Activation of *myoD* gene transcription by 3,5,3'-triiodo-L-thyronine: a direct role for the thyroid hormone and retinoid X receptors

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ABSTRACT

Thyroid hormones are major determinants of skeletal muscle differentiation *in vivo*. Triiodo-L-thyronine treatment promotes terminal muscle differentiation and results in increased *MyoD* gene transcription in myogenic cell lines; furthermore *myoD* and *fast myosin heavy chain* gene expression are activated in rodent slow twitch muscle fibers (Molecular Endocrinology 6: 1185–1194, 1992; Development 118: 1137–1147, 1993). We have identified a T_3 response element (TRE) in the mouse MyoD promoter between nucleotide positions -337 and -309 (5' CTG*AGGTCA*GTACA-*GGCTGG*AGGAGTAGA 3'). This sequence conferred an appropriate T3 response to an enhancerless SV40 promoter. *In vitro* binding studies showed that the thyroid hormone receptor α (TRα) formed a heterodimeric complex, with either the retinoid X receptor α or γ1 isoforms (RXRα, RXRγ), on the MyoD TRE that was specifically competed by other well characterised TREs and not by other response elements. Analyses of this heterodimer with a battery of steroid hormone response elements indicated that the complex was efficiently competed by a direct repeat of the AGGTCA motif separated by 4 nucleotides as predicted by the 3–4–5 rule. EMSA experiments demonstrated that the nuclear factor(s) present in muscle cells that bound to the myoD TRE were constitutively expressed during myogenesis; this complex was competed by the myosin heavy chain, DR-4 and PAL-0 TREs in a sequence specific fashion. Western blot analysis indicated that TRα1 was constitutively expressed during C2C12 differentiation. Mutagenesis of the myoD TRE indicated that the sequence of the direct repeats (AGGTCA) and the 4 nucleotide gap were necessary for efficient binding to the TRα/RXRα heterodimeric complex. In conclusion our data suggest that the TRE in the helix loop helix gene, *myoD*, is a target for the direct heterodimeric binding of TRα and RXRα/γ. These results provide a molecular mechanism/model for the effects of triiodo-L-thyronine on *in vitro* myogenesis; the activation of *myoD* gene expression in the slow twitch fibres and the cascade of myogenic events regulated by thyroid hormone.

INTRODUCTION

Thyroid hormone levels play a major role in regulating contractile protein isoform expression *in vivo* during adult muscle development as highlighted by myosin heavy chain isoform switches and the subsequent effects on the velocity of contraction (1). Thyroid hormones exert marked effects on cardiac and skeletal muscle, and result in a substantial reorganization of the myocyte which in turn, alters contractile protein performance and ion transport. In skeletal muscle, hyper-thyroidism results in an increase in type II fast fibers, whereas hypothyroidism results in a decrease in muscle mass and a reduced number of myofibres (1–6). Thyroid hormone regulates the steady-state levels of a many different contractile protein mRNAs (1,7,8 and references therein). Triiodo-L-thyronine treatment has been shown to activate or repress gene expression in a fiber type specific manner in adult rodents (1). Furthermore, embryonic and neonatal genes can be re-induced in adult muscle fibers by hypothyroidism (1). In general, long term hyper- and hypothyroidism precipitates in the precocious and retarded expression, respectively, of the normal contactile protein isoform profile that is modulated during embryonic, neonatal and adult development. The molecular basis for these observations (2–6,9,10) has not been elucidated.

MyoD and myogenin mRNAs preferentially accumulate in fast and slow twitch muscle respectively (9). Recently, it has been observed that triiodo-L-thyronine treatment of myogenic cell lines and adult rodents, promotes terminal muscle differentiation, results in increased *MyoD* gene transcription and the activation of *MyoD* gene expression in soleus slow twitch fibers (11,12). The MyoD protein belongs to a family of myogenic specific helix loop helix (HLH) proteins [MyoD, Myogenin, Myf-5, MRF-4/myf-6/herculin] that directs the fate of pluripotential mesodermal embryonic cells and indirectly/directly activate muscle specific genes involved in terminal differentiation and contraction. The MyoD gene family is differentially expressed

and temporally regulated during embryonic and adult mouse development. These proteins contain a 68 amino acid conserved basic/*myc*-like region that is necessary and sufficient for myogenic conversion. The basic region and adjacent HLH motif mediates DNA binding and dimerisation. The MyoD family forms heterodimers with ubiquitously expressed members of the HLH protein family, such as E12 and E47 (the alternatively spliced products of the E2A gene). The MyoD-E2A heterodimers bind to a consensus DNA binding sequence, the E-box motif, that includes a CANNTG motif, present in most muscle specific enhancers (reviewed in 13,14,15). The expression and function of these hierarchical regulators is modulated by growth factors, oncogene products, innervation and hormones (summarised and discussed in ref. 16).

The effects of thyroid hormones are mediated by the intracellular thyroid hormone receptors (TR), that are encoded by two distinct genes, c-*erbA* α and c-*erbA* β. The c-*erbA* α gene is alternatively spliced into α1 and α2 (hormone and non-hormone binding) isoforms respectively. Intriguingly, the c-*erbA* α locus has also been demonstrated to contained an overlapping transcription unit utilizing coding information on the opposite strand (rev-erb) . The α2 and rev-erb isoforms seem to regulate the function of c-*erbA*-α1 (17,18). TRs bind to response elements containing tandem direct [and degenerate] repeats, of the AGGT-CA N_x AGGTCA motif with spacings of 3 or 4 nucleotides and activate gene transcription (19−24). TR requires accessory/auxiliary factors for high affinity binding to their cognate sequences (25−27). The retinoid X receptor (RXR) family is one of these accessory proteins that is activated by 9-cis retinoic acid (9-*cis* RA). The RXRs heterodimerize with TRs and function to selectively target the high affinity binding of these receptors to their cognate elements (20,23,24,28,29,30).

Although TRs and RXRs are abundantly expressed in cardiac and skeletal muscle, the studies to date did not reveal whether the ligand activated thyroid hormone and retinoid X receptors directly activated MyoD transcription in myogenic cell lines and slow twitch fibers. In this report we document the identification of a thyroid hormone response element (TRE) in the mouse myoD gene that directly interacts with a thyroid hormone/retinoid X receptor heterodimer; and characterise the sequences and motifs in this TRE that are necessary for an efficient DNA−receptor interaction. These results indicate that T3 directly controls the expression of myogenic specific helix loop helix proteins and provide a mechanism for the changes in the contactile protein isoform profile observed in adult rodents exposed to T3.

