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Characterization of a Low Affinity Thyroid Hormone Receptor Binding Site within the Rat GLUT4 Gene Promoter

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ABSTRACT

Previous studies have demonstrated that thyroid hormone (T_3) stimulates insulin-responsive glucose transporter (GLUT4) transcription and protein expression in rat skeletal muscle. The aim of the present study was to define a putative thyroid hormone response element (TRE) within the rat GLUT4 promoter and thus perhaps determine whether T_3 acts directly to augment skeletal muscle GLUT4 transcription. To this end, electrophoretic mobility shift analyses were performed to analyze thyroid hormone receptor (TR) binding to a previously characterized 281-bp T_3-responsive region of the rat GLUT4 promoter. Indeed, within this region, a TR-binding site of the standard DR+4 TRE variety was located between bases −457/−426 and was shown to posses a specific affinity for *in vitro* translated TRs. Interestingly, however, the GLUT4 TR-binding site demonstrated a significantly lower affinity compared to a consensus DR+4 TRE, and only bound TRs appreciatively in the form of high affinity heterodimers, in this case with the *cis*-retinoic acid receptor.

In conclusion, these data demonstrated the presence of a specific TR-binding site within a T_3-responsive region of the rat GLUT4 promoter and thus support the supposition that thyroid hormone acts directly to stimulate GLUT4 transcription in rat skeletal muscle. Moreover, characterization of a novel TR-binding site with low affinity suggests an additional mechanism by which the intrinsic activity and responsiveness of thyroid hormone regulated genes may be modulated. (*Endocrinology* **138:** 1215–1223, 1997)

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THE ENTRY of glucose into mammalian cells is mediated by a family of tissue-specific plasma membrane transport proteins (GLUT1 through 4), the process of which represents the rate-limiting step in glucose metabolism (1, 2). In tissues that express the insulin-responsive glucose transporter isoform (GLUT4), *i.e.* skeletal muscle, adipose, and heart, the uptake of glucose is also largely dependent upon insulin (3). In normal individuals, insulin-mediated glucose disposal functions largely to normalize circulating plasma glucose levels after a meal, approximately 85% of which occurs within skeletal muscle (4). However, defects in this process result in persistent hyperglycemia and hyperinsulinemia, and represent the ultimate cause of insulin resistance in noninsulin-dependent diabetes mellitus (NIDDM) (3).

The molecular defect in glucose disposal leading to NIDDM has been suggested to reside within the insulin signaling pathway (5–7), although cause and effect have not been established in this disease. Nevertheless, elucidating positive regulators of GLUT4 as well as their mechanism(s) of action are of considerable interest as a possible treatment in NIDDM. Overexpression of GLUT4 in transgenic diabetic mice has been shown to be highly effective in ameliorating postprandial hyperglycemia, primarily by stimulating basal (noninsulin-mediated) glucose disposal (8). Previous studies from our laboratory (9) and that of Weinstein *et al.* (10) have demonstrated that thyroid hormone (T_3) stimulates basal and, to some extent, insulin-mediated glucose uptake in rat skeletal muscle. The mechanism for this stimulatory effect of T_3 was determined to be due predominantly to an induction of GLUT4 protein expression (9, 10). In the preceding manuscript, GLUT4 induction by T_3 was further defined to be primarily via transcriptional induction in red muscle, and a separate translational and/or posttranslational mechanism in white muscle fiber types (11). Moreover, in a previous study, constructs containing various regions of the GLUT4 upstream of a reporter gene, when transfected into C2C12 myotubes and treated with T_3, isolated a 281-bp region responsive to thyroid hormone (12). However, further experiments are required to determine whether T_3 acts directly on GLUT4 transcription and whether transcriptional induction of skeletal muscle GLUT4 by T_3 or a previously suggested (11) muscle-selective T_3-receptor agonist represents a viable therapeutic strategy in NIDDM.

The effects of T_3 on gene transcription are mediated directly via a family of nuclear receptor/transcription factors: c-*erb*Aα1, -β1, and -β2 (13–19). These thyroid hormone receptors (TRs) bind to specific thyroid hormone response elements (TREs) consisting of hexameric half-sites [consensus: AGGT(C/A)A], orientated in singlet or multiplex configurations within the promoter regions of target genes. A limited number of TREs have been elucidated; many consist of directly repeated half-sites separated by 4 bp (DR+4) (13), whereas, an everted half-site TRE separated by 6 bp (F2) has been reported to function as a silencer in the chick lysozyme

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promoter (20, 21). TRs bind with high affinity to their cognate DNA elements in both the presence and absence of T_3; however, TRs only function to stimulate (on positive TREs) or repress (on negative TREs) gene transcription in response to binding thyroid hormone (13–15, 17–19). In contrast, unoccupied TRs on positive TREs (*e.g.* DR+4 elements) actively repress basal transcription (22–27).

TRs bind to composite TREs as monomers; however, cooperative interactions favor the formation of homodimers. Moreover, TRs interact with coregulatory proteins or thyroid hormone auxiliary proteins *in vivo*, some of which have been characterized [*e.g.* the *cis*-retinoic acid receptors (RXRs)] (28–34). TR/RXR heterodimers demonstrate higher binding affinities than TR homodimers, are favored in the presence of T_3, and produce larger increases in T_3-induced gene expression (13, 14, 32, 33). Indeed, heterodimers are considered to be the primary complexes mediating T_3-regulated gene expression *in vivo*.

The aim of the present study was to determine and characterize a putative TRE within the rat GLUT4 promoter and thus perhaps establish whether T_3 acts directly to stimulate GLUT4 transcription. Sequential electrophoretic mobility shift assay (EMSA) analyses using a previously described 281-bp T_3-responsive region of the GLUT4 promoter (12) identified a TR-binding site, orientated in the classical DR+4 motif, between bases −450 and −434. However, in contrast to other established TREs, the GLUT4 TR-binding element demonstrated a significantly lower affinity, and as a likely consequence only bound TRs appreciatively in the presence of RXR. Therefore, these data are also suggestive of an additional level of complexity that may govern the inducibility and responsiveness of T_3-regulated genes.

