Nonbiased Identification of DNA Sequences That Bind Thyroid Hormone Receptor α1 with High Affinity*

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Thyroid hormone receptors (TRs) are ligand-dependent transcription factors that bind to specific DNA sequences and regulate gene expression in a ligand-dependent manner. Although thyroid hormone receptors are known to bind to the hexamer 5'-AGGTCA, it is not known if this represents the optimal binding site. Therefore, a nonbiased strategy was used to identify DNA sequences which bind thyroid hormone receptor α1 with high affinity. Such DNA sequences were isolated from a pool of random sequences using a strategy combining an electrophoretic mobility shift assay with the polymerase chain reaction. It was found that thyroid hormone receptor α1 binds with highest affinity to the octamer 5'-TAAGGTCA. Mutation of the two 5'-nucleotides decreased the affinity of thyroid hormone receptor α1 for this DNA sequence approximately 5-fold, and the importance of those nucleotides in receptor binding was confirmed by DNA footprinting. A single copy of the octamer sequence (but not the hexamer AGGTCA) could impart T3 responsiveness to a heterologous promoter in a transient transfection assay. The results indicate that the optimal binding site for thyroid hormone receptor α1 is 2 base pairs larger than previously thought, and that a single binding site can function as a response element. In addition, we speculate that the optimal binding sites for thyroid hormone, vitamin D, and retinoic acid receptors may not be identical, as had previously been thought.

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Thyroid hormone receptors (TRs) are ligand-dependent transcription factors which, along with the receptors for steroids, retinoic acid, and vitamin D, are members of the erbA superfamily of *trans*-acting proteins (1). TRs bind to triiodothyronine (T3) response elements (TREs) in the promoter regions of many genes and confer ligand-responsive transcriptional regulation. The molecular mechanism of transcriptional regulation is at present unclear. One important component of this regulation is the binding of the receptor onto the TRE. A modest number of T3 responsive genes have had their TREs localized (2-8). Brent et al. (9) performed an extensive mutational analysis of the TRE in the 5'-flanking region of the rat growth hormone (GH) gene, and based upon this study proposed a consensus TR binding hexamer 5'-AGGTCA/A (9). Most of the TREs characterized to date contain multiple copies of variations of this hexamer, although conservation is frequently weak. Recently, Kim et al. (10) reported data showing that sequences surrounding the hexamer influence TR binding and function. In order to understand how variations in DNA sequence affect TR binding and transcriptional activation, we believe that it is necessary to determine the optimal DNA binding sequence of TRs without the bias introduced by the use and alteration of known TREs.

By using a selection and amplification technique previously described for the determination of the DNA binding characteristics of myc (11), we have determined the optimal DNA binding sequence for TRα1 monomers. In the experiments described below we demonstrate that high affinity binding is conferred by an 8-base pair sequence, 5'-TAAGGTCA, and that nucleotide substitutions or chemical modifications of this sequence can be correlated with decreased TR binding and qualitative changes in the ligand responsiveness of reporter constructs in transient transfection assays.

MATERIALS AND METHODS

**DNA Construction**—Following the design of oligonucleotides described previously (11), oligonucleotide primers A (5'-TCCGAATTCCTACAG) and B (5'-AGACGGATCCATTGCA) were synthesized. Primer A contains an EcoRI site and primer B contains a BamHI site. In addition, a pool of oligonucleotides of 49 nucleotides in length was synthesized containing the primer A sequence at the 5'-end, an internal random sequence of 18 nucleotides, and the reverse complement of primer B at the 3'-end. This internally random sequence oligonucleotide pool was made double stranded by annealing with primer B and filling-in with a Klenow reaction. The double stranded pool was then purified by polyacrylamide gel electrophoresis.

