Novel location and function of a thyroid hormone response element

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We describe a novel thyroid hormone response element (TRE)-containing sequence, clone 144, isolated by immunoprecipitation of nuclear thyroid hormone receptor (TR)-DNA complexes from the rat pituitary tumor cell line GH4. These cells express several mRNAs of ~10 kb that hybridize to the TRE-containing genomic clone 144. These mRNAs are up-regulated at the transcriptional level in the absence of thyroid hormone (T3) and repressed in its presence. The sequence protected from DNase I digestion by TR in clone 144 contains two consensus TRE half-sites arranged as inverted palindromes. The clone 144 TRE is located in the 3' untranslated region (UTR) of several related mRNAs. A reporter construct transfected into 293 cells was responsive to TR regulation when the clone 144 TRE was inserted in the 3' UTR but not when inserted upstream of the promoter. As found for the endogenous 144 mRNAs, the 144 TRE reporter construct is activated by TR in the absence of T3, but not in its presence. Deletion analysis showed that clone 144 sequences flanking the TRE were necessary for TR-mediated regulation, suggesting that the mechanism by which TR regulates transcription through a TRE in the 3' UTR is different from that through the TREs located in the promoter region.

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Introduction

Thyroid hormones are important regulators of growth, development and homeostasis in vertebrates. Vertebrates contain two different thyroid hormone receptor genes, α and β , encoding a nuclear receptor which transduces thyroid hormone (T3) signals (Sap *et al.*, 1986; Weinberger *et al.*, 1986). These nuclear thyroid hormone receptors (TRs) belong to the superfamily of nuclear receptors, which function as ligand-dependent transcriptional regulators and share a similar structural organization (for reviews, see Evans, 1988; Beato, 1989). Ligand binding and dimerization functions are located in a C-terminal domain (for review, see Evans, 1988) and transactivation functions are found at the N-terminus as well as in the ligand binding domain (for reviews, see Evans, 1988; Green and Chambon, 1988; Beato, 1989). An N-terminal zinc-finger

domain that is well conserved among the members of this family allows binding to specific DNA sequences, the hormone response elements (Evans, 1988; Green and Chambon, 1988; Beato, 1989). These hormone response elements consist of two copies of a consensus core sequence, which are usually arranged as palindromic repeats (Evans, 1988; Green and Chambon, 1988; Beato, 1989). However, it has become evident that the thyroid hormone, retinoic acid and vitamin D receptors possess considerable flexibility regarding DNA recognition and are able to bind to half-sites arranged as direct, palindromic or inverted palindromic repeats (Glass *et al.*, 1988; Umesono *et al.*, 1988, 1991; Baniahmad *et al.*, 1990; Näär *et al.*, 1991; Carlberg *et al.*, 1993; Tini *et al.*, 1993). As the dyad nature of the response elements suggests, nuclear receptors bind to DNA as dimers. The members of the thyroid hormone/retinoic acid receptor family in particular can bind to DNA either as homodimers or as heterodimers with RXR (Yu *et al.*, 1991; Kliewer *et al.*, 1992; Leid *et al.*, 1992; Marks *et al.*, 1992; Zhang, X. *et al.*, 1992; Freedman *et al.*, 1994), the retinoid X receptor activated by 9-*cis* retinoic acid (Heyman *et al.*, 1992; Levin *et al.*, 1992) or possibly other nuclear proteins (Murray and Towle, 1989; Burnside *et al.*, 1990; Glass *et al.*, 1990; Liao *et al.*, 1990).

Whereas receptors such as glucocorticoid receptor or estrogen receptor become transcriptionally active upon ligand binding (for review, see Smith and Toft, 1993), TR can modulate transcription in the presence or absence of ligand. Although most thyroid hormone response elements (TREs) mediate repression in the absence of T3 and activation in its presence (Glass *et al.*, 1987; Brent *et al.*, 1989b; Damm *et al.*, 1989), others were described that mediate T3-induced repression (Chatterjee *et al.*, 1989; Carr *et al.*, 1992) or activation by unliganded receptor (Saatcioglu *et al.*, 1993; Bigler and Eisenman, 1994). At the present time, it is not well understood at the molecular level what determines activation or repression by TR. In some cases, the exact location of the TRE determines its mode of action, as is illustrated by the TREs mediating T3-induced repression. Although located in the promoter region, as are other TREs, their common feature is a position downstream from the TATA box (Chatterjee *et al.*, 1989; Carr *et al.*, 1992). In addition to location relative to the promoter, proteins with which TR can form heterodimers on DNA represent a further factor that may influence TR function. In *in vitro* assays, for example, TR heterodimers with RXR or other nuclear proteins are stably bound to DNA in the absence or presence of T3, whereas, on a number of TREs, TR homodimers are bound to DNA only in the absence of T3 but not in its presence (Andersson *et al.*, 1992; Forman *et al.*, 1992; Yen *et al.*, 1992a,b; Miyamoto *et al.*, 1993). In such cases, activation of a target gene by TR may depend on the presence not only of the hormone but also of RXR.

Although it is unusual for a transcription factor to have several modes of transactivation, there is evidence that all of them may play a physiological role. T3-inducible genes are expressed as soon as an organism produces thyroid hormone and are likely to represent a set of genes that are necessary throughout the life of the organism. Inducibility by unliganded receptor may play a role in early development, since it has been shown in *Xenopus laevis* that TR α is present prior to the onset of T3 production in the tadpole (Banker *et al.*, 1991; Eliceiri and Brown, 1994). At the same time in development, unliganded receptor may also function as a repressor of genes whose expression is needed later. T3-induced repression, as reported for thyroid-stimulating hormone (TSH) α and β (Chatterjee *et al.*, 1989; Carr *et al.*, 1992), seems to govern genes that are involved in regulating T3 production and which are subject to a feedback inhibition by T3.

The molecular aspects of TR action have been studied extensively, but we know only a few of the genes that are regulated directly by TR. We therefore developed a method, reported earlier, to detect T3-regulated genes based on the identification of endogenous TR binding sites (Bigler and Eisenman 1994). Here we describe a novel endogenous TR binding site that confers activation by unliganded TR and is located in the 3' untranslated region (UTR) of the TR target gene.