Materials and Methods

Plasmids and reticulocyte lysate-synthesized receptors

The previously described human complementary DNA clones for c-*erb*Aβ1 and c-*erb*Aα1 in pCMV (35) and for RXRα and retinoic acid receptor α (RARα) complementary DNAs in pSKXR3–1 (36) were used to program TNT T7-coupled reticulocyte lysates (Promega, Madison, WI). Unlabeled and [^{35}S]methionine-labeled receptors were synthesized in parallel using *in vitro* translation reactions, and the protein concentrations were determined from trichloroacetic acid-precipitable counts according to the manufacturer's instructions. The synthesis of the human α1 (hα1) TR, however, is not as efficient as those for β1 and RXRα and thus could not be used in sufficient amounts to give optimal results in EMSAs. In some experiments purified chick α1 TR was used to visualize α1 complexes more readily. This receptor was the gift of Dr. H. Samuels (New York University Medical Center, New York, NY). The isolation procedure for chick α1 has been described previously (37) and was used here in approximately equimolar ratios with hRXRα.

A plasmid containing a 281-bp *Eco*NI-*Bst*XI fragment of the GLUT4 promoter (bases −517 to −238), which accounts for full thyroid hormone responsiveness of 2212 bp from the transcriptional start site of GLUT4, was obtained from Dr. J. Pessin (University of Iowa, Iowa City, IA). Various double stranded DNA oligomers spanning this region were also designed to further localize the putative GLUT4 TRE. These were synthesized at the East Carolina University School of Medicine DNA-Core Facility.

Antibodies

The N-terminal polyclonal antibody specific for rat β1 (rβ1PAb, amino acids 62–92 in rat β1) and the C-terminal α1/β cross-specific polyclonal antibody (rα1/βPAb, amino acids 447–461 and 393–407 in rat β1 and α1, respectively) were provided by Drs. J. Oppenheimer and H. Schwartz (University of Minnesota, Minneapolis, MN). The hRXRα polyclonal antibody (RXRαPAb) was the generous gift of Dr. R. Evans (The Salk Institute, San Diego, CA).

Isolation and extraction of nuclei from rat skeletal muscle

Nuclei were isolated from rat skeletal muscle in the presence of protease inhibitors (0.5 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, 1 μg/ml aprotinin, and 10 mM EDTA) by the previously described method of Neufer *et al.* (38). Approximately 500 μg purified rat muscle nuclei were extracted using 100 μl cold (4 C) M4 extraction buffer [20 mM HEPES (pH 7.8), 0.4 M KCl, 2 mM dithiothreitol, and 20% glycerol] for 30 min (plus protease inhibitors), and the nuclear debris and DNA were pelleted at 12,000 rpm in a microfuge for 15 min. Nuclear extracts were assayed for protein concentration by the method of Brad-

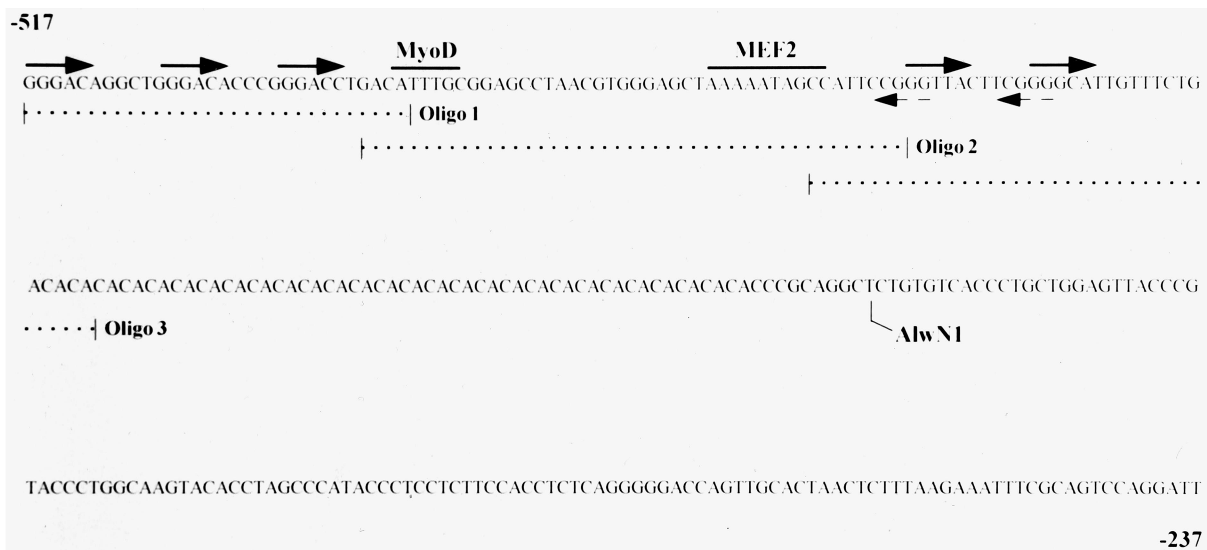

FIG. 1. Sequence of the previously described 281-bp thyroid hormone-responsive region of the GLUT4 promoter. *Arrows* show the putative TRE half-sites (consensus AGGT(C/A)A). MyoD, Consensus sequence for myogenic differentiation factor (MyoD); MEF-2, consensus sequence for MEF-2; AlwN1, cleavage site for restriction enzyme AlwN1. *Dotted lines* indicate the sequences included in double stranded DNA oligomers synthesized to determine the position of a putative GLUT4 TRE.