**Selection and Amplification Procedure**—Using the electrophoretic mobility shift assay (EMSA) described below, 100 ng of the random pool of double stranded DNA was incubated with 20,000 trichloroacetic acid-precipitable cpm of mouse TRα1 (12) reticulocyte lysate translation product and then briefly run into a polyacrylamide gel. The portion of the gel above the region of unbound DNA (as visualized by ethidium bromide staining of an adjacent lane lacking poly(dI-dC) was cut out and the small amount of DNA contained was eluted into 0.5 M NH₄OAc, 1 mM EDTA, 0.1% SDS, ethanol precipitated, and amplified by the polymerase chain reaction (PCR) using primers A and B. The PCR protocol utilized 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min. The selection process was repeated and in the third EMSA an [35S]TRα1 DNA complex could be visualized. For subsequent selections, the specific complex as revealed by autoradiography was excised from the dried gel, and the DNA was eluted and amplified by PCR.

**Cloning of TRα1-binding DNA Pools**—PCR-generated TRα1-binding DNA pools were ligated into either of the plasmid vectors pCR1000 (Invitrogen) or pBluescript (Stratagene). For the latter, the DNA was digested with EcoRI plus BamHI and gel purified prior to
ligation. Individual bacterial colonies were used to generate plasmid DNA, and PCR with primers A and B was then used to generate the contained 49-bp sequences. These were assayed for TRα1 binding activity by performing EMSAs with [35S]TRα1 (see below). Individual subclones were sequenced using the dyeoxynucleotide method with vector primers.

**EMSA**—[35S]Methionine-labeled TRα1 was synthesized by *in vitro* translation in rabbit reticulocyte lysate (13). Products were analyzed by SDS-PAGE and shown to be of appropriate size (data not shown). Matching *in vitro* translation reactions also were performed with nonradioabeled methionine. Protein-DNA binding reactions were performed in 35 μl of 20 mM HEPES, pH 7.8, 50 mM KCl, 1 mM dithiotreitol, 0.1% Nonidet P-40, and 20% glycerol. Incubations included ~12,000 trichloroacetic acid precipitable cm of translation product, 1.4 μg of poly(dI·dC), and 30 ng of PCR-generated DNA. Reactions were incubated at room temperature for 40 min prior to electrophoresis. Oligonucleotides 0.25 × TBE (22 mM Tris base, 22 mM boric acid, 0.5 mM EDTA), 6% polyacrylamide gels (29:1, acrylamide:bisacrylamide) at 4 °C. Gels were fixed in 30% methanol, 10% acetic acid, soaked in fluorography solution (Autofluor™, National Diagnostics), dried, and exposed to film for 1–3 days at ~70 °C.

In order to measure the relative *in vitro* affinity of TRα1 for individual sequences, a competition assay was devised. A 70-bp palindromic TRE (14) was self-annealed and labeled with 32P by a Klenow fill-in reaction. The full sequence of the single stranded oligonucleotide was CTAAGATCTCGGTCACTGAGATCAT (the palindromic TRE hexamer is underlined). Approximately 10,000 cpm of this labeled DNA were incubated with unlabeled in *vitro* translated TRα1 (1.1–2.0 μg; ~12,000 cpm; 40 ng volume) at room temperature prior to electrophoresis. Competition was assessed by determining how much unlabeled competitor DNA was required to decrease the intensity of the TRα1.TREcomplex by 50% (C50; defined by densitometry of autoradiograms). The rat GH TRE (bp 188 to 160) was used in a series of graded amounts to provide competition standardization between different electrophoretic runs.

To distinguish TRα1 monomer and dimer complexes, an EMSA was performed using various DNA probes. These studies utilized TRα1 binding sequences that had previously been ligated into the BamHI site of pUTKAT3 (15). The probes were generated by PCR with vector primers, digestion with EcoRI, and Klenow fill-in with [α-32P]dATP. The empty vector yields a 66-bp EcoRI fragment which served as a negative control. Sequences ligated into the BamHI site included (with the relevant TRα1 binding regions underlined, as explained under "Results"; top strand sequence only is shown): GATCCTAAGGTCTAG-GATCGAGGTCATGACCTGAGATCAT (the palindromic TRE hexamer is underlined). Approximately 10,000 cpm of this labeled DNA were incubated with unlabeled in *vitro* translated TRα1 (1.1–2.0 μg; ~12,000 cpm; 40 ng volume) at room temperature prior to electrophoresis. Competition was assessed by determining how much unlabeled competitor DNA was required to decrease the intensity of the TRα1.TREcomplex by 50% (C50; determined by densitometry of autoradiograms). The rat GH TRE (bp 188 to 160) was used in a series of graded amounts to provide competition standardization between different electrophoretic runs.