Results

A new T3-regulated mRNA in GH4 cells

We identified new T3-regulated genes by immunoprecipitation of solubilized chromatin with a TR-specific antiserum as previously described (Bigler and Eisenman, 1994). Chromatin was solubilized by digestion of GH4 cell nuclei with a restriction enzyme and eluted from the nuclei in a low salt buffer. The soluble complexes were then immunoprecipitated with an anti-TR antiserum. The immunoprecipitated DNA fragments were eluted from the complex and cloned into the appropriate restriction site of a plasmid vector. Approximately 1000 clones were then screened for TR binding sites using a gel mobility shift assay. Approximately 5% of the clones bound TR, among them clone 144. This genomic clone is 433 bp long and does not contain any obvious homology to previously identified sequences. It was of further interest because it hybridized to one or several mRNAs of ~10 kb isolated from GH4 cells. As Figure 1 indicates, the steady-state levels of these mRNAs vary in a T3-dependent fashion. The addition of T3 to the medium resulted in a decrease of 144 mRNA levels. In the absence of T3, however, the levels of 144 mRNAs were increased ~3.5-fold.

These results were similar to the ones we obtained with clone 122 from the same screen (Bigler and Eisenman, 1994), which hybridized to mRNAs that were up-regulated in the absence of T3. Consistent with this finding is our observation that clone 122 TREs conferred activation by unliganded TR upon a reporter construct. In general, TR activates expression upon ligand binding, and activation of expression by unliganded TR has been described only recently (Saatcioglu *et al.*, 1993; Bigler and Eisenman, 1994). These earlier observations, together with the preliminary data obtained for clone 144 from gel mobility shift assays (see below) and Northern analyses, suggested

Fig. 1. Northern analysis of GH4 mRNA. (A) Genomic clone 144 was used as a probe in Northern hybridizations of GH4 poly(A)⁺ RNA. GH4 cells grown in full serum (time point 0) were split into medium containing charcoal-treated serum with or without the addition of 100 nM T3 and were harvested at the indicated times. The blot was probed sequentially with clone 144, growth hormone and GAPDH. GAPDH was used to normalize the samples for the amount of poly(A)⁺ RNA loaded. Growth hormone mRNA, which is induced by T3, was used as a control for T3 effect. Exposure times were overnight for clone 144, 2 h for GAPDH and 5 days for growth hormone. (B) Quantification of Northern blot. The amount of hybridized probe was determined by PhosphorImager and GAPDH was used to normalize for the amount of poly(A)⁺ RNA loaded. The ratio of normalized RNA levels in the absence and presence of T3 is shown.

that this clone might contain a biologically relevant TRE. We therefore proceeded to characterize the TR binding properties and biological activity of this TR binding site in more detail.

TR binding characteristics of clone 144

To characterize clone 144 further, we examined the ability of TR to bind specifically to these sequences in a gel

Fig. 2. TR binding to clone 144 sequences is specific and limited to a particular region of the clone. Gel-purified, ^{32}P -labeled restriction fragments of clone 144 were analyzed by gel mobility shift assay using either control protein extract from Sf9 cells infected with wild-type baculovirus or extract containing TR α . To demonstrate TR in the protein–DNA complexes, anti-TR antiserum 5794 or the non-specific anti-max antiserum 7094 were added to the reactions. Autoradiography of the dried gels was overnight at -70°C . Open triangles indicate TR–DNA complexes and closed triangles indicate TR–DNA complexes supershifted with anti-TR antiserum.

mobility shift assay. For this purpose, clone 144 was divided into smaller fragments by restriction digestion and these labeled fragments were used as probes in gel mobility shift assays with baculovirus-expressed TR. Figure 2 shows that TR shifted only one of the three fragments tested. Since only the specific anti-TR antiserum, but not the anti-max antiserum which does not bind TR, caused a supershift of this complex, we concluded that TR was capable of specifically interacting with this genomic fragment.

DNase I footprinting of baculovirus-expressed TR α bound to clone 144 showed a protected region of ~ 30 bp (Figure 3A), which encompasses sequences which are related to the AGGTCA consensus TRE half-site (TCTCT-CCTTAATGGGAGGTGATCG). The two half-sites are arranged as inverted palindromes in the same manner as the half-sites of the F2 TRE in the chicken lysozyme promoter and the Rous sarcoma virus (RSV) long terminal repeat (Baniahmad *et al.*, 1990; Saatcioglu *et al.*, 1993). Methylation interference experiments showed that residues interfering with TR α binding to 144 sequences are located within the consensus TRE in both half-sites, as well as in the spacer (Figure 3B and C). A second, fainter footprint can be detected 3' of the TR binding site when TR is included in the binding reactions, but not with the control

extracts. Methylation interference experiments indicated that methylated residues in that region did not interfere with TR binding. Furthermore, a fragment that contains the region of the second footprint, but not the TR binding site, is not bound by TR in a gel mobility shift assay (data not shown). We therefore presume that the additional footprint may be due to a factor that can be recruited by TR to bind to this sequence.

Figure 4 shows a gel mobility shift assay comparing the TR binding properties of oligonucleotides representing the TR binding sites of clone 144 with the growth hormone TRE (Brent *et al.* 1989a), the palindromic TRE (TREpal; Glass *et al.*, 1988) and the DR4 element (Umesono *et al.*, 1991). For all three binding sites, we observed predominantly homodimeric complexes. TR bound to the clone 144 binding site with a higher affinity than the growth hormone TRE, but with a lower affinity than the TREpal and the DR4 element. The results from these binding studies show that the TR binding site in clone 144 exhibits sequence specificity and binding characteristics that are similar to previously described TREs.

