Both HNP3 and NP5 bound to T3REs only in the absence of T3, suggesting that the changes in responsiveness may have been due to changes in basal activities rather than decreased levels of induced activities. HNP3 was tentatively characterized as a repressor of TR's repressor function; mutation of the putative binding site for HNP3 caused a decrease in basal activity and a comparable increase in T3 responsiveness. The action of NP5 resembled that of a group of TR cofactors known as co-repressors because it augmented the silencing function of TR. Interestingly, binding of NP5 required nucleotides just 3' of the downstream T3RE 5 half-site; to our knowledge this is the first report of 3'-flanking sequences playing a role in the function of T3REs.

NP5 differs from other co-repressors in that it binds specifically to the DNA and fails to supershift upon the addition of TR antibody. Other TR co-repressors interact directly with TR to enhance the silencing function when T3 is absent (18, 19, 48–50). NCoR may be an exception because it binds to both DNA and TR. The region to which NP5 bound was necessary for its binding to T3RE 5 but was not sufficient to confer binding of NP5 in the context of T3RE 3 (results not shown); additional specific sequences may be required for NP5 binding.

NP4 may be a co-activator of T3RE function; increased binding is correlated with increased T3 responsiveness. In addition, NP4 bound to DR4, and DR4 shows a high degree of T3 responsiveness in transfected hepatocytes (results not shown). The binding site for this protein was not localized, because most mutations used to localize the site disrupted its binding. Binding of NP4 may be dependent on the binding of RXR/TR and/or nucleotide sequence of the T3RE. RXR/TR forms in solution through an interface in the ligand binding domain of each receptor and forms a second interface in the DNA binding domains; the second interface restricts the dimer to specific direct repeat sequences separated by four nucleotides (13). The orientation of the dimer on the element is critical for high affinity binding and subsequent transactivation by ligand; changes in conformation of the element upon binding of RXR/TR dimers may be critical to effective transactivation. DNA bending may be the way by which the element acquires the required conformation (5, 14, 49).

When bound by RXR/TR, T3RE 2, T3RE 4, and DR4 may have similar conformations that are recognized by NP4 and possibly NP2. Interestingly, DR4 competed with T3RE2 for the binding of NP2 and competed with T3RE 4 for the binding of NP4. By contrast, T3RE 2 and T3RE 4 (or 4mD) were unable to compete for the binding of NP4 and NP2, respectively. This suggests that the structural conformation of the T3RE may be important for the binding of NP2 and NP4 to DR4 and that additional features may be involved in conferring the specificity of NP2 and NP4 binding to T3RE 2 and T3RE 4, respectively.

The T3 responsiveness of T3RE 7 was decreased when "favorable" nucleotide substitutions were made, whereas that for other T3REs increased. This T3RE has an unusual nucleotide in position VI of each half-site as well as an unusual C in position I of the downstream half-site. Other T3REs also contain such unusual nucleotides; the Sp1 and NF-κB motifs in the human immunodeficiency virus type-1 long terminal repeat can bind to and be transactivated by cTRκ (51). The viral position of the RXR/TR heterodimers is denoted by a bracket. Binding of a novel low molecular weight protein specific for T3RE 4 is indicated by an arrowhead. D, double-stranded competitor DNAs were added to the binding reactions at a molar excess of 1–500-fold over probe as indicated. Gel electrophoretic mobility shift analyses were conducted using [α-32P]dATP-labeled double-stranded probe (30 nt). Nuclear extracts were prepared from hepatocytes that were treated with T3; each reaction contained 7 μg of nuclear protein. DNA-protein complexes were visualized on a 6% non-denaturing acrylamide gel. Radioactivity in the individual complexes of the dried acrylamide gel was quantitated using an InstantImager (Packard); radioactivity present in the lane without nuclear extract was subtracted as background.
protein Tat requires specific DNA sequences to convert a functionally inactive TR binding site in the Sp1 element to an active site, confirming the importance of other factors in T3RE function.

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