MATERIAL AND METHODS

Cell culture and transfection

Mouse myogenic C2C12 cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 20% fetal calf serum (FCS) in 6% CO_2 as described previously (43). This cell line was induced to biochemically and morphologically differentiate into multinucleate myotubes by mitogen withdrawal [DMEM supplemented with 2% fetal calf serum in 6% CO_2]. Differentiation was essentially complete within 72−96 hours with respect to isoform switching in the actin multigene family (22). However, these cells will spontaneously differentiate at a very high confluence (100%) in the presence of mitogens. COS-1 cells were grown in DMEM supplemented with 10% FCS in 6% CO_2.

COS-1 cells were cultured for 24h in T3 and T4 deficient medium containing 5% charcoal stripped FCS in DMEM prior to transfection. Each 60mm dish of COS-1 cells (60−70 % confluence) was transiently transfected with 4−5 μg of reporter plasmid DNA expressing Luciferase, mixed with 0.01−2μg of receptor expression vectors and pUC18 plasmid to a total of 10 μg in each transfection experiment by the DOTAP (Boehringer Mannheim) mediated procedure as described previously (7,8). The DNA/DOTAP mixture was added to the cells in 6 mls of fresh culture (T3 and T4 deficient) medium. After a period of 24 hours, fresh medium ±T3/ 9-cis RA (10^{-7} M) was added to the cells. The cells were harvested for the assay of Luciferase enzyme activity 24 h after the transfection period. Each transfection experiment was performed at least three times in order to overcome the variability inherent in transfections (7,8).

Luciferase assays

Cells from 60 mm cell culture dishes were washed twice in PBS and harvested in 200 μl 1× cell culture lysis reagent (25 mM Tris−phosphate pH 7.8, 2mM DTT, 2mM 1,2-diaminocyclo-hexane-N,N,N′, N′-tetraacetic acid, 10% glycerol , 1% Triton and 1 mg/ml BSA. 5−20 μl of the cell extract was mixed with 100 μl of reconstituted Luciferase assay reagent (20 mM Tricine, pH 7.8, 1.07 mM [$MgCO_3$]$_4$Mg[OH]$_2$-5H$_2$O, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 0.27 mM coenzyme A, 0.47 mM luciferin and 0.53 mM ATP). The reactions were placed in a luminometer after 20−45 second incubation, followed by 5×5 second measurements.

Plasmids

The plasmid pGL-2-promoter [an enhancerless SV40 promoter linked to luciferase in a pUC19 backbone] was purchased from Promega. The plasmid, pCMV-rTRα expressing the rodent c-*erbA* α gene in the eucaryotic expression vector CMV 4, containing the cytomegalovirus promoter and SV40 origin of replication was described by Zilz *et al.*, 1990 (31). The plasmids, pSG 5-RXRα and γ expressing the mouse RXRα and γ gene in the eucaryotic expression vector, pSG5 containing the early SV40 promoter; β-globin intron II for splicing and SV40 origin of replication was described by Leid *et al.* 1992 (28). The pGEX-1-cTRα plasmid was described by Muscat *et al.* 1993 (7). We constructed pGEX3-mRXRγ1 by excising the mouse RXRγ1 cDNA from the pSG 5 expression vector by Eco R1 digestion. This cDNA was cloned in frame into EcoR1 cleaved pGEX-3. Sense and anti-sense orientation clones were identified by two double digests *Pst* 1/*Hind* III and *Pst* 1/*Xba* 1. The pGEX-2T-hRXRα was described in Manglesdorf *et al.*, 1991 (20). The sense and anti sense strands of the myoD TRE sequences with gatc ends were annealed, phosphorylated with T4 polynucleotide kinase and self ligated with T4 DNA ligase. These products were then cloned into the Bgl II site in the pGL-2-promoter vector from Promega and initially screened for inserts by Hind III /Sac I digestion. Clones identified by restriction analyses were sequenced by double stranded sequencing to determine the orientation and number of copies cloned. The clone utilised in these studies pMyoD-GL-p contained three copies of the MyoD TRE (−339/−307) in the following orientation ← ← ← (−307/−339) with respect to the direction of luciferase transcription.

Expression and purification of receptors

Human RXRα, mouse RXRγ and chicken TRα were expressed as fusions with glutathione-S-transferase (GST) using the pGEX-2T, pGEX-3 and pGEX-1 bacterial expression vectors. BL21(DE3)pLysS cells or DH5α cells containing these expression vectors were induced for 1−2 hours with 0.4 mM IPTG after the cells had grown to an OD_{600} of ~0.6. The GST fusion proteins were affinity purified with glutathione−agarose columns as described previously by Muscat and Downes et al., 1993 (7, 8). Quantification of TR and RXR after affinity purification was achieved by Bradford determination with the Biorad protein assay reagent, followed by SDS PAGE to determine the purity of the preparation (these proteins were usually 20−50% pure).

Nuclear extracts and gel mobility shift assays

Nuclear extracts were prepared as decribed previously (7,8). Each binding mixture (25−30 μl) contained 1−2 ng of a T4 Polynucleotide Kinase labeled DNA fragment, 1−30 pmol of purified receptors or 5−10 μg of crude nuclear extract, and 1−2 μg of poly dI-dC as a non-specific competitor [only when crude nuclear extract was used] in Dignam buffer C. The assays were incubated at room temperature for 20 min. and electrophoresed through a 6% (20:1 polyacrylamide:bisacrylamide) gel in 80 mM Tris borate and 2 mM EDTA. Gels were briefly soaked in 10% acetic acid, dried and autoradiographed.

Western blots

Antigen containing cellular extracts (10 μg) were elecrophoresed on 10% SDS PAGE, electroblotted/transferred onto Hybond ECL. Non specific binding sites were blocked by immersing the membranes in 5% dry milk, 1×PBS and 0.02 % NaN_3 for 1−2h. The membrane was subsequently washed in 1×PBS containing 0.1% Tween 20, followed by an incubation with a 1 in 100 dilution of the antibody in blotto for 16h at 4°C with shaking. Further steps involving secondary antibodies and the catalyzed oxidation of luminol were carried out with the Amersham ECL western blotting detection reagents according to the manufacturer's protocols.