TR α and TR β can bind to clone 144

It was shown recently (Saatcioglu *et al.*, 1993) for the RSV TRE that there are differences in the ability of TR α

Fig. 3. (A) DNase I protection analysis of clone 144 sequences. 32 P-end labeled DNA fragments were incubated with 2 μ l of either a control extract from Sf9 cells infected with wild-type baculovirus (C) or an extract from Sf9 cells expressing TR α , or no extract (-). After DNase I digestion, the fragments were separated on a 5% sequencing gel and the dried gels were autoradiographed for 72 h. The solid bars indicate the TR α -specific protected region and the bracket a non-TR footprint. (B) Methylation interference of clone 144 sequences. Bound and free fragments were cleaved with piperidine and the products separated on a 5% sequencing gel. B: bound fragments; F: free fragments. The dots indicate methylated residues which interfere with TR α binding. (C) Schematic presentation of the results from DNase I protection and methylation interference experiments. Dashed line: sequences protected from DNase I digestion; dots: methylated residues that interfere with binding of TR α ; arrows indicate sequences related to the AGGTCA consensus binding site for TR.

and TR β to transactivate through this response element, which shares the inverted palindromic arrangement of half-sites with the clone 144 TR binding site. Such functional differences between the two receptors have not been described for the F2 TRE located in the chicken lysozyme promoter, which is the third known TRE to contain inverted palindromic half-sites, or for any other

TREs. It appears that N-terminal sequences upstream of the DNA binding domain, which are divergent between the otherwise very well conserved α and β forms of TR, enable TR α , but not TR β , to bind and transactivate through the RSV TRE (Saatcioglu *et al.*, 1993). Furthermore N-terminal sequences were shown to influence sequence specificity of TR binding to response elements arranged

Fig. 4. Comparison of TR binding to clone 144, the growth hormone TRE, TREpal and DR4. 32 P-labeled oligonucleotides representing the above TR binding sites were incubated with 0.5 μ l of an extract from Sf9 cells infected either with wild-type baculovirus (C) or baculovirus expressing TR α (TR). The complexes were separated on a 5% polyacrylamide gel and the dried gels autoradiographed for 16 h at -70°C .

as direct repeats (Chen *et al.*, 1993). Since the clone 144 TR binding site and the RSV TRE share the inverted palindromic arrangement of the half-sites, we tested in a gel mobility shift assay whether TR α and TR β bound with different properties to the clone 144 site. Figure 5 shows binding of TR α , TR β and TR α p40, from which the sequences upstream of the DNA binding domain were deleted (Bigler *et al.*, 1992), to the clone 144 TR binding site. TR α as well as TR β bound to this site, a prerequisite for transactivation. The weaker binding of TR β was, in part, due to lower amounts of protein in the reaction. Thus, in terms of TR binding, the clone 144 TR binding site shows the same properties as the F2 TRE and none of the TR subtype specificity of the RSV TRE. More surprising, however, was the very weak binding of the N-terminally truncated TR α form. With TREs like F2 or TREpal, such differences were not observed (Bigler *et al.*, 1992; Wahlström *et al.*, 1992; and data not shown). This indicates that the N-terminal sequences may be necessary for the stabilization of the TR-DNA complex on this particular binding site.

A further characteristic of TR is its ability to form heterodimers with RXR. Although heterodimerization seems to occur on all TREs, a stabilization of TR-RXR heterodimers as compared with TR homodimers (Yu *et al.*, 1991; Kliewer *et al.*, 1992; Leid *et al.*, 1992; Zhang, X. *et al.*, 1992) is not observed for every binding site (Saatcioglu *et al.*, 1993; Bigler and Eisenman, 1994).

Figure 5 shows that, as expected, TR can form heterodimers with RXR on the clone 144 binding site. However, there are subtle differences between TR α , TR β and TR α p40 in the nature of their interaction with RXR α . With clone 144, TR α -RXR α heterodimers did not display enhanced binding as compared with TR α homodimers, since the amount of probe shifted was approximately the same for homodimer and heterodimer. For TR β and TR α p40, however, there was a significant increase in stability of the heterodimer versus the homodimer. Although the heterodimer formed by the truncated TR has a similar mobility to that of a background band from the reticulocyte lysate, binding in the presence of anti-TR antiserum was abolished only in the lane containing both receptors, but not in the lane containing unprogrammed lysate (Figure 5B). The formation of heterodimers between TR α , TR β , or TR α p40 and RXR α on the 144 TR binding site occurred in the absence or presence of either T3 or 9-*cis* retinoic acid or both ligands. TR homodimers, however, were not formed by receptor molecules that had T3 bound (data not shown). These observations are consistent with binding characteristics reported for other TREs.

The clone 144 TR binding site is located in the 3' UTR

We now wanted to determine whether the clone 144 binding site was capable of conferring T3 regulation upon a reporter construct. The TREs described to date are located in the promoter region or at some distance 5' of the promoter. Genomic clone 144 contained a TR binding site and hybridized to mRNA as well, implying that it encompassed the promoter. TATA box-like sequences were present in both orientations, which prompted us to insert this fragment in both orientations upstream of a promoterless CAT construct. However, neither one of these constructs yielded measurable amounts of CAT activity. Although unexpected, there were at least two possible explanations for this result: either this fragment did not contain a functional promoter or the promoter functions only in a very tissue-specific manner.