**Nonbiased Selection Reveals a Highly Conserved 8-Base Pair DNA Footprint**—Using the EMSA and PCR amplification of gel eluted DNA, an original pool of double stranded DNA sequences with an internal span of 18 random nucleotides was selected for those species capable of binding TRα1. Fig. 1 shows the specific [35S]TRα1 -DNA complexes formed after four rounds of selection, as analyzed by EMSA. Radiolabeled TRα1 that is not complexed to DNA is retained at the top of the gel. The nature of the diffuse haze in the middle of lane 1 is unknown, as this haze is only present with some batches of [35S]TRα1. While the original pool of DNA sequences does not bind sufficient amounts of [35S]TRα1, to form a visible band on the autoradiograph of the EMSA gel (lane 2), the fourth round selected pool is enriched for TRα1 binding sequences and reveals a specific TRα1-DNA complex (lane 4).

To assay for the importance of the 5-methyl groups of thymine bases for the protein-DNA interaction, a uracil interference procedure was utilized (18). Deoxyuracil incorporation into the DNA sequence was accomplished by spiking a PCR reaction with a small amount of dUTP. The deoxyuracil incorporated DNA was labeled in a manner similar to that used for the then clonal interference studies. This material was then incubated with TRα1 and subjected to EMSA as described above. The deoxyuracil incorporated DNAs were eluted from the wet gel, digested with uracil DNA glycosylase in PCR buffer for 1 h at 37 °C, ethanol precipitated, and cleaved with piperidine. The samples were resolved on the same sequencing gels as used for the gmine methylation interference studies.

**Cell Culture and Transfections**—JEG-3 cells were grown in 90% Eagle's medium plus 10% iron-enriched calf serum supplement (Life Technologies Inc.) and were transfected using standard calcium-phosphate precipitation (13). TRα1 was expressed from the vector pCDM (13). Transfections included 3 μg of pCDMTRα1 (or vector) plus an additional 3 μg pCDM as "filler" plasmid. Ligand responsive reporter plasmids were constructed by ligating putative TRE sequences into pUTKAT3 (13) at a unique BamHI site located just upstream of the minimal herpes simplex virus thymidine kinase promoter directing chloramphenicol acetyltransferase (CAT) expression (described above in the "EMSA" methods). Reporter plasmids were transfected at a dose of 4 μg/Petri dish. To control for transfection efficiency, each transfection also included 0.5 μg of pRSVGH, in which the Rous sarcoma virus promoter directs expression of human GH. Cells in 60-mm Petri dishes were transfected in the presence of 10% charcoal stripped iron-enriched calf serum supplemented with 100 nM dexamethasone (1.6 pmol/μl volume) for 3 days at ~75% cell saturation dose of TRα1 (100 nm). Cell lysates were assayed for CAT activity and media for human GH as previously described (13). Ligand responsiveness is defined as CAT/human GH for cells cultured with ligand divided by CAT/human GH for cells cultured without ligand. Results are presented as the mean ± S.E. for at least four transfections per construct.

Transfections also were performed in COS cells. The technique was similar to that described above, except the cells were grown in 90% Dulbecco's modified Eagle's medium plus 10% fetal bovine serum.

**RESULTS**

**Nonbiased Selection Reveals a Highly Conserved 8-Base Pair Sequence**—Using the EMSA and PCR amplification of gel eluted DNA, an original pool of double stranded DNA sequences with an internal span of 18 random nucleotides was selected for those species capable of binding TRα1. Fig. 1 shows the specific [35S]TRα1 -DNA complexes formed after four rounds of selection, as analyzed by EMSA. Radiolabeled TRα1 that is not complexed to DNA is retained at the top of the gel. The nature of the diffuse haze in the middle of lane 1 is unknown, as this haze is only present with some batches of [35S]TRα1. While the original pool of DNA sequences does not bind sufficient amounts of [35S]TRα1, to form a visible band on the autoradiograph of the EMSA gel (lane 2), the fourth round selected pool is enriched for TRα1 binding sequences and reveals a specific TRα1-DNA complex (lane 4).