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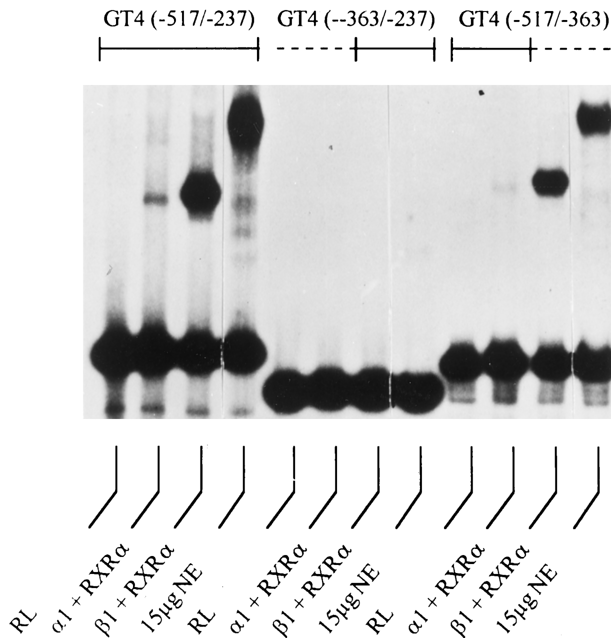

FIG. 2. EMSA using *in vitro* translated TR and skeletal muscle nuclear extracts with the full-length (−517/−237) and AlwN1 restriction fragments (−517/−363 and −363/−237) of the thyroid hormone-responsive GLUT4 promoter region. Lane 1, Reticulocyte lysate control; lane 2, hα1 TR and RXRα; lane 3, hβ1TR and RXRα; lane 4, 15 μg skeletal muscle nuclear extract.

ford (39) and were used either immediately for analysis or stored at −80 C until required.

EMSAs

EMSAs were performed as previously described (40). Briefly, approximately 100 ng (60,000 cpm) of a ^{32}P-labeled double stranded DNA oligomer were incubated with either 1 μl (10 fmol) reticulocyte lysate synthesized receptor (1 μl hβ1 and/or hRXRα) or 5 μg nuclear extract for 30 min at room temperature in a total volume of 20 μl reaction buffer [25 mM Tris (pH 7.8), 0.5 mM EDTA, 88 mM KCl, 1 mM dithiothreitol, 150 μg/ml poly(dI·dC)-poly(dI·dC), 0.05% Triton X-100, and 12.5 μg/ml sonicated salmon sperm DNA]. For supershift experiments, 1 μl polyclonal antisera was incubated with *in vitro* translated receptors for 30 min at room temperature before addition of the DNA oligomer. Reaction mixtures were subjected to electrophoresis on a 5% nondenaturing polyacrylamide gel at 4 C and 40 mA (200 V) for approximately 2.5 h, and the dried gels were exposed with intensifying screens to x-ray film for 16–48 h at −80 C.

Results

Identification of TR-binding site within a T_3-responsive region of the GLUT4 promoter

To identify a putative TRE within the rat GLUT4 promoter, a series of electrophoretic mobility shift analyses were performed using a 281-bp fragment of the rat GLUT4 promoter (Fig. 1), previously delineated in transient transfection studies to be T_3 responsive (12). EMSA using this fragment was sufficient to first establish the presence of a TR-binding site within the GLUT4 promoter. The binding of reticulocyte lysate-synthesized α1 and β1 TR/RXRα heterodimers to this 281-bp fragment is shown in the four *left lanes* of Fig. 2 (complex composition confirmed in Fig. 6). In addition, complexes were formed using rat skeletal muscle nuclear ex-

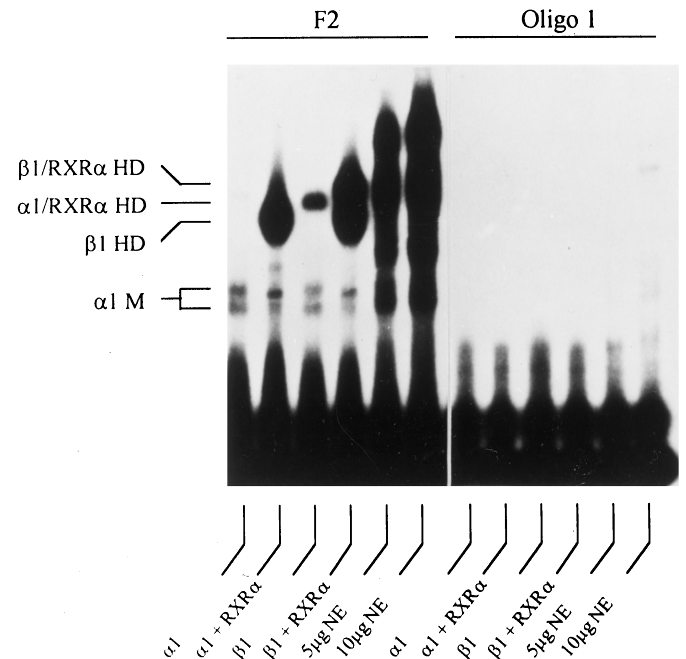

FIG. 3. EMSA using *in vitro* translated TRs and skeletal muscle nuclear extracts with the chicken lysozyme F2 TRE and sequences −526/−485 (oligo 1) of the thyroid hormone-responsive GLUT4 promoter region. Lane 1, hα1; lane 2, hα1 TR and RXRα; lane 3, hβ1; lane 4, hβ1TR and RXRα; lane 5, 5 μg skeletal muscle nuclear extract; lane 6, 10 μg skeletal muscle nuclear extract. M, Monomer; HD, homodimer or heterodimer.

tracts, in particular one that migrates closely with the human *in vitro* translated α1/RXRα heterodimer (Fig. 2, *left lanes* 4 and 2, respectively) and one highly abundant species with very low mobility. The lesser degree of hα1 TR binding to the GLUT4 sequence as well as other TREs used throughout this report is due to the lower efficiency of the *in vitro* translation reaction for hα1, such that equimolar ratios with hβ1 and RXRα could not be used in this assay.