To address whether clone 144 contains a promoter, we decided to use cDNA clones to define the structure of the transcription unit more accurately. We used an oligo(dT)-primed cDNA library from GH4 cells to isolate cDNA clones hybridizing to clone 144. Figure 6 shows the alignment of the genomic sequences with the four different cDNA clones obtained from the GH4 cDNA library. All cDNA clones contain a region that is highly homologous to the genomic sequence. In cDNA clones 144-111, 144-152 and 144-153, this region of homology is located near the 3' end, and in clone 144-052 it is ~1 kb upstream of the 3' end of the cDNA, a location highly unusual for a transcription factor binding site. These cDNA clones of 1.2–3 kb represent only partial cDNAs, since the mRNAs to which clone 144 hybridized appeared to be at least 9 kb long (Figure 1). The fact that (i) the cDNA library was constructed using an oligo(dT) primer, (ii) all the cDNA clones contained polyadenylation signals as well as poly(A) tails, and (iii) there were no long open reading frames present in the cDNA clones, implied that these cDNA clones contained exclusively 3' untranslated sequences. The reason for the presence of several highly

Fig. 5. Different TR forms bind to clone 144 and heterodimerize with RXR. (A) Five μ l of chicken TR α , rat TR β and TR α p40, translated *in vitro* in reticulocyte lysate, with or without 5 μ l of *in vitro* translated human RXR α , were incubated with 32 P-labeled oligonucleotide representing the clone 144 TR binding site. The complexes were separated on a 5% polyacrylamide gel and the dried gel was autoradiographed for 16 h at -70°C . C: unprogrammed reticulocyte lysate; HD: TR–RXR heterodimer; D: TR homodimer; M: TR monomer. (B) To distinguish the non-specific band due to reticulocyte lysate and the TR α p40–RXR α heterodimer, anti-TR antiserum was added to the binding reactions and the complexes were separated on a 5% polyacrylamide gel.

related but distinct cDNAs, none of which contains a sequence 100% homologous to the genomic clone, may lie, in part, in the aneuploidy of the GH4 cells. We obtained further support for this view from a quantitative PCR analysis, which indicated the presence of ~ 10 copies of clone 144 sequences per GH4 genome (data not shown). Alternatively, it is possible that the different cDNAs originated from a family of highly related genes.

The TR binding site is conserved in all the cDNA clones (Figure 6B). Clones 144-052, 144-111 and 144-153 contain a polymorphism in the 3' half-site and a further polymorphism outside the core binding domain (Figure 6B). However, these base changes do not appear to impair TR homodimer binding, whereas the mutations introduced into both the TRE half-sites in the 144mut3 binding site effectively abolish TR binding (Figure 6C). Interestingly, there is a difference in TR–RXR heterodimer formation on the 144 binding site as compared with the 144-052 binding site. Although there does not appear to be a significant stabilization of TR–RXR heterodimers over TR homodimers on the 144 TRE, heterodimers are readily formed. On the 144-052 binding site, however, the pre-

dominant complexes are TR homodimers, even in the presence of RXR. The fact that the polymorphisms present in the TR binding site in the different cDNAs do not seem to impair TR binding suggests that all these clones contain a functional TR binding site.

The clone 144 TRE mediates activation by unliganded TR

The results from the alignment of genomic clone 144 sequences with their corresponding cDNA sequences allowed us to construct the appropriate CAT reporter plasmid. p144UTR-CAT was created by inserting the 230 bp *Afl*III–*Hae*III fragment containing the TR binding site into the 3' UTR upstream of the polyadenylation signal of pGLCAT4 (see Materials and methods). When transiently transfected into 293 cells, this construct mediated activation by TR in the absence of T3, while only basal CAT activity was observed in the presence of T3 (Figure 7). This result was consistent with the up-regulation of endogenous 144 mRNAs in the absence of T3 (Figure 1).

In accordance with the results from the gel mobility

Fig. 6. Genomic clone 144 is contained in the 3' UTR of the corresponding mRNAs. (A) Alignment of the genomic clone 144 with four different cDNA clones that were obtained from an oligo(dT)-primed GH4 cDNA library. The hatched box indicates the location of the sequence homologous to genomic clone 144. The numbers indicate the percentage of conservation of the sequences contained in the cDNA compared with the genomic clone. The numbers in the white boxes indicate the percentage of conservation among the cDNA clones as compared with clone 144-152. Clone 144-153 appears to be an alternatively spliced form that lacks an exon as compared with the other cDNAs. (B) Comparison of the TRE sequences of the different cDNA clones. Nucleotides that differ from the genomic clone 144 are in italics. 144mut3 is a synthetic mutant designed not to bind TR. (C) Gel mobility shift assay comparing TR binding with the immunoprecipitated genomic clone 144 TRE with binding to the alternate TRE sequence found in clones 144-052, 144-111 and 144-153, as well as the synthetic mutant 144mut3. Baculovirus-expressed TR α was incubated with reticulocyte lysate that did or did not contain *in vitro* translated RXR α . Bound and free complexes were separated on a 5% polyacrylamide gel and the dried gel autoradiographed for 16 h at -70°C . HD: TR-RXR heterodimer; D: TR homodimer.

shift assays, which showed binding of TR α as well as TR β to the 144 TRE, both receptor forms were able to transactivate this reporter TRE, as shown in Figure 7.

TR α p40, which bound only very weakly to the 144 element in gel mobility shift assays, was unable to transactivate p144UTR-CAT (data not shown). As a control for the T3 effect, we used the point mutant TR α H379G, which has a significantly reduced ability to bind T3 (Bigler *et al.*, 1992). This mutant receptor, when used with T3-inducible TREs, constitutively repressed expression of the reporter gene in the absence and presence of ligand (data not shown). With the 144 TRE linked to the reporter gene, we observed only a slight, statistically insignificant, reduction in activation by TR α H379G in the presence of T3, further supporting the notion that this TRE mediates T3-dependent regulation. The alternate TRE sequence found in three different cDNA clones containing 144 sequences (Figure 6B) was also able to mediate activation by unliganded receptor (Figure 7B), whereas the synthetic mutant TRE144mut3 failed to display TR- and T3-dependent transcriptional regulation, consistent with the lack of TR binding to this site.

In *in vitro* binding assays, TR-RXR heterodimers can form complexes with DNA that are more stable than TR homodimers. This enhanced binding is reflected in a higher degree of transactivation when both receptors are present as compared with TR alone (Yu *et al.*, 1991; Zhang, X. *et al.*, 1992). Although we were able to reproduce this effect with TREpal and the F2 TRE, the presence of RXR in addition to either TR α or TR α p40 had no discernible effect on transactivation when p144UTR-CAT was used as a reporter (data not shown). The notion that RXR may play no role in transactivation through the 144 TRE is supported by the results from the 144-052 TRE, which preferentially binds TR homodimers *in vitro* (Figure 6) and is able to mediate activation by unliganded TR.