RESULTS

The retinoid X and thyroid hormone receptors form heterodimers that bind with high affinity to the mouse myoD TRE

We scanned the mouse *myoD* gene promoter (33) for a putative thyroid hormone response element (TRE) that could account for the *in vitro* and *in vivo* effects of 3,5,3' triiodo-L-thyronine (T_3) treatment on MyoD expression. Using the established DR-4 binding motif, AGGTCA [or a degenerate consensus motif; RGG^A/$_T$YN; R= purine, Y=pyrimidine, N=nucleotide), recognised by TR/RXR complexes, we identified a putative TRE between nucleotide positions −337 and −309 (5' CTG-AGGTCAGTACAGGCTGGAGGAGTAGA 3'). This putative TRE sequence was arranged as a direct repeat of the core binding motif with a 4 nucleotide gap and was accommodated by the tandem direct repeat model [3−4−5 rule] proposed by Evans and Rosenfeld (21,22,29,30). We characterized the putative mouse myoD TRE between nucleotide positions −337/−309 by demonstrating that it bound heterodimers of the thyroid hormone and retinoid X receptor with high affinity.
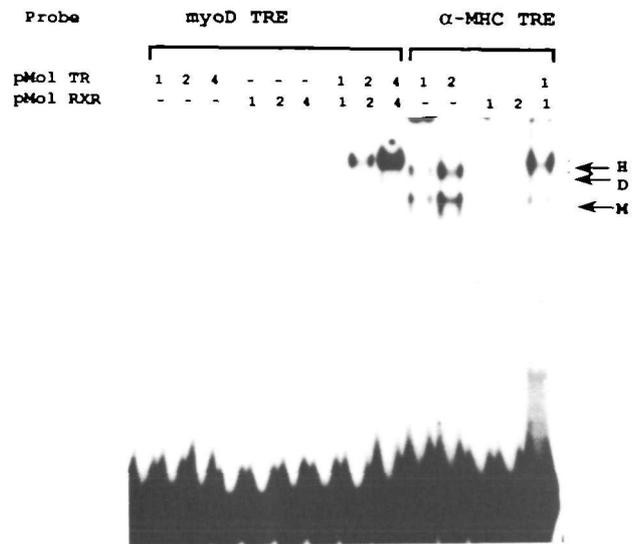

Figure 1. The thyroid hormone and retinoid X receptors form heterodimeric complexes on the myoD and α-MHC TREs. *E.coli* expressed and affinity purified TRα and RXRα were incubated with the myoD and α-MHC TRE. H, D and M denote heterodimer, dimer and monomer formation on the probe respectively.
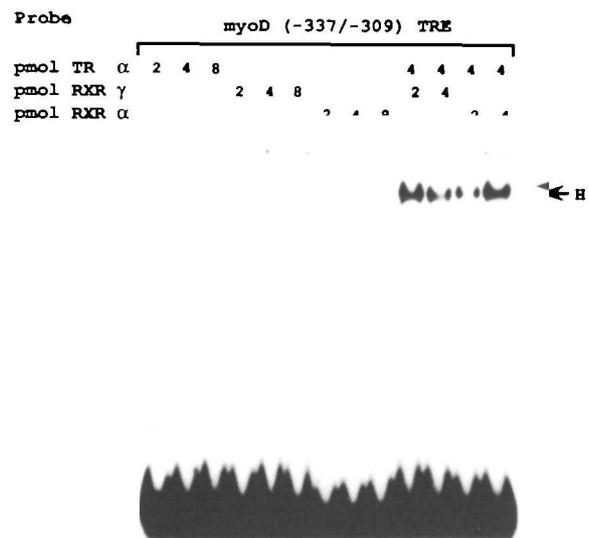

Figure 2. The thyroid hormone receptor form heterodimeric complexes with either the α or γ isoform of the retinoid X receptor on the myoD TRE. *E.coli* expressed and affinity purified TRα and RXRα/γ were incubated with the myoD TRE. H, D and M denote heterodimer, dimer and monomer formation on the probe respectively.

A large body of evidence indicates that the RXR/TR heterodimer rather than the TR homodimer is the entity that recognises functional thyroid hormone response elements (TRE) (19,20,23,24,28,29,30). We investigated the ability of bacterially expressed thyroid hormone and retinoid X receptors (TRα and RXRα) to interact with the putative myoD TRE. We used the well characterised rodent α-myosin heavy chain (MHC) TRE as a heterodimerisation control; three groups have demonstrated that RXR heterodimerizes with TR and markedly enhances the
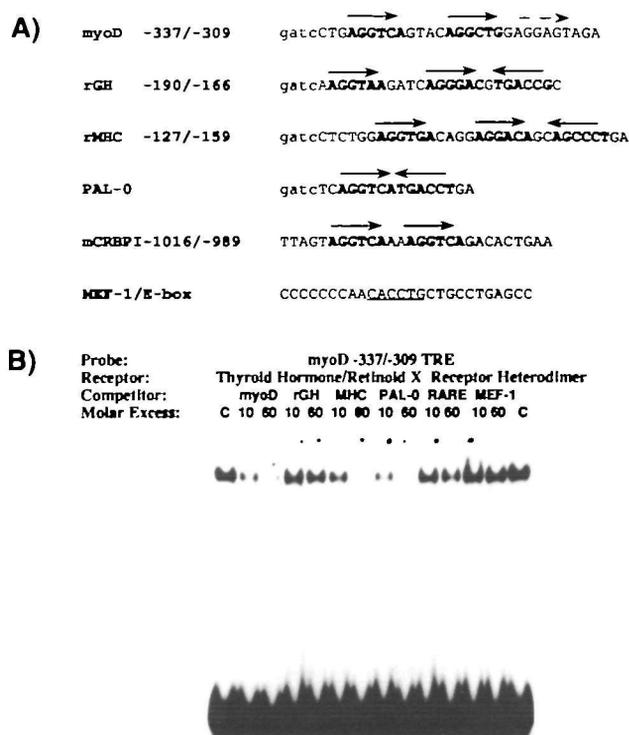
Figure 3. A. The thyroid hormone response element (TRE) in the mouse myoD promoter and from a number of other characterized genes. The synthetic direct repeat response elements based on the 3−4−5 rule are also depicted. The sequences of one strand of each double-stranded oligonucleotide probe are depicted. The direction and location of the TREs are indicated by solid arrows. The dashed arrow represents the location of a weak TRE in the MM promoter. These sequences match the core sequence binding motif for the thyroid hormone receptor: RGG$^{T}/_{A}$CN (R = purine, N = nucleotide). PAL-0 is a palindromic/inverted repeat arrangement of motifs that confers a transcriptional response to RXR, TR and RAR. CRBP I is a retinoic acid response element (RARE) in the cellular retinol binding protein I. MEF-1/E-box, Myocyte enhancer factor 1; interact with basic helix loop helix proteins. The nucleotide positions are indicated with respect to the transcriptional start site at +1. **B.** The myoD TRE interacts with TRα/RXRα heterodimer in a sequence specific fashion. The effect of competition (10−60 fold molar excess), by self, GH, MHC and PAL-0 characterized TRE response elements and other non-TRE DNA binding sites (MEF-1, mCRBP I RARE) on the complex formed between the probe myoD (−337/−309) TRE and the TRα/RXRα heterodimer [4 pmol of each receptor]. The molar excess of each DNA competitor is indicated. C, denotes the control binding reaction in the absence of any unlabelled competitor.