Using two AlwN1 restriction fragments of the 281-bp promoter sequence, the GLUT4 TR-binding element was further localized to the 5′-fragment (Fig. 2). In addition, consistent with the presence of a MyoD and/or myocyte enhancer factor-2 (MEF-2) consensus binding site within the 5′-restriction fragment (Fig. 1), both the full-length and 5′-AlwNI sequences bound low mobility complex produced with muscle nuclear extracts (*left and right four lanes* of Fig. 2, respectively). Based on these data, three oligomers were synthesized to span the 5′-AlwN1 restriction fragment: oligo 1 (bases −526/−485), oligo 2 (bases −491/−452), and oligo 3 (bases −460/−418) (Fig. 1), to further define the position of the GLUT4 TRE as well as establish the putative binding of MyoD and/or MEF activities within this thyroid hormone-responsive region of the GLUT4 promoter. Specifically, oligo 1 encompassed the three upstream putative TRE sequences, oligo 2 covered the MyoD and MEF consensus binding sites, and oligo 3 spanned the remaining near-consensus TRE sequence within this AlwN1 restriction fragment (Fig. 1). Oligo 3 was not synthesized to cover the residual downstream sequences in the 5′-AlwN1 fragment, because this region is simply composed of a stretch of AC microsatellite dinucle-

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otide repeats and thus is highly unlikely to contain a specific TR-binding element.

The bindings of *in vitro* translated TRs and skeletal muscle nuclear extracts to oligo 1 and a previously characterized TRE (the chicken lysozyme F2 TRE) (20, 21) were compared in Fig. 3. These data clearly demonstrated that bases −526/−485 of the GLUT4 promoter do not contain a TR-binding element, although the putative TRE half-sites within this region (highlighted in Fig. 1) are close to the consensus AGGT(C/A)A. The experiment shown in Fig. 4, *left panel*, directly compared the binding of TRs and skeletal muscle nuclear extracts to oligos 2 and 3. Consistent with the respective putative consensus elements within these oligos (Fig. 1), binding of *in vitro* translated TRs was localized to oligo 3 (bases −460/−418), whereas the postulated MEF-2 and/or MyoD binding complex was defined within oligo 2. Moreover, competition of the oligo 2 binding species with a large excess of an unlabeled MEF-2, but not a MyoD consensus binding site, confirmed this DNA-binding species to be composed solely of MEF-2 (Fig. 4, *right panel*).

In summary, these data clearly demonstrated the presence of a TR-binding site within the GLUT4 promoter, the position of which was determined to be within bases −460/−418. However, two overlapping near-consensus DR+4 TRE sequences could equally be envisaged to be present within this region (Fig. 1, *solid and dashed arrows*, and Fig. 5, *top panel*). Therefore, to further define the boundaries and orientation of the GLUT4 TRE, two additional oligomers were synthesized with mutations designed to abrogate TR binding dependent upon which sequence represented the true TR-binding site (Fig. 5). Mutations within the 5′- to 3′-orientated putative TRE were shown to abolish TR binding, whereas mutations within the oppositely orientated sequence did not (Fig. 5). Therefore, these data unambiguously established the location of the GLUT4 TR-binding site between bases −457/−426, composed in a classical DR+4 arrangement orientated in the 5′ to 3′ direction.

Characterization of the GLUT4 TR-binding site

A series of experiments was performed to characterize in detail the specificity, affinity, and identity of TR complexes able to form on this newly distinguished TR-binding site. Supershift experiments (Fig. 6, lanes 1–6) first demonstrated that the TR complexes formed using *in vitro* translated TRs and hRXRα on the GLUT4 TRE were composed of TR/RXRα heterodimers. Moreover, the successful competition of these species with a large excess of an unlabeled oligo containing the F2 TRE, but not with a mutated (non-TR-binding) version of this element (M2; Fig. 6, lanes 7–12), demonstrated the specificity of the GLUT 4 sequence for TRs.

All EMSA experiments described thus far were performed for the most part by coincubating *in vitro* translated TRs with hRXRα, principally with the intent of visualizing the maximum possible number of TR/TRE interactions. However, only a single species has consistently been observed in these assays, *i.e.* DNA binding was unable to be demonstrated by TRs in the absence of hRXR. These data, therefore, suggest that binding of TRs to the GLUT4 TRE may be dependent upon heterodimerization. Hence, the GLUT4 TRE was next