T3 effect on expression levels is transcriptional

Although the regulation that we observed with the 144 TRE inserted in the 3' UTR was dependent on both TR and T3, it was conceivable that this regulation occurred at a post-transcriptional level. There is some precedent for this, as estrogen recently was found to induce expression of a 3' UTR binding protein that recognizes a sequence which is important for the stability of the target RNA (Dodson and Shapiro, 1994). Furthermore, since circular plasmids were used in the transactivation assays, it was impossible to determine whether the clone 144 TRE functioned as a 3' element or as a far 5' element.

In order to address the latter question, we stably integrated a CAT reporter construct with or without the 144 TRE in the 3' UTR position into the GH4 cells. For this purpose, the CAT reporter constructs were co-transfected with a plasmid carrying the neomycin resistance gene as a selectable marker. Of the neo-resistant colonies, 10% expressed functional CAT protein. Since these cells express sufficiently high levels of endogenous TR α , it was not necessary to transfect the receptor. The CAT-containing cell lines were then grown in the absence or presence of 100 nM T3. Figure 8A shows that these stably integrated reporter constructs were regulated in a T3- and TRE-dependent manner. As in the transient transactivation assays, CAT activity was induced in the absence of T3. Thus, it appears that a TRE can function when located in the 3' UTR.

To determine whether the repression of endogenous 144

Fig. 7. The clone 144 TRE mediates activation by unliganded TR. (A) 293 cells were transfected with the reporter plasmid p144UTR-CAT containing the clone 144 TRE in the 3' UTR, and either empty expression vector (C) or expression vector containing cDNAs encoding chicken TRα, rat TRβ or mutant chicken TRαH379G, which is deficient for T3 binding. (B) p144UTR-CAT constructs that contained either the 144-052 or the 144mut3 TRE in place of the 144 TRE were transfected into 293 cells together with either empty expression vector (C) or chicken TRα (TR). The CAT activity obtained in the absence of TR and T3 was arbitrarily set at 1. The numbers represent the average of at least three experiments $\pm$ standard deviation.

Fig. 8. (A) T3 regulation of stably integrated CAT reporter constructs. GH4 cells containing stably integrated CAT reporter constructs with or without the clone 144 TRE inserted in the 3' UTR (cf. Figure 7) were grown in the absence or presence of 100 nM T3 for 40 h. The CAT activity represents the average $\pm$ standard deviation from a representative experiment done in triplicate. (B) Nuclear run-on transcription of endogenous genes from untransfected GH4 cells grown in the presence or absence of 100 nM T3. The autoradiograph shows a representative experiment and the numbers for the rate of transcription represent the average of three experiments $\pm$ standard deviation. BS: pBluescribe(+); GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

mRNAs levels in the presence of T3 was transcriptional, we performed nuclear run-on assays (Groudine *et al.*, 1981), measuring the transcription rates of 144 mRNAs in untransfected GH4 cells in the presence and absence of T3. Figure 8B shows that the endogenous 144 mRNAs were transcribed at a higher rate in the absence of T3 than in its presence. Note that the rate of transcription of the control mRNAs encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and histone 2B was unchanged under these conditions. These results show that T3 regulates 144 mRNA expression at the transcriptional level.

Is transactivation from the 3' UTR specific for the 144 TRE?

The transactivation assays with stably transfected reporter genes and the nuclear run-on assays described above

indicated that TR was able to modulate transcription through a TRE located in the 3' UTR. These results raised two questions: (i) can the 144 TRE function upstream of the promoter, where TREs are normally located, and (ii) can other TREs function when placed in the 3' UTR? In order to address these questions, we inserted several TREs either upstream of the promoter or in the 3' UTR. In addition to an oligonucleotide representing the clone 144 TRE (see Materials and methods) and the 230 bp *AflIII*-*HaeIII* fragment used in the previous experiment, we chose TREpal, F2 TRE and the DR4 element (Umesono *et al.*, 1991) representing palindromic, inverted palindromic and direct repeat arrangements of the TRE half-sites, respectively. Figure 9A shows the transactivation potential of the reporter constructs with the TREs inserted upstream of the promoter. As expected, TREpal, F2 TRE and the DR4 element conferred T3 inducibility upon the

A

B

Fig. 9. Effect of location of the TRE on its ability to regulate transcription. The TREs TREpal, F2, DR4, 144TRE and 144 (see Materials and methods) were inserted either upstream of the promoter (A) or in the 3' UTR (B) of the CAT reporter construct and transfected into 293 cells either in the presence (TR) or absence (control) of co-transfected TR α . The numbers represent the average $\pm$ standard deviation from at least three independent experiments. The hatched boxes in the schematic diagrams of the vectors indicate the position of the TREs.

reporter construct, although DR4 mediated only very weak transactivation. Somewhat surprisingly, the constructs containing the 144 TRE oligonucleotide or the 230 bp fragment were not functional in this position. When the TREs were inserted in the 3' UTR, as shown in Figure 9B, no transactivation was obtained with the F2 TRE or DR4. Unliganded TR activated the construct containing the 230 bp fragment of clone 144 and, to a lesser extent, the construct carrying the 144 TRE oligonucleotide. A slight activation by unliganded receptor was also observed with TREpal.

The 230 bp *AflIII*–*HaeIII* fragment containing the 144 TRE was also inserted into the CAT construct 3' of the poly(A)⁺ site. Transcriptional activation obtained with unliganded TR was comparable with the activation levels obtained when the same fragment was located in the 3' UTR. The clone 144 TRE without its normal flanking sequences had no transcriptional effect 3' of the poly(A)⁺ site (data not shown).

The results from these transactivation experiments indicate that location of the TRE relative to the promoter may, in part, determine whether it can regulate transcription, since the 144 TRE, either as the oligonucleotide alone or with flanking sequence, conferred significant T3 regulation only when located in the 3' UTR. TREpal mediated greatly reduced activation when located in the 3' UTR, and F2 and DR4 activated transcription in response to T3 only upstream of the promoter but not in the 3' UTR.