binding to the MHC TRE. Electrophoretic mobility shift analyses (EMSA) was used to evaluate the homodimeric and heterodimeric binding of RXRα and TRα to the myoD TRE. Increasing amounts (1, 2 and 4 pmol) of RXRα and TRα were incubated with fixed quantities of the myoD TRE and the defined MHC TRE (Figure 1). This data demonstrated that TRα in pmol qauntities did not interact with the myoD TRE, however, addition of equivalent amounts of RXRα induced heterodimerization and markedly enhanced binding of the receptor complexes to their cognate sequences similar to the α-MHC TRE heterodimerization control (Figure 1). The data here suggests that RXR may play an important role in modulating the TR interaction to this sequence. This data raises the possibility that the myoD TRE −337/−309 may be a site for direct cross-talk between two different hormonal signals (9-cis RA and T$_3$) at the receptor level.

The RXR family (α-, β-, γ-) is abundantly expressed in a variety of adult tissues (34,35). RXRα and β are expressed in many tissues but most abundantly in liver, lung, kidney and cardiac/skeletal muscle. The RXR γ gene encodes two isoforms γ1 and γ2 (35). The γ1 isoform is specifically and abundantly expressed in adult skeletal muscle (35). Hence, we examined the ability of RXRγ1 relative to the RXRα isoform to induce heterodimerization and markedly induce binding of TR to the MyoD TRE. It was found that RXRα and RXRγ1 behaved in a very similar fashion with respect to the formation of heterodimers on the myoD TRE (Figure 2).

The interaction of the TR and RXR heterodimer with the mouse myoD TRE [−337/−309] is specifically competed by classical TREs

We conducted electrophoretic mobility shift analysis (EMSA) competition studies using the classical and well characterized natural rodent growth hormone (rGH) and α-myosin heavy chain (αMHC) TREs, and the synthetic PAL-0 TRE sequences to assess the sequence specific binding of the TRα/RXRα heterodimer to the myoD [−337/−309] sequences. The sequence of the oligonucleotides used in this study are listed in figure 3A with the TRE orientations defined by arrows. The competition studies were carried out at 10 and 60 fold molar excess of oligonucleotide with respect to the myoD TRE −337/−309 probe (Figure 3B). These studies demonstrated that the complex formed between the −337/−309 sequences and TRα/RXRα heterodimer could be specifically competed by the established wild type TREs (that have been demonstrated to interact with heterodimeric receptor complexes). The rGH TRE only competed at > 100-fold excesses (data not shown). However, the CRBP I RARE (cellular retinol binding protein I, retinoic acid response element) and MEF-1/E-box (Myocyte enhancer factor 1 binding sequences; that interact with muscle specific helix loop helix -MyoD family of proteins) did not compete for the formation of the heterodimeric complex on the TRE. These results indicated that the −337/−309: TRα/RXRα interaction was highly specific and involved in the triiodo-thyronine signalling pathway.

Direct repeats of the sequence RGGTCA N$_x$ RGGTCA with spacings of 4 and 5 specifically competed the formation of the TRα/RXRα heterodimer on the MyoD TRE

A functional relationship among the RXR, Perixosome proliferator activated receptor (PPAR), COUP-TF, vitamin D receptor (VDR), TR and RAR has recently been described in which these receptors bind and activate through direct repeats of the AGGTCA motif separated by 1, 3, 4 and 5 (x = 2, also binds RXR and RAR). This rule applies to most of the natural hormone response elements. In an effort to further characterize the myoD TRE: TRα/RXRα complex we synthesized as described in Kliewer *et al.* (32) an array of synthetic hormone response elements as predicted by the 3−4−5 rule, arranged as direct repeats of the AGGTCA motif with spacings of 1, 2, 3, 4 and 5 nucleotides [designated DR-1, DR-2, DR-3, DR-4 and DR-5 respectively] (Figure 4A).

We tested these hormone response elements in EMSA competition assays to assess the specificity and gap/spacing preference of the TRα: RXRα heteromeric binding to the myoD −337/−309 sequence. DR-1, DR-2, DR-3, DR-4 and DR-5 were used in the binding reactions at 10 and 60-fold molar excess with respect to the myoD TRE probe (Figure 4B). This data indicated that DR-4/DR-5 > DR-3 > DR-2 > DR-1
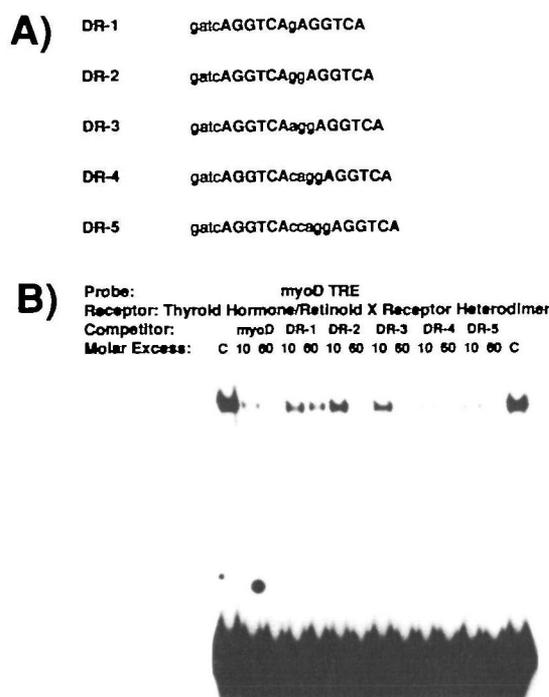
A)

DR-1	gatcAGGTCAgAGGTCA
DR-2	gatcAGGTCAggAGGTCA
DR-3	gatcAGGTCAaggAGGTCA
DR-4	gatcAGGTCAcaggAGGTCA
DR-5	gatcAGGTCAccaggAGGTCA

B)

Probe: myoD TRE
Receptor: Thyroid Hormone/Retinoid X Receptor Heterodimer
Competitor: myoD DR-1 DR-2 DR-3 DR-4 DR-5
Molar Excess: C 10 60 10 60 10 60 10 60 10 60 10 60 C