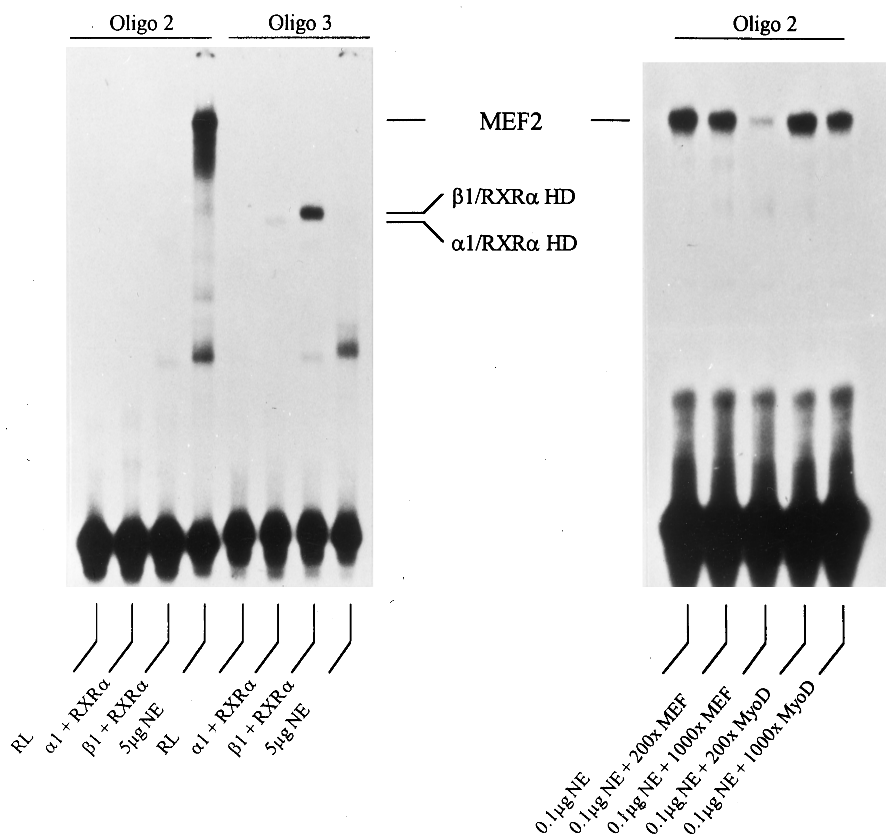

FIG. 4. EMSA using TRs, MEF-2A, and skeletal muscle nuclear extracts with oligomers spanning sequences −491/−452 (oligo 2) and −460/−418 (oligo 3) of the thyroid hormone-responsive GLUT4 promoter region. Lanes 1 and 5, Reticulocyte lysate control; lanes 2 and 6, purified chick α1 TR and RXRα; lanes 3 and 7, hβ1TR and RXRα; lanes 4 and 8, 15 μg skeletal muscle nuclear extract; lane 9, 0.1 μg nuclear extract (NE); lane 10, 0.1 μg NE and a 200-fold excess of a MEF-2 consensus DNA-binding element; lane 11, 0.1 μg NE and a 1000-fold excess of MEF-2 DNA; lane 12, 0.1 μg NE and a 200-fold excess of a MyoD consensus DNA-binding element; lane 13, 0.1 μg NE and a 1000-fold excess of MyoD DNA.

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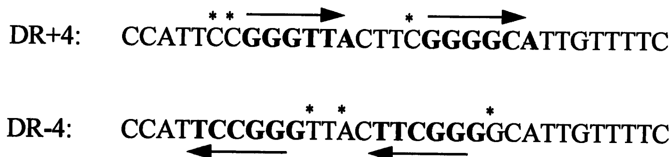

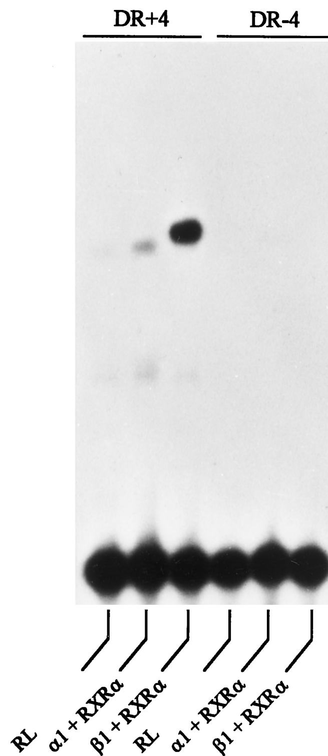

FIG. 5. EMSA using two mutated oligomers spanning the sequence −457/−426 determined the orientation of the GLUT4 TRE. Sequence and EMSA of oligos DR+4 and DR−4. lanes 1 and 4, Reticulocyte lysate control; lanes 2 and 5, purified chick α1 TR and RXRα; lanes 3 and 6, hβ1 TR and RXRα. *, Mutation sites [all replaced with an adenine (A), except the one original A, which was replaced with a thymine].

compared to a canonical DR+4 TRE sequence composed of perfect consensus half-sites to determine the relative ability of these sequences to bind TRs (Fig. 7; both elements labeled to identical specific activities). Complexes on both elements demonstrated the characteristic downshift of heterodimeric TR/TRE complexes and dissociation of TR homodimers in the presence of T_3 (Fig. 7). However, TR binding to the GLUT4 TRE was overall considerably lower, and as a likely consequence, homodimer and even monomeric binding evident for β1 TRs on the canonical DR+4 element, was not detectable on the GLUT4 TR-binding site.

This phenomenon was next studied in more detail, initially by comparing a titration curve for the binding of increasing amounts of *in vitro* translated β1 TR with a constant concentration of RXRα to the GLUT4 and canonical DR+4 TR-binding sites. The results of this experiment (Fig. 8) demonstrated that unlike the canonical DR+4, only at the highest β1TR concentration was TR homodimer marginally apparent on the GLUT4 TRE.

With regard to a potential mechanism for this observation, as the binding affinity/specificity of TRs is presumably determined by the DNA sequence of the binding site, a final experiment was performed using a series of oligos synthesized with single base substitutions to determine a crucial base or sequence dictating the lower affinity and perhaps putative heterodimer-selective binding of the GLUT4 TR-binding element (Fig. 9). Specifically, these mutations were designed to replace nonconsensus half-site bases with consensus nucleotides. In addition, various substitutions were introduced within the intervening and flanking regions of the GLUT 4 TR-binding element, *e.g.* mutant oligo 7 was synthesized, replacing all the intervening and flanking bases with those present in the canonical DR+4 TRE.

The results of the binding of *in vitro* translated β1 and β1+RXRα receptors to these mutant GLUT4 TREs are shown in Fig. 9. Interestingly, bases within the intervening and/or flanking sequences were also shown to be crucial for TR binding; in particular intervening bases 2 and 3. In contrast, the mutations in oligo 5 and oligo 6 (which converts the first base of the 3′-TRE half-site to the consensus) apparently imparted an additional affinity to these sequences, the degree of which and perhaps complex formation on would seem to be comparable with those of the canonical DR+4 TRE. Therefore, in combination with the previous titration experiment, these data suggest that it is the overall lower affinity of the GLUT4 sequence that accounts for the visualization of only the more avid heterodimer in these experiments, *i.e.* a quantitative, rather than qualitative, phenomenon. This conclusion would also be supported by the reduced binding and formation of only heterodimer observed by mutant 7, wherein all the flanking and intervening bases were substituted with those from the canonical TR-binding element.

In conclusion, these data demonstrated that the newly identified GLUT4 TRE has a specific affinity for TRs. However, in contrast to other established TREs, GLUT4 TRE was shown to have a much lower affinity, and this property appears to be responsible for the observation that only high affinity TR/heterodimeric receptors bind appreciatively on this TRE. Moreover, combined with the results of a previous study that demonstrated this region of the GLUT4 promoter to confer thyroid hormone responsiveness in transfection studies (12), these data indicate that the effects of T_3 on GLUT 4 transcription are likely to be mediated directly by this TR-binding element. However, formal proof of this hypothesis requires additional functional analyses using mutations within this TR-binding element.