Sequences flanking the 144 TRE determine TR activity in the 3' UTR

Although both the 144 TRE oligonucleotide and the 230 bp fragment were able to mediate activation by unliganded TR, there was a difference in transactivation potential between those two sequences, which suggested that the additional sequences contained in the larger fragment might contribute to the TR activity in the 3' UTR. In order to address this question, we deleted the TRE from the 230 bp fragment of clone 144 and replaced it with either TREpal, F2 or DR4, and inserted this chimera into the 3' UTR of the CAT reporter construct. As shown in Figure 10A, all three TREs mediated a T3 transcriptional response when they were flanked by clone 144 sequences, although the degree of transactivation, with the exception of DR4, was less than in their natural position upstream of the promoter. Interestingly, all three chimeric constructs still confer T3 inducibility, as they do when positioned upstream of the promoter. Activation by unliganded receptor, which is characteristic for the 144 TRE, is not detected. Thus, it appears that 144 sequences flanking the TRE may provide a binding site for a factor that is instrumental in transcriptional regulation by TR in the 3' UTR. In order to test this idea, we deleted increasing amounts of 144 flanking sequence. Figure 10B shows that deletion of 80 bp has no effect on the ability of the 144 TRE to mediate transactivation, but deletion of a further 56 bp results in the loss of transactivation. These results support the notion that TR needs to interact with another factor in order to regulate transcription.

Discussion

We used an immunoprecipitation method whereby TR complexed with chromatin was precipitated with a specific anti-TR antiserum to identify new endogenous binding sites for TR. Clone 144 possessed the properties that we would expect of a sequence that can function as a TRE in the cell: (i) it bound TR specifically and with an affinity comparable with previously identified TREs; (ii) it conferred T3-dependent regulation upon a reporter construct; and (iii) it was part of an mRNA that was regulated by T3 in the cells from which it was isolated.

The TRE in clone 144 consists of two consensus half-

Fig. 10. Sequences flanking the 144 TRE allow TR to function. (A) Reporter constructs where TREpal, F2 or DR4 were substituted for the 144 TRE were transfected into 293 cells either in the presence (TR) or absence (control) of co-transfected TR α . (B) 144UTR-CAT reporter constructs containing variable amounts of flanking sequences were transfected into 293 cells either in the presence (TR) or absence (control) of co-transfected TR α . The fold activation was obtained by calculating the CAT activity -T3/CAT activity +T3. The numbers represent the average $\pm$ standard deviation from at least three independent experiments.

sites arranged as inverted palindromic repeats and mediates activation by unliganded TR. This mechanism of transactivation has been described so far only for two other TREs (Saaticioglu *et al.*, 1993; Bigler and Eisenman, 1994) and is distinct from the T3-induced repression described for the negative TREs regulating TSH α and β (Chatterjee *et al.*, 1989; Carr *et al.*, 1992). Unliganded TR has no

effect on a negative TRE, but transcriptional activity is repressed below basal levels in the presence of T3. However, when under the control of the 144 TRE, a reporter construct shows enhanced transcriptional activity in the presence of unliganded receptor, while the addition of T3 results in basal transcriptional activity.

The clone 144 TRE shares the TRE half-site arrangement with two other TREs, the F2 and RSV TREs (Baniahmad *et al.*, 1990; Saaticioglu *et al.*, 1993), which, despite the same half-site arrangement, have different functional properties. The RSV TRE mediates activation by unliganded receptor and is the only known TRE that is responsive only to TR α but not TR β . The F2 TRE is responsive to both receptor forms and mediates T3-induced activation. The clone 144 TRE has properties in common with both of these sites: it mediates activation by unliganded receptor like the RSV TRE, but is responsive to both TR α and TR β , like the F2 TRE. The very weak binding of TR α p40 to the clone 144 TRE and its inability to transactivate through this TRE are unusual and may be functionally significant, since TR α p40 is expressed in certain cell types at levels equal to the full-length form (Bigler and Eisenman, 1988).

At the present time, it is not understood what properties of the DNA binding site or the receptor govern the mechanism of transactivation mediated by TR. Location of the TRE, presence or absence of T3 and binding of TR to a TRE as a homodimer or heterodimer with RXR or other nuclear factors may play a role. In addition, interactions with as yet uncharacterized proteins may also influence activity. It appears that TR, as well as retinoic acid receptor and vitamin D receptor, functions only when bound to a response element as a heterodimer with either RXR (for review, see Stunnenberg, 1993) or other nuclear factors. TR-RXR heterodimers on the 144 TRE were formed readily in *in vitro* assays. It is noteworthy, though, that transactivation of the 144 TRE by TR in the absence of T3 but not in its presence is more consistent with the *in vitro* DNA binding properties of TR homodimers than of TR-RXR heterodimers. TR homodimers are bound to the 144 TRE in the absence of T3, which would allow the receptor to activate transcription. In the presence of T3, on the other hand, TR is unable to bind to this site (data not shown) and therefore does not affect transcription. TR-RXR heterodimers are bound to the 144 TRE in the absence as well as in the presence of ligand but, in transactivation assays (data not shown), RXR neither enhanced activation by unliganded TR nor did it allow activation in the presence of T3 by preventing dissociation of TR from DNA. These results lead to the suggestion that TR may regulate transcription through the 144 TRE as a homodimer and not as a heterodimer with RXR. This notion is supported further by the *in vitro* DNA binding and transactivation properties of the alternate 144 TRE found in the cDNA clones 144-052, 144-111 and 144-153, which preferentially binds TR homodimers and mediates transactivation like the 144 TRE. This TRE potentially represents a TR-specific binding site. In addition, since the clone 144 TRE is located in the 3' UTR, TR may interact with a different set of factors, resulting in transactivation mechanisms different from those of other TREs located in the promoter region.

