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Characterization of a Novel Thyroid Hormone Receptor α Variant Involved in the Regulation of Myoblast Differentiation

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The regulation of gene expression by thyroid hormone (T_3) involves binding of the hormone to nuclear receptors [thyroid hormone receptor (TR)] acting as T_3-dependent transcription factors encoded by TRα (NR1A1) and TRβ (NR1A2) genes. Several TRα variants have already been characterized, but only some of them display T_3 binding activity. In this study, we have identified another transcript, TRα-ΔE6, produced by alternative splicing with microexon 6b instead of exon 6. This splicing leads to the synthesis of a protein devoid of a hinge domain. The TRα-ΔE6 transcript is detected in all mouse tissues tested. Although TRα-ΔE6 did not bind DNA, its expression induced a TRα1 sequestration in the cytoplasm. Functional studies demonstrated that TRα-ΔE6 inhibits the transcriptional activity of TRα1 and retinoic X receptor-α, but not of retinoic acid receptor-α. We also found that TRα-ΔE6 efficiently decreased the ability of TRα to inhibit MyoD transcriptional activity during myoblast proliferation. Consequently, when overexpressed in myoblasts, it stimulated terminal differentiation. We suggest that this novel TRα variant may act as down regulator of overall T_3 receptor activity, including its ability to repress MyoD transcriptional activity during myoblast proliferation. (*Molecular Endocrinology* 20: 749–763, 2006)

T_3 EXERTS A PLEIOTROPIC effect on development and on adult homeostasis. T_3 action is mediated by ligand-inducible transcription factors that are members of the steroid/thyroid hormone receptor (TR) superfamily. There are two types of TRs encoded by TRα and TRβ genes (NR1A1 and NR1A2 according to nuclear hormone receptor nomenclature) (1, 2). Both TR loci are complex, and numerous TR proteins are produced by alternative promoter usage, alternative splicing, and the use of internal initiation codon. These processes lead to the synthesis of four nuclear receptors (TRα1, TRβ1, TRβ2, and TRβ3) (3–7), three mitochondrial receptors (p43, p28, and TRβ0) (8–10), and five nonreceptor isoforms (TRα2, TRα3, TR$\Delta\alpha$1, TR$\Delta\alpha$2, and TR$\Delta\beta$3) (7, 11–13). Because of this complexity, numerous models of TR knockout have been developed to understand the specific function of each protein. Phenotype analyses have revealed significant differences, despite redundancy among the various TRs, in the observation that the loss of one variant can be overcome by the activity of other isoforms in many tissues (14). However, TRα appeared to be crucial for postnatal development and cardiac function, whereas TRβ mainly controls thyroid hormone levels, liver metabolism, and development of retinal and auditory functions (14).

In earlier studies we have shown that T_3 stimulates myoblast differentiation through inhibition of activator protein 1 (AP1) activity (15). This T_3 myogenic influence mediated by TRα1 is only functional at a particular stage of myoblast progression in the myogenic program characterized by RXR expression. In addition, we provided evidence that TRα1 is involved in a mechanism preserving the duration of myoblast proliferation by repressing MyoD (myogenic determination factor 1) and myogenin transcriptional activity independently of the presence of the hormone (16).

In this report, using a RT-PCR approach in murine C2C12 cells or in mouse liver, we have identified a new TRα transcript produced by alternative splicing in which a microexon (exon 6b) is used instead of exon 6 (TRα-ΔE6). This splicing leads to generation of a TRα1 protein without a hinge domain. Furthermore, this transcript is detected in various mouse tissues. We found that TRα-ΔE6 did not bind DNA but induced a TRα1 sequestration in the cytoplasm. Functional studies demonstrated that this TRα variant inhibits the transcriptional activity of TRα1 and RXRα, but not of RARα. Last, its overexpression stimulates QM7 myoblast differentiation by restoring MyoD transcriptional activity through inhibition of the functional TRα1/MyoD in-

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Abbreviations: AchRα, Acetylcholine receptor α-subunit; AF-2, activation function 2; CAT, chloramphenicol acetyltransferase; DBD, DNA-binding domain; DR, direct repeat; EGFP, enhanced green fluorescent protein; IR, inverted repeat; RAR, retinoic acid receptor; RXR, retinoic X receptor; TR, thyroid hormone receptor.

teraction. These data suggest that TRα-ΔE6 might exert a regulatory role during mouse muscle development.