Discussion

The primary aim of this study was to analyze the binding of TRs and nuclear extracts to a previously identified thyroid hormone-responsive region of the GLUT4 promoter (12) and thus perhaps determine whether thyroid hormone acts directly to stimulate GLUT4 transcription in rat skeletal muscle (11). Indeed, consistent with this hypothesis, a series of EMSA analyses clearly demonstrated the presence of a spe-

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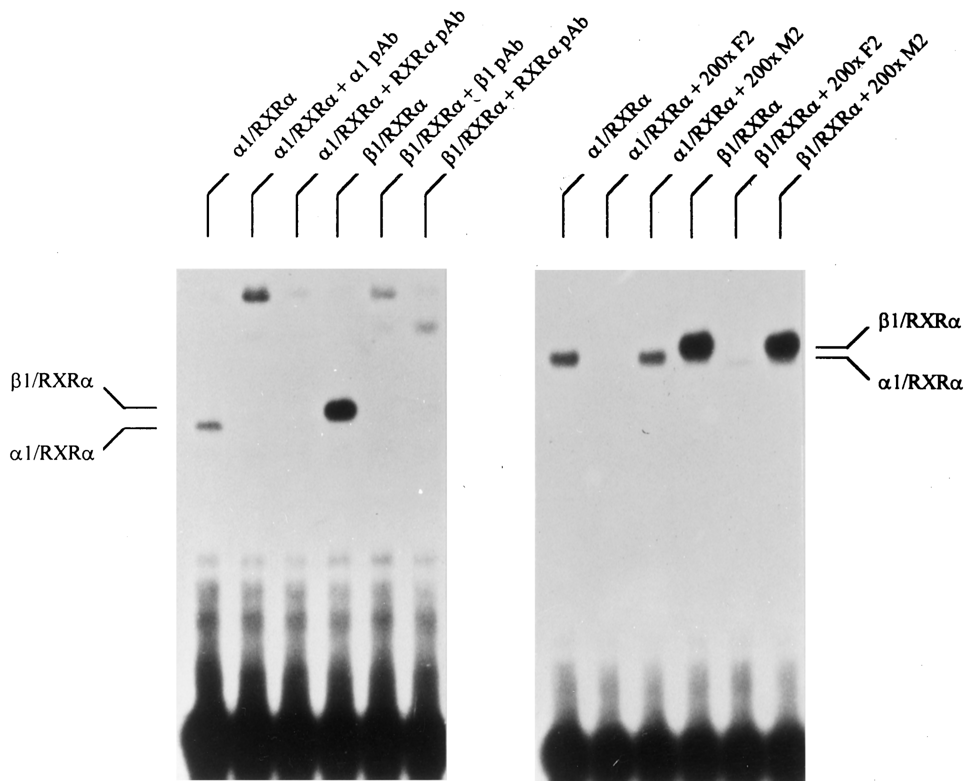

FIG. 6. EMSA to determine the identity and binding specificity of TR complexes formed on the GLUT4 TRE. Lanes 1–6, EMSA supershift experiment using TR isoform-specific antibodies to determine the composition of TR/GLUT4 TRE complexes. Lane 1, Purified chick α1 and hRXRα; lane 2, purified chick α1, hRXRα, and α1 polyclonal antibody; purified chick α1, hRXRα, and RXRα polyclonal antibody; lane 4, hβ1, hRXRα, and β1 polyclonal antibody; hβ1, hRXRα, and RXRα polyclonal antibody. Lanes 7–12, EMSA with competing DNA to determine the binding specificity of the GLUT4 TRE for TRs: lane 7, purified chick α1 and hRXRα; lane 8, purified chick α1, hRXRα, and a 200-fold excess of cold F2 TRE; lane 9, purified chick α1, hRXRα, and a 200-fold excess of a cold mutant (non-TR binding) TRE (M2); lanes 10–12, same as for lanes 7–9, except using *in vitro* translated β1 TR and RXRα.

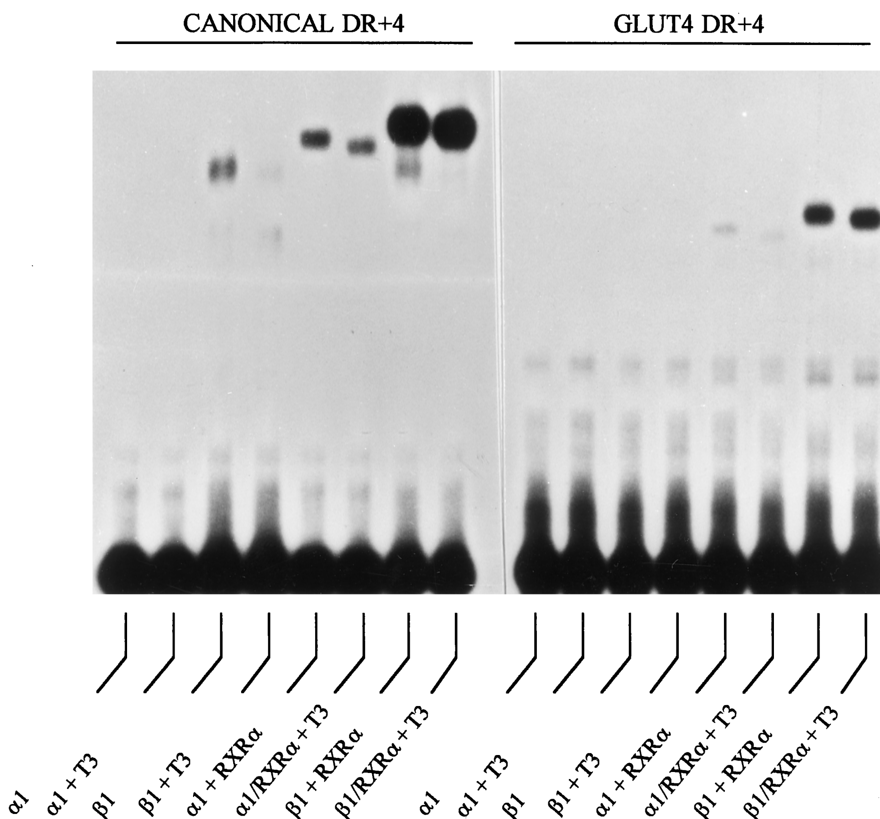

FIG. 7. EMSA comparing the relative affinity and TR complexes formed on the GLUT4 TRE with a canonical DR+4 TRE sequence. GLUT4 and DR+4 TREs were labeled to an identical specific activity, and equal counts were used for EMSA analysis. Lane 1 and 9, Purified α1 TR; lanes 2 and 10, purified α1 TR and T_3 (1×10^{-7} M); lanes 3 and 11, hβ1; lanes 4 and 12, hβ1 and T_3; lanes 5 and 13, purified α1 and RXRα; lanes 6 and 14, purified α1, RXRα, and T_3; lanes 7 and 15, hβ1 and RXRα; lanes 8 and 17, hβ1, RXRα, and T_3.