Figure 4. A. The synthetic Direct Repeat response elements based on the 3−4−5 rule are depicted. The sequences of one strand of each double-stranded oligonucleotide probe are depicted. The direction and location of the TREs are indicated by solid arrows. DR-1, DR-2, DR-3, DR-4 and DR-5, are arranged as direct repeats of the 'AGGTCA' motif with spacings of 1, 2, 3, 4 and 5 nucleotides. A functional relationship among the RXR, VDR, TR and RAR and has recently been described in which these receptors bind and activate through tandem direct repeats AGGTCA N_x AGGTCA with spacing of 1,3,4 and 5 nucleotides respectively ($x=2$, mediates a positive response to RA and negative response to T3). B. The MM TRE interacts with TRα/RXRα heterodimer and was preferentially competed by direct repeats with a 4 or 5 nucleotide gap. The effects of competition by a battery of hormone response elements, designated DR-1, DR-2, DR-3, DR-4 and DR-5, on the complex formed between the myoD TRE probe and TRα/RXRα [4 pmol of each receptor]. The molar excess of each DNA competitor is indicated. C, denotes the control binding reaction in the absence of any unlabelled competitor.

preferentially competed for the formation of the TRα/RXRα heteromeric complex on the myoD TRE. These experiments rigourously demonstrated that the TRα and RXRα heterodimer complex on the myoD TRE was selectively competed by direct repeats of the AGGTCA half site motif separated by 4 nucleotides and provided strong evidence that the −337/−309 sequences functioned as a TRE site according to the 3−4−5 rule. These analyses were analogous to the work of Umesono *et al.* (22) that showed DR-4/DR-5 > DR-3 preferentially competed for the formation of the TRb heterodimeric complex on a sequence that functioned as a TRE in transfection studies.

The myoD TRE interacts with a factor(s) from myogenic nuclear extracts that is specifically competed by characterised TREs and DR-4 sequence motifs

We conducted electrophoretic mobility shift analysis (EMSA) competition studies using well characterized natural and synthetic TRE sequences to assess the sequence specific binding of myogenic nuclear factors to the myoD [−337/−309] sequence. The sequence of the oligonucleotides used in this study are listed in figure 3A with the TRE orientations defined by arrows.
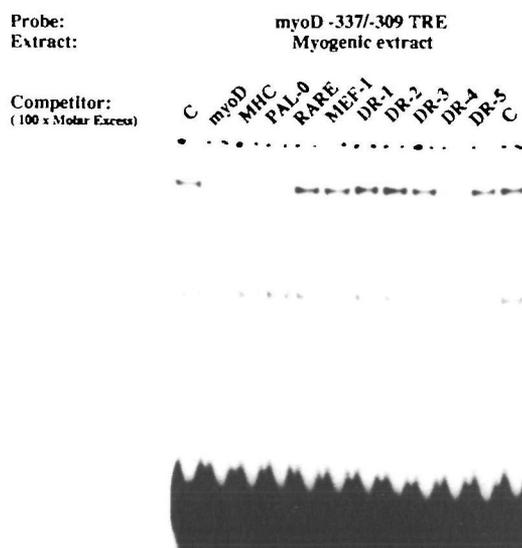
Probe: myoD -337/-309 TRE
Extract: Myogenic extract

Competitor: C myoD MHC PAL-0 RARE MEF-1 DR-1 DR-2 DR-3 DR-4 DR-5 C
(100 x Molar Excess)

Figure 5. The myoD TRE interacts with factor(s) from myogenic extracts in a sequence specific fashion: The myoD TRE sequence interacts with factor(s) in myogenic cextracts that are specifically competed by thyroid hormone response elements. Myogenic extracts were derived from C2C12 cells that were differentiated by 24h of mitogen withdrawal. The effect of competition by a number of AGGTCA containing hormonal response elements on the complex formed between the myoD TRE probe and myogenic factors. The molar excess of each DNA competitor is indicated. C, denotes the control binding reaction in the absence of any unlabelled competitor. MEF-1, myocyte enhancer factor-1.

Furthermore, we tested the battery of synthetic hormone response elements (DR-1, DR-2, DR-3, DR-4 and DR-5) in EMSA competition assays to assess the specificity and gap/spacing preference of the myogenic nuclear factor(s) from differentiated C2C12 cells that interact with the myoD −337/−309 sequences. These competitors were used in the binding reactions at ~ 100-fold molar excess with respect to the myoD TRE probe (Figure 5). These studies demonstrated that the complex formed between the −337/−309 sequence and the myogenic nuclear factor(s) could be specifically competed by self, the established/characterized α-MHC, PAL-0 and DR-4 TREs. However, the CRBP I RARE, MEF-1/E-box (Myocyte enhancer factor 1 binding sequences; that interact with muscle specific helix loop helix -MyoD family of proteins); DR-1, DR-2, DR-3 and DR-5 did not compete for the formation of the myogenic factor on the myoD TRE. These results indicated that the sequence specific interaction of the −337/−309 sequences with nuclear factors was involved in the triiodo-thyronine signalling pathway.

Nuclear extracts were prepared from mouse myogenic C2C12 myoblasts and myotubes in different stages of differentiation to assay the developmental regulation of TRE-bound proteins in muscle. Specifically, extracts were isolated from proliferating myoblasts (PMB), confluent myoblasts (CMB), and myotubes (MT-1). These extracts were assayed for the levels of Oct-1, which is a constitutively expressed factor and used to standardise the amount of nuclear extracts used in the experiments. The myoD TRE sequences interacted with a nuclear factor denoted as MTBF [MyoD TRE Binding Factor(s)] *in vitro* that was constitutively expressed during the differentiation of mouse myogenic cell lines relative to the ubiquitously expressed Oct-1 protein (Figure 6A). Similarly, it has been observed that the human skeletal α-actin and mouse myogenin TREs interact with

myogenic nuclear factors that were constitutively expressed during myogenesis (7,8). We further characterised the expression of TRα1 [the hormone binding form] during myogenesis by Western blot analyses using the the commercially available antibody, PA1-211, from Affinity Bioreagents. The polyclonal antibody, PA1-211, specifically recognises TRα1 (48KDa) from humans and rodents and not TRα2 or TR β. It was derived from a synthetic peptide (aa 403−410, extreme C-terminal end) that is downstream from the point of sequence divergence from the α-2 isoform. PA1-211 detected a ~ 50 KDa band in all stages of muscle differentiation and in the COS-1 cells transfected with pCMV-rTRα (Figure 6B). These experiments indicated that TRα-1 protein was constitutively expressed during myogenesis. The antisera also recognised a 130 kDa band in myogenic extracts which was not present in the pCMV-rTRα transfected COS-1 cells, that indicated this band was due to non-specific binding. These data correlate with the results of Carnac *et al.* (12) who observed the constitutive expression of c-erb A α1 and α2 mRNAs that encode TRα1 and TRα2 during the differentiation of mouse myogenic cell lines.