Transcription regulatory elements are frequently located

in the promoter region or upstream of it, but enhancers were also identified in the 3'-flanking region of the gene they regulate (e.g. Choi and Engel, 1986; Trainor *et al.*, 1987; Donoghue *et al.*, 1988; Langston and Gudas, 1992). The 3' UTR is an unusual location for a binding site for a transcription factor. These regions are better known for harboring sequences determining efficiency of translation as well as mRNA stability and degradation (for review, see Atwater *et al.*, 1990; Sachs, 1993; Decker and Parker, 1994). There are, however, indications that 3' UTRs may be carriers of additional functions. The expression of the 3' UTR of several muscle structural genes *in trans* was found to lead to the activation of other muscle-specific genes, as well as the suppression of proliferation in 10T1/2 cells (Rastinejad and Blau, 1993). The 3' UTR of α -tropomyosin, in particular, was able to suppress tumor formation by a myogenic cell line in mice (Rastinejad *et al.*, 1993). It has not been established yet by what mechanisms these 3' UTRs exert their effect as regulators of proliferation. Evidence that 3' UTRs may contain transcriptional regulatory elements or may regulate expression levels has been reported for several genes (Dale *et al.*, 1992; Kruys *et al.*, 1992; Zhang, W. *et al.*, 1992; Inoue *et al.*, 1993; Tazawa *et al.*, 1994). The presence in some of these genes of either an estrogen response element (Inoue *et al.*, 1993), a cyclic AMP response element (Tazawa *et al.*, 1994) or a TRE (Zhang, W. *et al.*, 1992) led to the assumption that transcriptional effects were responsible for the regulation of these genes by estrogen, cAMP and T3, respectively. Nevertheless, regulation at the transcriptional level was not rigorously established, and other regulatory mechanisms, such as changes in mRNA stability, could explain the observed effects. Regulation by T3 through the 144 TRE in transactivation assays is dependent on the presence of both the receptor and the response element. The endogenous mRNAs in GH4 cells containing the 144 TRE were transcribed at a higher rate in the absence of T3 than in its presence, as determined by nuclear run-on assays, indicating that regulation by TR through this TRE in the 3' UTR occurs at the transcriptional level. The ability to regulate transcription of a target gene through response elements in the 3' UTR may be a property common to other transcription factors, and the identification of further, naturally occurring response elements will be needed to confirm this mechanism of regulation.

The notion that TR, when bound to the 144 TRE in the 3' UTR, interacts with other factors is supported by our analysis of clone 144 sequences flanking the TRE. When TREs were inserted into the 3' UTR of the reporter construct by themselves, activation by TR was either absent or very weak. However, all TREs tested gained T3-dependent regulatory activity in the 3' UTR when flanked by clone 144 sequences. Deletion analysis suggested that clone 144 sequences downstream from the TRE are instrumental in TR transcriptional regulation through a TRE in the 3' UTR. The effect of the flanking sequences is not necessarily specific for a location in the 3' UTR, since the 144 flanking sequences can confer function to a TRE 3' of the transcription unit as well (data not shown). Thus, the additional factor may allow a TRE to regulate transcription when it is located a certain distance from the promoter. These sequences appear to

act by establishing a permissive context for TR activity, allowing TR to transactivate in their presence, but not affecting the mode of transactivation, i.e. activation by unliganded receptor or activation by T3. The adjoining sequences may provide binding sites for additional regulatory factors which could influence TR activity. Evidence for the modulation of the activity of a transcription factor by other DNA binding proteins was reported recently for the dorsal switch protein 1, which determines whether the transcription factors Dorsal and NF- κ B function as activators or repressors (Lehming *et al.*, 1994).

In summary, our data suggest that the location of a TRE potentially plays a crucial role in determining TR function, since various flanking sequences could juxtapose TR to specific regulatory proteins. Our studies provide further evidence for the versatility of TR, and suggest that factors interacting with TR may, to a large extent, determine its mode of action.

Materials and methods

Plasmid constructs

The genomic fragments containing TR binding sites were cloned into the *Sma*I site of pBS(+) (Stratagene) (Bigler and Eisenman, 1994). The corresponding, partial cDNA clones, p144-052, p144-111, p144-152 and p144-153 were isolated from an oligo(dT)-primed GH4 cDNA library and contained inserts from 1.3 to 3 kb (to be described elsewhere).

Reporter plasmids for transactivation assays were constructed by inserting a 225 bp fragment of clone 144 containing the TR binding site either into the *Hpa*I site or the *Hind*III and *Xba*I sites of the CAT reporter plasmid GLCAT4 (Nyborg *et al.*, 1990), positioning the TR binding site 5' of the poly(A)⁺ site or upstream of the promoter, respectively. For the reporter constructs TREpal-UTRCAT, F2-UTRCAT and DR4-UTRCAT, oligonucleotides representing the respective sites (Glass *et al.*, 1988; Baniahmad *et al.*, 1990; Umesono *et al.*, 1991) were inserted into the *Hpa*I site of pGLCAT4. pTREpal-CAT and pF2-CAT were as described previously (Bigler *et al.*, 1992) and for pDR4-CAT the oligonucleotide was inserted into the *Sal*I site upstream of the tk promoter in pGLCAT4. In order to substitute heterologous TREs for the clone 144 TRE, a 70 bp *Bgl*II-*Syl*I fragment was deleted from the clone, the overhangs blunt-ended with the large fragment of DNA polymerase I, *Xho*I linkers added and oligonucleotides representing 144mut3, TREpal, F2 and DR4 ligated into the *Xho*I site. For deletions, the 144 fragment was digested with *Afl*III and either *Ava*II or *Alu*I, which removed 80 and 136 bp of 144 flanking sequences, respectively.

Plasmids expressing chicken TR α and rat TR β 1 were constructed by insertion of the corresponding cDNAs (Murray *et al.*, 1988; Sap *et al.*, 1990) into the *Eco*RI site of pEMSV (Harland and Weintraub, 1985). The plasmids encoding chicken TR α mutants TR α H379G and TR α p40 were described previously (Bigler *et al.*, 1992).