RESULTS

Identification of a New Transcript Encoded by the TRα Gene

To test the possible existence of new TRα variants, RT-PCR experiments were performed on total RNA extracts from murine C2C12 cells using a set of specific primers covering the TRα1-coding region (Fig. 1A). Using primers designed to hybridize to the extremities of the TRα1 transcript, we detected a PCR product of the expected size (Fig. 1B). Surprisingly, a second PCR product, approximately 200 bp shorter, was systematically obtained in the same PCR reactions (Fig. 1B). To test the amplification specificity of the shorter product, we purified the two cDNAs to perform additional PCR experiments. Using primer pairs −4/1250, 107/1250, −4/927, 107/927, we systematically observed the difference of approximately 200 bp between the cDNA encoding the TRα1 transcript and the shorter form (Fig. 1C). However, when the primer pairs 444/1250 included a primer hybridizing to a sequence corresponding to exon 6 (444), only the cDNA corresponding to the full-length TRα1 was amplified (Fig. 1C). Lastly, the use of primers pairs 733/927 and 733/1250 gave rise to a PCR product of similar size for both purified cDNAs (Fig. 1C). This set of data clearly supported the possible existence of a new TRα transcript lacking a sequence of about 200 bp, including a part of exon 6.

Consequently, we cloned and sequenced the purified shorter cDNA product. We found that the TRα variant lacked the 368 to 608-bp sequence corresponding to the exon 6 encoding region (numbering based on mouse nucleotide sequence GenBank accession no. X07750). However, 10 nucleotides were substituted for the exon 6 sequence relative to the TRα1 transcript (Fig. 2). Interestingly, these changes do not result in a frame shift introducing a premature stop codon but could lead to the synthesis of a TRα1 protein without a hinge domain (TRα-ΔE6). To rule out the possibility that this transcript could be a peculiarity of the C2C12 cell line, similar experiments were conducted using total RNAs extracted from mouse liver. Identical results were obtained, thus leading to the conclusion that a novel TRα transcript is expressed in mice.

Analysis of the Genomic Context Leading to the Generation of TRα-ΔE6

To understand the events leading to alternative splicing of exon 6 associated with the introduction of a

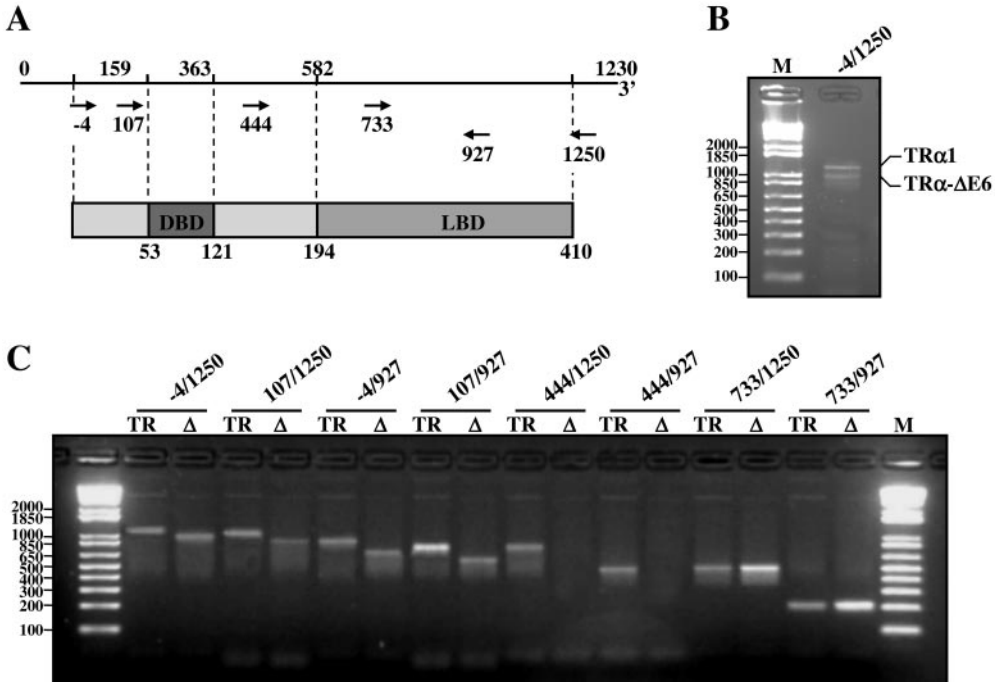

Fig. 1. Identification of a New TRα Transcript

A, Schematic representation of TRα1 coding sequence and position of the primers (*arrows*) used for RT-PCR analysis. *Numbers below arrows* indicate the first 5′-nucleotide of corresponding primers. B, RT-PCR analysis on RNA isolated from C2C12 cells. Total RNA was extracted, reverse transcribed, and amplified using primers designed to hybridize to the extremities of the TRα1 transcript. C, RT-PCR analysis on RNA isolated from C2C12 cells. Total RNA was extracted, reverse transcribed, and amplified using the indicated pairs of primers as described in *Materials and Methods*. TR, TRα1; Δ, TRα-ΔE6; LBD, ligand-binding domain.