Mutagenesis of the myoD TRE identifies the RGGTCA motifs as essential TRα, RXRα binding sites

We characterized the nucleotides in the myoD TRE that interact with TRα and RXRα heterodimer by mutating the myoD TRE sequentially from the 5′ to 3′ direction by six mutations that spanned the putative half site motifs. These mutant TREs were designated, M1−M5 (See Figure 7A) and used in EMSA competition analyses to ascertain their ability to disrupt the complexes formed between the myoD TRE and TRα/RXRα heterodimer. We independently incubated wild type myoD TRE [−337/−309] probe with cTRα/RXRα heterodimer (Figure 7B) and competed with 10, and 60 fold molar excesses of the M1, M2, M3, M4 and M5 mutant TREs. Figure 7B depicts the ability of the mutant myoD TRE oligos, M1−M5, to compete for binding to the heterodimer. The mutant myoD TREs M1, M3, and M5 competed efficiently for binding to the TRα/RXRα heterodimer. This demonstrated that the sequences flanking the two half-site RGGTCA motifs and the sequence of the 4nt gap were not important for binding to the complex. In contrast, the M2 and M4 mutant myoD TREs did not compete for binding to the heterodimeric complex. This data strongly implicated the importance of the half site sequence and requirement for the direct repeats [i.e. two half sites] in the efficient formation of a TR/RXR heterodimer on the myoD TRE.

Sequences between nucleotide position −337 and −309 in the mouse myoD promoter confer T3 regulation to an enhancerless simian virus 40 promoter

The formation of TR/RXR heterodimers that strongly and selectively interact with a target sequence, correlate with a functional T3 dependent trans-activation *in vivo*. An important question to address is whether the strong and specific heterodimeric binding between TR and RXR on the myoD TRE can be directly correlated with T3 dependent trans-activation *in vivo*. We then cloned the −337/−309 sequences into an enhancerless SV40 promoter linked to the firefly luciferase gene, [pGL-2-promoter, Promega] and conducted experiments to see if the myoD TRE conferred appropriate hormonal regulation to a heterologous promoter. The resultant plasmid, pmyoD-GL-p, contained three copies of the myoD tRE; similar multiple copy plasmids have been utilized by Chambon, Evans, Phafl and their
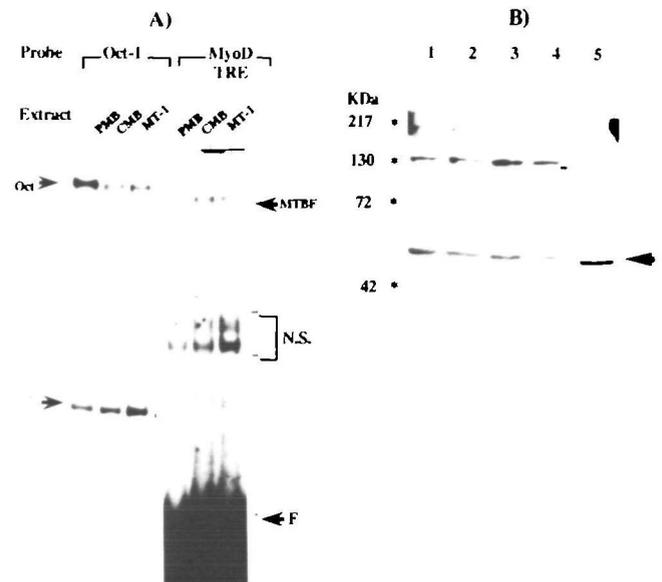

Figure 6. A. The myoD TRE sequence interacts with constitutively expressed factor(s) in myogenic extracts. PMB and CMB denote extracts derived from proliferating and confluent C2C12 myoblasts. MT-1 denote extracts derived from myotubes, 1 day after mitogen withdrawal. Oct-1, is a factor that is constitutively expressed and unaffected by the mitotic state of the cell. MTBF, denotes MyoD TRE Binding Factor(s). F, denotes free probe. N.S, denotes non-specific bands as a results of single stranded DNA binding proteins. B. The thyroid hormone receptor α1 is constitutively expressed during the differentiation of mouse myoblasts. Whole cell extracts were derived from mouse C2C12 cells. PMB and CMB denote extracts derived from proliferating and confluent myoblasts. MT-1 and MT-4 denote extracts derived from differentiated myotubes, 1 and 4 days after mitogen withdrawal by 24h of mitogen withdrawal. Lane 1, PMB; Lane 2, CMB; Lane 3, MT-1; Lane 4, MT-4 and Lane 5, COS-1 cells transfected with pCMV-TRα. Western blot analyses was performed with the Affinity Bioreagents antibody PA1-211 that specifically recognises TRα1 from humans and rodents.