![Fig. 1. Selection of TRα1 binding sequences by EMSA](image-url)
3). Subcloning of this pool revealed that some individual DNA sequences would bind to \[^{35}S\]TRα1 (lane 4) but others would not (lane 5).

To determine the relative affinities of specific DNA sequences for TRα1, a competition EMSA was devised using a reproducible TRα1-\[^{32}P\]DNA complex as a baseline. The labeled DNA sequence consisted of the well-characterized synthetic palindrome TRE\textsubscript{pal} (5'-TCAGGTCTATGACCTGTA). To assess TRα1 affinity, varying amounts of unlabeled DNA of each of the subclones were added to binding reactions and the decrease in the intensity of the baseline TRα1-TRE\textsubscript{pal} complex was determined. For comparison, varying amounts of the rat GH gene promoter TRE were used to assess relative affinity and standardize results across several electrophoretic runs.

Using this assay, a C\textsubscript{so} (50% competition) value for various TRα1-binding DNA sequences was determined (Fig. 2). For the rat GH gene TRE, the C\textsubscript{so} was 3.6 ng. Ten individual subclones were characterized with C\textsubscript{so} values clustered around 1.5 ng (1.5 ± 0.2, mean ± S.E.). A C\textsubscript{so} analysis for representative subclone A3 is shown in Fig. 2. Sequencing of these 10 subclones demonstrated that they all contained the 5-bp sequence 5'-TAAGGTCA. Performing competitions with a single dose of 5 ng of DNA, an additional subclone was identified which gave ~80% competition at that dose. Based upon the slope of the C\textsubscript{so} curves for the other 10 subclones, these 4 subclones also would be predicted to have C\textsubscript{so} values of 1–2 ng. All 4 of these subclones contained the sequence 5'-TAAGGTCA. Thus, a total of 14 high affinity binding subclones were identified, and all contained this conserved octamer. However, among these 14 subclones, no sequence conservation was detected in the nucleotides surrounding this octamer. In addition, the position of the octamer within the random 18-mer was not conserved, nor was the orientation of the octamer relative to the primers. All other subclones had C\textsubscript{so} values greater than 3 ng. Several of these were sequenced, and all contained variations of the octamer sequence (Table I). Overall, the results suggest that the optimal binding sequence for TRα1 is 2 bp larger than previously believed.

**Mutation of the First Two Bases of the TRE Octamer**

![Fig. 2. Affinity of TRα1 for various DNA sequences analyzed by competition EMSA. \[^{32}P\]TRE\textsubscript{pal} was incubated with nonradiolabeled TRα1 plus various amounts of nonradiolabeled competitor DNAs, and protein-DNA complexes were analyzed by EMSA. The dose of competitor DNA that competes 50% of the TRα1-\[^{32}P\]TRE\textsubscript{pal} complex (C\textsubscript{so}) was determined by densitometry and used as a measure of relative affinity. rGH is the rat GH gene TRE (bp −188 to −160), A3 is a representative subclone that binds TRα1 with high affinity, A3M is a mutant version of A3, and 52 and 53 are subclones that do not bind TRα1. The three lanes at the far right were derived from a separate experiment using a shorter electrophoretic run than the other lanes.

| Sequence          | C\textsubscript{so} |
|-------------------|---------------------|
| TAAGGTCA          | 1.5                 |
| TAAGGACA          | 4.2                 |
| GCAGGTCA          | 7.7                 |
| TGAGGTGA          | 7.7                 |
| GGAGGTAC          | 11.0                |
| CAAGGTCG          | 35.0                |

**TABLE I**

Sequence and C\textsubscript{so} values for the consensus octamer TAAGGTCA and derivatives thereof.