Chicken TR α and human RXR α constructs used for *in vitro* transcription were described previously (Bigler *et al.*, 1992; Bigler and Eisenman, 1994). A rat TR β cDNA encompassing the sequences from -37 to +2165 (Murray *et al.*, 1988) was inserted into pBluescribe(+) and transcribed with T7 RNA polymerase.

Northern analysis

For Northern analysis, GH4 cells were transferred into Dulbecco's modified Eagle medium (DMEM) supplemented with charcoal-stripped fetal bovine serum (5%) and charcoal-treated horse serum (12.5%) with or without the addition of 100 nM T3. Cells were harvested at the indicated times and poly(A)⁺ RNA was obtained following standard procedures. poly(A)⁺ RNA (2 μ g) were separated on a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane according to standard procedures. The blots were hybridized with 5 $\times$ 10⁶ c.p.m. of random primed clone 144 probe in 50% formamide at 42°C overnight. As controls, a rat GAPDH cDNA clone and a genomic clone containing the first exon of human growth hormone were used. The amount of probe in the bands was quantified using a PhosphorImager 400A from Molecular Dynamics (Sunnyvale, CA) (Johnston *et al.*, 1990) and the program ImageQuant.

Gel mobility shift assays

Genomic DNA fragments digested with restriction enzymes were gel purified and end-labeled with T4 polynucleotide kinase and [γ - 32 P]ATP (ICN; 7000 Ci/mmol). Synthetic oligonucleotides representing the clone 144 TR binding site (5' TCGAGATCTCTCTCCTTAATGGGAGGTG-ATCGTGTCCACCTAGTACGTCGA 3') as well as the rat growth hormone TRE (Brent *et al.*, 1989a), the palindromic TREpal (Glass *et al.*, 1988) and the DR4 element (Umesono *et al.*, 1991) were labeled with the large fragment of DNA polymerase I, [α - 32 P]dCTP and [α - 32 P]dTTP (NEN; 800 Ci/mmol).

The binding reactions were done as described (Bigler and Eisenman, 1994), using ~0.5 ng of labeled DNA and 0.5 μ l of partially purified baculovirus-expressed TR α or control protein from Sf9 cells infected with wild-type baculovirus or 5–10 μ l of *in vitro* translated proteins. In samples containing antiserum, 1.5 μ g of affinity-purified anti-TR antibodies 5794 (Bigler and Eisenman, 1988) or anti-max antibodies 7094 (Blackwood *et al.*, 1992) were added after 5 min and incubation continued for 15 min at room temperature. The DNA–protein complexes were separated on a 5% polyacrylamide gel in 25 mM HEPES buffer pH 7.5.

DNase I footprinting and methylation interference

Gel-purified fragments were uniquely end-labeled with T4 polynucleotide kinase and [γ - 32 P]ATP (ICN, 7000 Ci/mmol). DNA binding reactions were set up as described for gel mobility shift assays, using 1–2 μ l of partially purified baculovirus-expressed TR α or baculovirus control protein and ~1 ng of end-labeled DNA (~20 000 c.p.m.). Incubation was at room temperature for 10 min. DNase I digestion was terminated with phenol–chloroform (50:50) after 4 min. Labeled DNA was ethanol precipitated in the presence of 10 μ g of carrier yeast tRNA. The samples were separated on 5% sequencing gels next to G+A Maxam–Gilbert reactions (Bencini *et al.*, 1984) of the same DNA fragment.

For methylation interference assays, uniquely end-labeled DNA fragments were methylated for 50 min following standard procedures. Bound and free fragments were separated on a 5% polyacrylamide gel in 0.5 $\times$ Tris–borate buffer. The extracted fragments were cleaved with piperidine and separated on a 5% sequencing gel.

Transfections

Transient transactivation assays were carried out essentially as described previously (Bigler *et al.*, 1992), except for using 293 cells, which were maintained in DMEM supplemented with 10% fetal bovine serum. For transactivation assays, the cells were grown in medium supplemented with 10% charcoal-treated fetal bovine serum with or without 25 nM T3. The cells were transfected overnight in 6 cm dishes with 250 ng of reporter plasmid, 500 ng of expression plasmid and 25 ng of plasmid pCH110 (Pharmacia) expressing β -galactosidase and harvested 24 h after transfection.

GH4 cell lines stably expressing the reporter constructs pGLCAT4 and p144UTR-CAT were created by electroporating 1×10^7 GH4 cells in phosphate-buffered saline in the presence of 20 μ g of the appropriate CAT reporter plasmid as well as 1 μ g of pRSVneo as a selectable marker. The settings for the electroporation with a Bio-Rad GenePulser were 250 μ F and 400 V. Clones were selected in the presence of 200 μ g/ml G418 (Gibco/BRL) and then screened for CAT expression.

Sequencing

Genomic DNA was sequenced using the Taq Dye Primer cycle sequencing kit (Applied Biosystems) and the sequencing products were analyzed on an ABI 373A DNA sequencer. Genbank (release 87.0) and EMBL (release 41.0) database searches were performed at NCBI using the blast network service (Altschul *et al.*, 1990). The programs Bestfit and Pile up of the GCG package version 7 were used for sequence alignments (Devereux *et al.*, 1984).

Nuclear run-on transcription

For nuclear run-on transcription, GH4 cells were transferred to medium supplemented with charcoal-treated serum and incubated in the absence or presence of 100 nM T3 for 28 h. The isolation of nuclei from GH4 cells for run-on transcription, nuclear transcription and hybridization were performed as described (Groudine *et al.*, 1981; Linial *et al.*, 1985). *In vitro* transcribed RNA from GH4 cells was hybridized to 5 μ g of denatured, double-stranded plasmid DNA which was bound to Hybond N membranes (Amersham) using a slot blot apparatus. The probes used were a plasmid containing a 3 kb cDNA insert homologous to genomic clone 144 and representing 3' untranslated sequences, chicken TR α cDNA and, as controls, the cDNAs for rat GAPDH (Tso *et al.*, 1985)

and human histone 2B. The amount of RNA hybridizing was quantified using a PhosphorImager and the program ImageQuant (Molecular Dynamics, Sunnyvale, CA).

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