colleagues in these types of experiments. (19,24,36,37,38). This plasmid, pmyoD-GL-p was transfected into COS-1 cells and grown in thyroid hormone and retinoid deficient medium in the presence of co-transfected TRα (2 μg). The myoD TRE sequences conferred a ~4 to 5 fold induction after T3 treatment to the enhancerless SV40 promoter (Figure 8A, lanes 5 and 6), whereas the basal vector, pGL-2-p was not stimulated by T3 (figure 8A, lanes 1 and 2). The basal vector, pGL-2-p, did not show any significant induction in the presence of RXRα (2 μg) alone and 9-*cis* RA (Figure 8A, lanes 3 and 4), however, the level of expression from pmyoD-GL-p was significantly repressed, relative to the basal vector (Figure 8A, lanes 7 and 8). Furthermore, RXRα (2 μg) completely suppressed the T3 dependent trans-activation (Figure 8A, lanes 9 and 10). We investigated this effect in more detail by co-transfecting the reporter, pmyoD-GL-p, in the presence of increasing quantities of RXRα (10−300 ng) with a fixed quantity of TRα (2 μg). This experiment indicated that >10 ng of co-transfected RXRα expression vector repressed the T3 dependent trans-activation (Figure 8B, lanes 1−5). These observations are supported by the recent report from the Phafl group (39) that demonstrated RXRs in excess in the presence of 9-cis RA form homodimers that sequester RXR from RXR-TR heterodimers and lead to the repression of the T3 response by ligand (9-cis RA) induced squelching. These results confirmed that the cis-acting region
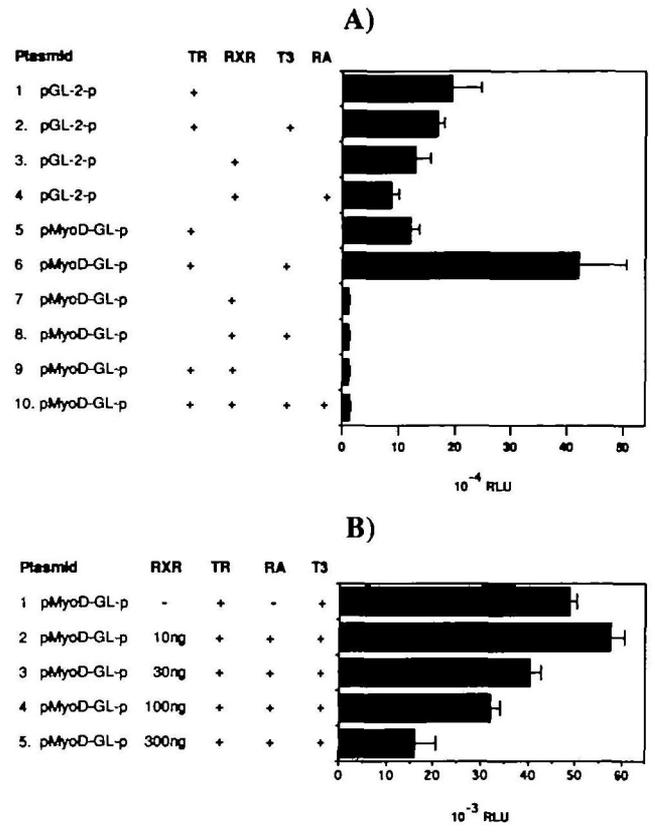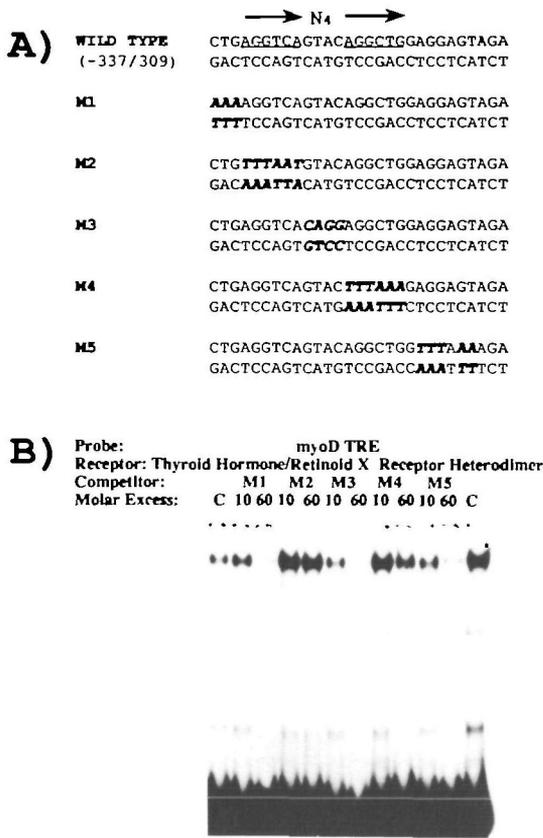
A)

			N4	
WILD TYPE	CTGAGGTCAGTACAGGCTGGAGGAGTAGA			
(-337/309)	GACTCCAGTCATGTCCGACCTCCTCATCT			
M1	**AAA**AGGTCAGTACAGGCTGGAGGAGTAGA			
	TTTTCCAGTCATGTCCGACCTCCTCATCT			
M2	CTG**TTTAAT**GTACAGGCTGGAGGAGTAGA			
	GAC**AAATTA**CATGTCCGACCTCCTCATCT			
M3	CTGAGGTCA**CAGG**AGGCTGGAGGAGTAGA			
	GACTCCAGT**GTCC**TCCGACCTCCTCATCT			
M4	CTGAGGTCAGTAC**TTTAAA**GAGGAGTAGA			
	GACTCCAGTCATG**AAATTT**CTCCTCATCT			
M5	CTGAGGTCAGTACAGGCTGG**TTTAAA**GA			
	GACTCCAGTCATGTCCGACC**AAAT TTT**CT			

B)

Probe: myoD TRE
Receptor: Thyroid Hormone/Retinold X Receptor Heterodimer
Competitor: M1 M2 M3 M4 M5
Molar Excess: C 10 60 10 60 10 60 10 60 10 60 C

Figure 7. **A.** Pictorial representation of the various site specific mutation in the mouse myoD TRE. The wild type TRE sequence is depicted and the mutations in the M1, M2, M3, M4 and M5 TREs are denoted by bold italic text. **B.** Mutational analyses of the mouse myoD TRE. The core receptor binding motifs in the myoD TRE are important for binding. The effects of competition by a battery of mutations in the myoD TRE, designated M1, M2, M3, M4 and M5 on the complex formed between the myoD TRE probe and TRα/RXRα heterodimer. The molar excess of each DNA competitor is indicated. C, denotes the control binding reaction in the absence of any unlabelled competitor.

A)

Plasmid	TR	RXR	T3	RA
1. pGL-2-p	+			
2. pGL-2-p	+		+	
3. pGL-2-p		+		
4. pGL-2-p		+		+
5. pMyoD-GL-p	+			
6. pMyoD-GL-p	+		+	
7. pMyoD-GL-p		+		
8. pMyoD-GL-p		+	+	
9. pMyoD-GL-p	+	+		
10. pMyoD-GL-p	+	+	+	+

10^{-4} RLU

B)

Plasmid	RXR	TR	RA	T3
1. pMyoD-GL-p	-	+	-	+
2. pMyoD-GL-p	10ng	+	+	+
3. pMyoD-GL-p	30ng	+	+	+
4. pMyoD-GL-p	100ng	+	+	+
5. pMyoD-GL-p	300ng	+	+	+

10^{-3} RLU

Figure 8. The myoD TRE confers hormonal regulation to an enhancerless SV40 promoter. The mean RLU's, relative light units, are shown for the plasmid, pMyod-GL-p, containing the myoD TRE sequence cloned into the pGL-2-promoter vector (See Materials and Methods for specific cloning details). Results are shown for transient transfections in COS-1 cells. Mean values and standard deviations were derived from a triplicate experiment. **A.** The myoD TRE sequence confers a T_3 response to the SV40 promoter in the plasmid pMyoD-GL-p. T3, denotes triiodo-L-thyronine and RA, denotes 9-cis RA. **B.** Over-expression of RXR represses the trans-activation of pMyoD-GL-p by TR/T_3. T_3, denotes triiodo-L-thyronine and RA, denotes 9-cis RA.

between nucleotide positions −337/−309 functions as a positively acting TRE in the muscle specific mouse myoD promoter.