**TRα1 Binding**—In order to confirm that the two nucleotides upstream of the classical TRE hexamer are important for TRα1 binding, the following experiment was performed. A representative subclone (A3) was selected and a "mutant" version (A3M) was synthesized that contained substitutions in two bases upstream of the TRE hexamer, altering the sequence to 5'-GCAGGTCA. When the original sequence and the mutant sequence were compared by EMSA for their abilities to compete away the TRα1-\[^{32}P\]TRE\textsubscript{pal} complex, it was observed that the substitution of the two nucleotides increased the C\textsubscript{so} approximately 5-fold, from 1.5 to 7.7 ng (Fig. 2). Fig. 2 also demonstrates the lack of competition by two subclones (52 and 53) that do not bind TRα1.

**TRα1 Binds as a Monomer to the Sequence TAAGGTCA**—It was important to demonstrate that TRα1 binds only as a monomer to the sequence TAAGGTCA. Therefore, an EMSA was performed comparing the binding of TRα1 to various DNA sequences, including TAAGGTCA, in the context of a 66-bp EcoRI fragment derived from the vector pUTKAT3 (Fig. 3). TRα1 does not bind to the 66-bp vector probe (lane 2), indicating there is no receptor binding site inherent to this region of pUTKAT3. Binding of TRα1 to the palindromic TRE yields both monomer and dimer bands (TKPal, lane 4), which are not competed by nonspecific DNA (lane 5), but are competed by nonradiolabeled TRE\textsubscript{pal} (lane 6). As expected, binding to the sequence TAAGGTCA yields only the monomer band (TKM, lane 7), which again shows specific competition (lanes 8 and 9). When the TRα1 binding site was altered to GCAGGTCA, which contains only the traditional single half-site hexamer, binding of TRα1 was weakened, but the mobility of the monomer complex was unaltered (TKM1, lane 10). This weakened binding is consistent with the competition data presented above (Fig. 2), which indicates that, in the context of the full 49-bp A3 clone, TRα1 binds to the sequence TAAGGTCA with approximately 5-fold greater affinity that to the mutated sequence GCAGGTCA.

**Methylation Sensitivity Confirms That the Binding Sequence Is an Octamer**—DNA footprinting studies were performed to confirm that TRα1 did indeed bind to the full octamer, and also that important contacts were not made with surrounding bases. Critical guanine residues were identified by methylation interference, and critical thymine residues by uracil interference. As can be observed in Fig. 4, TRα1 exhibits markedly reduced binding when any of the guanine residues from either strand in the octamer sequence is methylated. Importantly, the loss of the thymine 5-methyl group by replacement with deoxyuracil markedly reduces the affinity of TRα1 for the DNA only when the thymine affected is part of the upstream "TA" of the octamer (Fig. 4). In addition it is clear that chemical modification of nucleotides outside the octameric sequence has no major effect on TRα1 binding. These data reinforce the observation that the two nucleotides upstream of the classical TRE hexamer play an integral role in the formation of the protein-DNA complex, and that TRα1
binds TRal (albeit with reduced affinity). The lack of T3 responsiveness with pTKMl is not surprising, since studies with the rat GH gene TRE indicate that T3 responsiveness is absent if only one of the three hexamers is intact (9).

To confirm the above results, transfections also were carried out in COS cells. Following transfection with TRa1, T3 induced CAT expression from pTKMA in 5' flanking region of the rat a-glycoprotein subunit gene negative TRE appears to contain just one (29). Presentation (pTKMB), ligand responsiveness decreased significantly. This suggests that the single octamer requires a specific orientation relative to the initiation site to function as a TRE.

When the mutant octamer 5'-GCAGGTCA was inserted into the same reporter construct to create pTKM1, T3 responsiveness was lost (Fig. 5). This mutant sequence retains the idealized TRE hexamer sequence and, as noted earlier, still binds TRa1 (albeit with reduced affinity). The lack of T3 responsiveness with pTKM1 is not surprising, since studies with the rat GH gene TRE indicate that T3 responsiveness is absent if only one of the three hexamers is intact (9).