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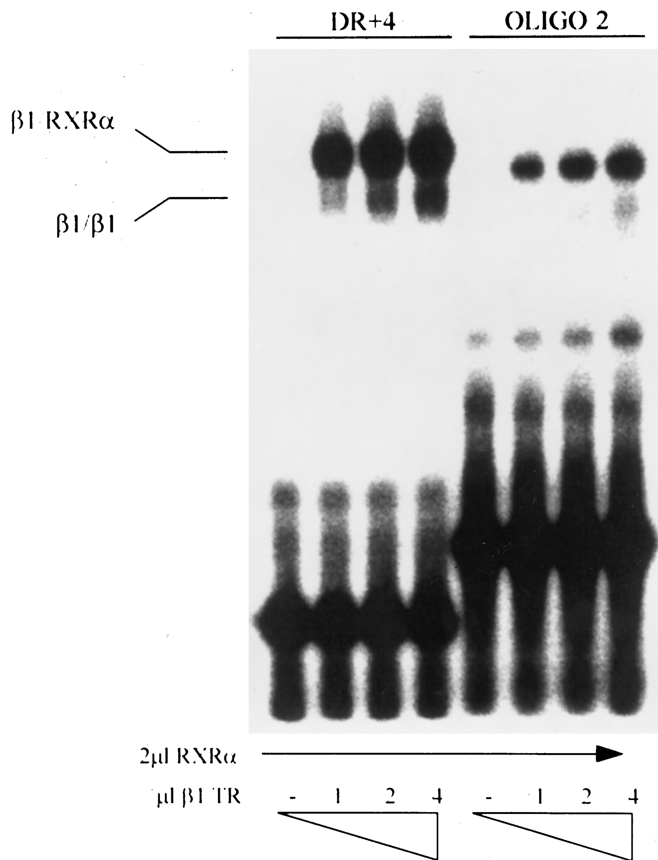

FIG. 8. EMSA titration curve to determine the specificity of the GLUT4 TRE (oligo sequence −452/−431; TCCGGGTTACT-TCGGGGCATTG) for TR/RXR heterodimers compared to the canonical DR+4 TRE. Lanes 1 and 5, 2 μl RXRα; lanes 2 and 6, 2 μl RXRα and 1 μl β1TR; lanes 3 and 7, 2 μl RXRα and 2 μl hβ1; lanes 4 and 8, 2 μl RXRα and 4 μl hβ1TR.

GLUT4 TRE (-452/-431)	TCC**GGGTTA**CTTC**GGGGCA**TTG
Mutant oligo 1:	TCC*A***GGTTA**CTTC**GGGGCA**TTG
Mutant oligo 2:	TCC**GGGTTA***T*TTC**GGGGCA**TTG
Mutant oligo 3:	TCC**GGGTTA**C*A*TC**GGGGCA**TTG
Mutant oligo 4:	TCC**GGGTTA**CT*A*C**GGGGCA**TTG
Mutant oligo 5:	TCC**GGGTTA**CTT*A***GGGGCA**TTG
Mutant oligo 6:	TCC**GGGTTA**CTTC*A***GGGCA**TTG
Mutant oligo 7:	*CTG***GGGTTA***CAGG***GGGGCA***GAG*

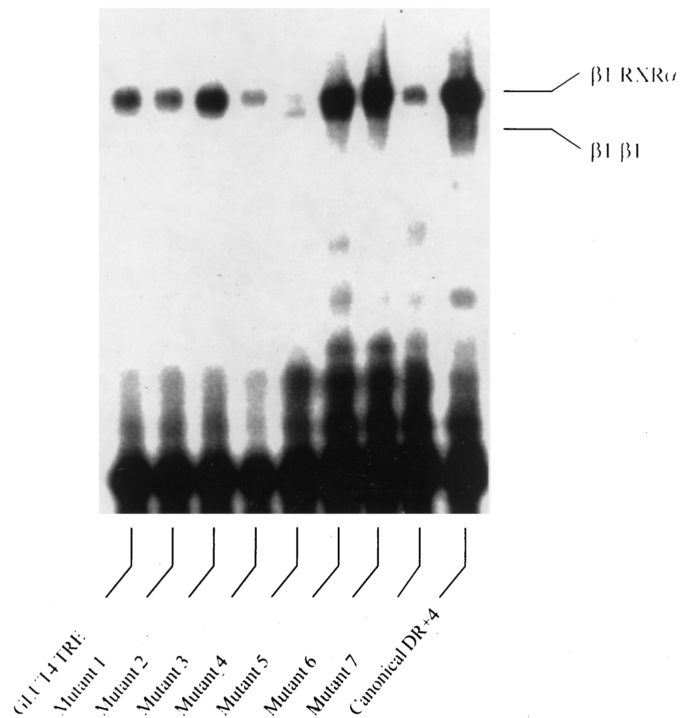

FIG. 9. EMSA using a series of GLUT4 TRE mutants to determine a putative base(s) imparting specificity for TR/RXR heterodimers. The sequences of GLUT4 TRE mutants (*italic*, mutated base) and EMSA using the GLUT4 TRE and GLUT4 TRE mutants 1–7 (lanes 1–8, respectively) are shown. Lane 9, Canonical DR+4 TRE.

cific DR+4 TR-binding site between bases −457/−426 of the GLUT4 promoter fragment. However, given the similar deviations from the consensus [AGGT(C/A)A] of this confirmed TR-binding site and the other three putative TRE half-sites located further upstream, it is not immediately clear why the latter sequences did not also display TR-binding activity. Conceivably, the second and third half-sites of the latter tandem sequence can be discounted due to their nonoptimal separation distance (3 bp). However, the first and second elements form an apparently viable DR+4 element. Nevertheless, this can probably be explained by the fact that neither of these half-sites contains a consensus thymine [AGGT(C/A)A] at the fourth position.