DISCUSSION

Thyroid hormones are major determinants of skeletal muscle differentiation and play a role in regulating contractile protein isoform expression during muscle maturation. Triiodo-L-thyronine treatment of the myogenic cell line, C2.7, increased the steady state level of myoD mRNA and resulted in increased *MyoD* gene transcription in myoblasts. Furthermore, myogenin, myosin light chain 1A and troponin T, were precociously induced, and indicated that T_3 promotes terminal differentiation (12). Recently, it has been demonstrated that MyoD selectively accumulates in fast twitch fiber types, however, plantaris muscle, predominantly fast twitch, accumulates considerable myogenin mRNA. There transgene data suggested that MyoD activity was restricted to a subset of non IIA fast fibres (i.e IIx and/or IIb) (11). Furthermore, Peterson and colleagues showed that thyroid hormone treatment results in the significant induction of MyoD and fast IIA myosin heavy chain mRNA in slow twitch soleus

fibers and a slight activation of fast II B MHC mRNA (11). Soleus muscle from euthyroid animals contains fast IIA MHC , but not MyoD or II B fast MHC (1,11).

Our study, has shown that the T3 influenced induction of myoD mRNA in rodent muscle and myogenic cell lines involves direct transcriptional mechanisms. Transfection experiments and EMSA were used to identify a functional T_3 response element (TRE) in the mouse myoD gene. This TRE is located between nucleotide positions −337 and −309 (5′ CTGAGGTCAGTACAGGCTGGAGGAGTAGA 3′) and is accommodated by the DR-4 TR/RXR binding motif, **RGGTCA**. This TRE sequence conferred a ~ 4−5 -fold induction to an enhancerless SV40 promoter, after T3 treatment in a TR-dependent manner. EMSA experiments showed that *Escherichia coli* expressed/affinity purified TRα and RXRα/γ formed a heterodimeric complex that bound very efficiently to the myoD TRE. This is in agreement with all the recent data that defines the heterodimer rather than the homodimer as the entity that efficiently recognises thyroid hormone response elements. Futhermore, the TR/RXR complex on the myoD TRE was specifically and selectively competed by other natural and synthetic TREs; in contrast a natural RARE

and the DR-1, DR-2 and DR-3 sequences that interacted with a wide variety of other steroid receptors (41) [RXR/PPAR, COUP-TF and VDR]; and the MEF-1/E-box sites did not compete for the formation of the TR/RXR complex on the myoD TRE. These *in vitro* binding data correlated with the ability of these receptors after hormone treatment to trans-activate the myoD TRE *in vivo*, in agreement with a variety of other studies. Mutagenesis of the myoD TRE indicated that the two core RGGTCA binding motifs were necessary for efficient TRα/RXRα heterodimer formation. The myoD TRE interacted with MTBF from myogenic nuclear extracts that was specifically competed by the characterized the α-MHC, PAL-0 and DR-4 TREs but not by other cis-acting sequences. Our experiments have demonstrated that the −337/−309 cis-acting sequence interacts with the heterodimeric TR/RXR receptor complex with high affinity and sequence specificity similar to the well characterised α-MHC TRE, and that this sequence conferred an appropriate T3 response. This data satisfies the criteria that have evolved to define thyroid hormone response elements.

Our experiments suggest that the RXRs may modulate the interaction of TRα to the myoD TRE and that the −337/−309 sequence in the mouse *myoD gene* is a target for direct cross-talk between two different hormonal signals (T$_3$ and 9-cis RA) at the receptor level. This correlates with the abundant levels of c-erbAα (TRα) and RXR α/γ in adult muscle and myogenic cultures. To our knowledge this is the first demonstration of heteromeric binding of TRα and RXRα/γ to a TRE in the myoD gene, expressed specifically in skeletal muscle. Our results provide a molecular explanation for the increased *myoD gene* transcription observed after T$_3$ treatment of C2C12 myogenic cultures and the activation of myoD expression in slow twitch muscle fibres.

How does T$_3$ activate myoD expression in slow fibres and induce fast contractile protein gene transcription?

The effects of thyroid hormone levels in adult animals are only apparent in long term intoxication (9,10). T$_3$ treatment results in an increase in myoD mRNA in soleus muscle and type II fast fibers, whereas hypothyroidism reduces the number of these fibres (9,10). In hypothyroid rats expression of adult fast myosin is drastically reduced and neonatal myosin is abnormally high. The multigene MHC family is repressed and activated by thyroid hormone in a tissue and fiber type specific manner (1). These findings suggest that the hormonal influences on HLH expression and fiber type are quite complex and involve a variety of differentially regulated factors. We suggest that the differential expression and steady state levels of TR accessory factor isoforms, e.g RXR α, β and γ genes, in different muscle twitch types may mediate fiber type specific activation events. Although, differences in the uptake or processing of T3/T4 in different skeletal muscle types may account for some of these differences (42). All TREs interact efficiently with TR/RXR heterodimers *in vitro*, however, specific TREs display distinct RXR isoform preferences with respect to functional *trans*-activation (see 37). Furthermore, some TRE heterodimer complexes are very sensitive to RXR levels (7, 39). In support of our suggestion, it was recently shown that the RXR isoforms α, β and γ are expressed at different levels in skeletal muscle (34,35) and differentially modulated by T$_3$ (43) (RXR β and γ steady state mRNA levels are increased and repressed respectively). Our present study supports the hypothesis that T$_3$ directly targets the hierarchical HLH regulators. Our recent identification and

characterization of the TRE in the skeletal α-actin gene (7) that mediates hormonal induction implies that T$_3$ simultaneously targets the hierarchical HLH regulators and the contractile protein genes during the regulation of terminal differentiation. Fine tuned expression is most probably achieved by the MyoD family and other intermediate regulators such as MEF-2/RSRF family (44,45,46) that are part of a positive autoregulatory loop that maintain the required threshold levels of these muscle specific transcription factors. We are currently examining the fiber type specific distribution of the RXR isoforms and the relative levels of other c-erb A α gene products TRα2 and rev-erb A α which are known to modulate the function of TR.

ACKNOWLEDGEMENTS

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