To confirm the above results, transfections also were carried out in COS cells. Following transfection with TRa1, T3 induced CAT expression from pTKMA 5.6 ± 0.9-fold \( (n = 4) \), whereas the induction with pTKM1 was only 2.2 ± 0.2-fold \( (n = 4) \). Furthermore, unliganded TRa1 did not suppress CAT expression in either cell line. In both COS and JEG cells, the normalized CAT activity for cells transfected with pTKMA and TRa1 and cultured without T3 was 90% of that for cells transfected with empty vector in place of TRa1. Thus, the T3 induction of CAT activity from pTKMA represents a true increase in gene expression over the basal state, not just a relief of repression induced by unliganded receptor.

**DISCUSSION**

Despite great interest in the molecular mechanism of transcriptional regulation by erbA superfamily members, it is still unclear how these proteins interact with DNA and other trans factors to produce ligand specific regulation of gene expression. Although up to 5–10% of liver proteins may be regulated by T3 (20, 21), only a small number of TREs have been extensively studied by techniques such as footprinting and mutational analysis (2, 4, 7, 9). Perhaps the most extensively studied TRE is that derived from the 5' flanking region of the rat GH gene. This TRE contains three binding sites (commonly called half-sites) for TR. Starting with the wild type sequence, Brent et al. (9) performed an extensive mutational analysis that demonstrated all three half-sites are important for full T3 responsiveness. Furthermore, these studies led to the proposal that the optimal TR binding sequence is 5'-AGGTCA/AA. Indeed, most TREs identified to date contain one or more variations of this hexamer, although rarely is the exact hexamer found. However, even though it is clear this sequence binds TR, it is not clear it represents the optimal binding sequence. The sequence 5'-AGGTCA/AA was derived with a pre-existing bias as to what a TRE sequence should look like (the rat GH TRE), and practical considerations limited the number of specific mutations that could be tested. The potential importance of sequences outside the hexamer was not investigated, possibly because of a preconceived bias derived from steroid hormone response elements (22) that binding half-sites are likely to be hexamers. Not only do TREs vary in half-site sequence, but the half-sites can be oriented as direct repeats (4), palindromes (4, 14), or inverted palindromes (7, 23). Furthermore, the spacing between half-sites is not easily predicted. While directly repeated half-sites usually are separated by 4 bp (24, 25), exceptions exist (26, 27). Palindromic TREs can have spacing of 0–5 bp (4, 14, 27, 28), and inverted palindromic TREs have been reported with spacings of 2 and 4 bp (7, 23). Most of the TREs studied to date appear to contain 2 or 3 half-sites, but the a-glycoprotein subunit gene negative TRE appears to contain just one (29).

In addition, considerable uncertainty exists as to the nature of the actual protein that binds to each of the half-sites of any TRE in vivo. Using the rat GH TRE as a model, it has...
been shown that TR can bind all three half-sites in vitro, and the effects of mutations in any half-site on TR binding in vitro correlate closely with the effects on T3-dependent reporter gene activation in transfected cells (3, 9). These data suggest TR occupies all three half-sites in vivo. However, TR binds to previously characterized positive TREs (all of which contain at least two half-sites) in vitro with a higher affinity as a heterodimer with the retinoid X receptor (RXR) than as a monomer or homodimer (30-34), and mutations that disrupt heterodimerization also impair T3-dependent gene activation (35). Furthermore, cotransflecting cells with RXR enhances T3-dependent reporter gene expression (30-34, 36). Thus, it is currently felt that TR-RXR heterodimerization may play a central role in T3-dependent gene activation, although it is unclear whether specific TRE half-sites bind TR or RXR in vivo.

Clearly, a better understanding of the TR-DNA interaction is required to understand the mechanism of T3-dependent gene activation. We wished to address this problem without any preconceived bias derived from studies of the few already characterized TREs. In addition, we felt that the great variation in number, orientation, and spacing of half-sites in known TREs, as well as the uncertainty as to which half-sites actually are occupied by TR, would seriously confuse the evaluation of TR binding sequences selected for homodimer, heterodimer, or multimer binding properties. Rather, we felt the initial unbiased characterization of TR binding sites would be easiest to interpret if we focused on single half-sites that could bind TR monomers. This information could then be used to reassess what factors actually bind to naturally occurring TREs with multiple half-sites, and could be used as a frame of reference to interpret sequences selected in the future for binding TR homodimers, TR-RXR heterodimers, and possibly multimers.