The exquisite specificity of base sequences directing protein-DNA interaction(s) was similarly demonstrated in the mutation experiment, which revealed that intervening and flanking sequences surrounding the GLUT4 half-sites were also crucial for mediating TR binding. Indeed, we may have been fortunate in that the mutations introduced within the intervening and flanking regions to discern the orientation of the GLUT4 TRE did not also abrogate TR binding. However, none of the mutations analyzed in Fig. 9 directly altered the propensity of the GLUT4 TRE to bind heterodimers. Moreover, certain substitutions (in particular the conversion of the first base in the 3′ half-site to the consensus adenine; mutant oligo 6) that enhanced the affinity of the GLUT4 TRE, resulted in binding properties similar to those of the canonical DR+4 TRE. Of note, increased binding to the mutation oligo 6 would be consistent with the fact that TRs in heterodimeric complexes are considered to bind this particular half-site. In contrast, mutant oligo 7, in which all bases other than those within the GLUT4 TRE half-sites were replaced with the corresponding sequences in the canonical DR+4, demonstrated properties comparable to those of the wild-type GLUT4 TRE. These data, therefore, suggest that the apparent heterodimer specificity observed for the GLUT4 TR-binding site is simply a function of the overall lower affinity of the half-sites within this element. Indeed, the titration experiment demonstrated the GLUT4 sequence to be equally de-

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CONSERVATION OF THE GLUT4 TRE

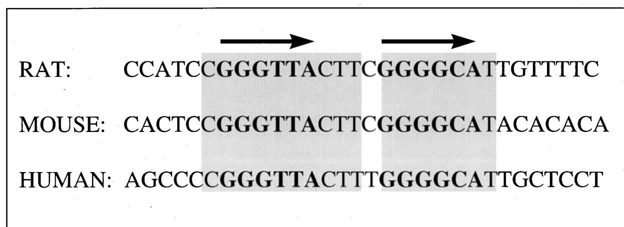

FIG. 10. Evolutionary conservation of the GLUT4 TRE. Comparison of the base sequences within the GLUT4 promoter region containing the GLUT4 TRE in mice, rats, and humans.

ficient in binding both homodimers and heterodimers compared to a canonical DR+4 TR-binding element (estimated to be approximately 5-fold from densitometric scanning).

Finally, characterization of a low affinity TR-binding site has a number of implications for thyroid hormone-regulated genes, the first of which would presumably be the inducibility of responsive genes. Indeed, the degree of transcriptional induction observed for the GLUT4 gene (~2.5 under chronic hyperthyroid *vs.* hypothyroid conditions) (11) compared to genes containing high affinity TRE(s) (*e.g.* malic enzyme; transcription stimulated 3- to 4-fold in the liver of euthyroid rats) (43) is consistent with this hypothesis. Secondly, as 1) TRs bind DNA in the presence and absence of thyroid hormone, 2) unoccupied TRs actively repress basal transcription, and 3) heterodimers demonstrate higher affinities over homodimers and are favored in the presence of T_3 (13), a low affinity TRE would have special relevance for thyroid hormone-regulated genes. For example, one can speculate that although a low affinity TRE would not illicit as large a T_3 induction compared to a high affinity TRE, by the same token, in the absence of T_3 a low affinity TRE would not be subjected to such a great degree of basal repression.

A previous study suggested a criterion by which different combinations of weak and strong artificial TRE half-sites modulate TR complex formation and T_3 responsiveness of thyroid hormone-regulated genes (44). In this classification, the GLUT4 TRE presumably falls into the category of either a weak/weak (w/w) or, more likely from the high affinity imparted to the mutant 6, a strong/weak half-site composition. Interestingly, consistent with our observations, both of these previously described TREs were weakly T_3 responsive (44). Moreover, distinct from all other TRE combinations, these elements were also dependent upon RXR to mediate T_3 induction in transfection assays. Unfortunately, however, the basal repression properties of a w/w or strong/weak TRE were not investigated in this study (44).

Interestingly, both the half-site sequences, the immediately flanking bases, and three of the four intervening sequences of the GLUT4 TRE are absolutely conserved among humans, mice, and rats (Fig. 10). Their conservation would, therefore, further suggest the probable functionality of this low affinity TRE as well as the importance of sequences other than those within the TR half-sites for mediating TR binding. Indeed, one can envisage that during the evolution of genes, the properties of promoter regulatory elements, *e.g.* affinity, may be similarly selected so that they compliment the function of the particular gene product, *i.e.* for a TRE; a low affinity TRE(s) may predominate in genes that need to be constitutively expressed, but under certain conditions need to be up-regulated to some extent, *e.g.* GLUT4 (11). In contrast, a higher affinity TREs would be highly responsive to thyroid hormone, but would also be subjected to a greater degree of active repression by unoccupied heterodimers and/or homodimers. Such a TRE would, therefore, be highly sensitive to changes in T_3 status/availability and may be advantageous for imparting high inducibility on, for instance, temporally expressed genes (Fig. 11).

In conclusion, these studies demonstrated the presence of a specific TR-binding element within a region of the GLUT4 promoter previously found to impart T_3 responsiveness (12). These data, therefore, corroborate the supposition that the effects of T_3 on GLUT4 transcription are direct. However, formal demonstration of this hypothesis must await proof of nonfunctionality in transient transfection assays of mutants within the GLUT4 TR-binding site. Nevertheless, the GLUT4 TR-binding element was also shown to possess a significantly lower affinity than a conventional DR+4 TR-binding element and only bound TRs significantly in combination with RXR. These data, therefore, suggest another mechanism by which the intrinsic basal activity and T_3 responsiveness of thyroid hormone-regulated genes may be modulated.

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