Our data indicate that the highest affinity binding site for TRa1 is the octamer 5'-TAAGGTCA. It is interesting to ask whether the unexpected 5'-nucleotides (TA) are found in any of the already well characterized TREs. A review of the rat GH (4, 9), malic enzyme (4, 5), and a-myosin heavy chain (4, 6, 37) TREs indicates that these elements contain a total of 8 half-sites, with the initial Thd being present in 4 and the following Ado in but 1. The remaining nucleotides 3-8 of the octamer (AGGTCA) all are present in at least 4 of these 8 half-sites. Thus, although the initial TA may be less well conserved than the traditional hexamer in these TREs, it remains possible that half-sites which lack this initial Thd and/or Ado are the ones that prefer to bind RXR, not TR, in vivo.

All currently known TREs that induce gene expression contain at least two half-sites. In addition, mutation of any two of the three rat GH gene TRE half-sites totally abolishes T3 induction (3, 9). Thus, it has been thought that positive TREs require at least two half-sites. Given this, we were interested to test the T3 responsiveness of a single octamer half-site. Surprisingly, these studies indicate this octamer can confer a modest 4-5-fold T3 induction upon a neutral heterologous basal promoter. This response is abolished by mutation of the 5'-TA' within the octamer. It is important to emphasize that the wild type rat GH TRE, which has three imperfect half-sites, is no more active than this single octamer (2, 19). Indeed, our EMSA data indicate TRa1 binds a single octamer with a higher affinity than it binds the intact rat GH TRE with all three half-sites. Thus, although wild type TREs...
can be mutated and polymerized to create constructs that show 25 or more fold T₃ induction (9), the single octamer TRE yields an hormonal response similar to that of a naturally occurring TRE. The ability of the octamer to function as a TRE was orientation dependent, suggesting that when TRα1 occupies the element in reverse orientation it cannot interact favorably with other transcription factors. This is not surprising considering that the octamer is not a palindrome.

The conclusion that a single octamer is a functional TRE would be compromised if a cryptic TRE half-site had been introduced into pTKMA during construction of this reporter vector. However, this remote possibility is excluded by the EMSA presented in Fig. 3, which shows that only a TR monomer-DNA complex forms when TRα1 is incubated with an 80-bp EcoRI fragment from pTKMA that includes a minimum of 23 bp of sequence flanking each side of the TRE octamer.

These data lead to the prediction that certain naturally occurring positive TREs may consist simply of a single octamer half-site. Since TR would presumably bind as a monomer to these TREs, the T₃ response probably would not be dependent on the presence of RXR. Thus, genes with this class of TRE might be regulated in a very different manner than the currently known genes with positive TREs.

Since TRs, retinoic acid receptors, vitamin D receptors, and RXRs all bind to the sequence AGGGTCA, it is likely all will bind to the octamer TAAAGGTC. However, we would speculate that only TRα1 would bind this octamer with the highest affinity, and that alterations in the first two octamer nucleotides may create optimal binding sites for these other eRα superfamily members. Thus, although it has been proposed that half-site spacing determines whether a response element is specific for TRs, retinoic acid receptors, or vitamin D receptors (24, 25), it is possible that the full half-site sequence (octamer, not hexamer) also helps determine receptor (and hormone) specificity.

Recent studies by Kim et al. (10) bear relevance to our work. These authors studied a sequence near the TATA box of the rat GH promoter that can function as a negative TRE. By performing a mutational analysis of the TR binding site in this region, they proposed that an optimal half-site is 10 bp long, with 2 extra bp both 5′ and 3′ to the traditional hexamer. Our nonbiased selection strategy supports the assignment of one of their four novel bases, the most prominent sequence (octamer, not hexamer) also helps determine receptor (and hormone) specificity.

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