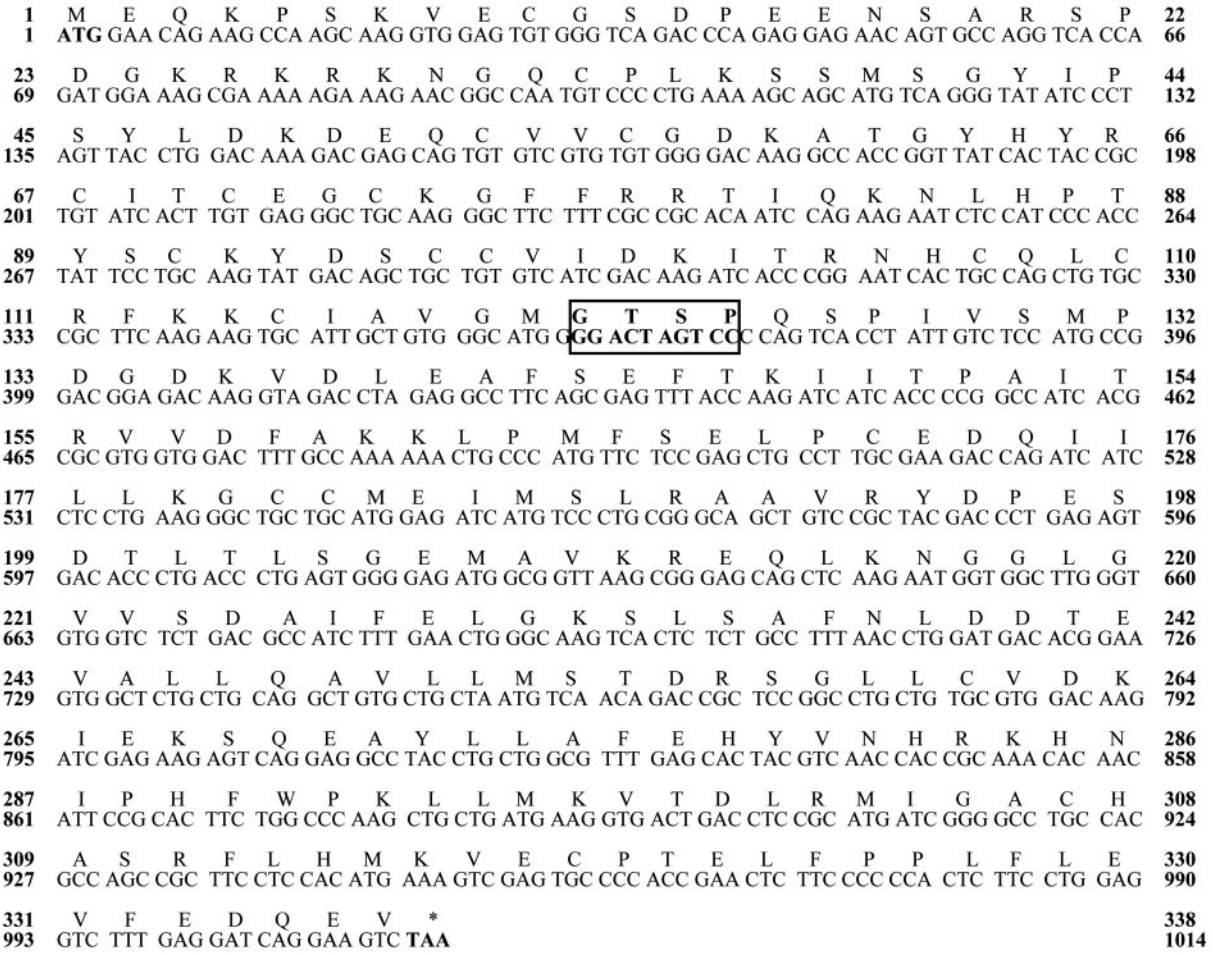

Fig. 2. Sequence Alignment of TRα Variant Transcript and Its Predictive Protein

The TRα variant transcript lacks the exon 6 encoding sequence but displays 10 additional nucleotides encoding for four additional amino acids (nucleotides *encircled*).

small additional sequence, we cloned and sequenced the genomic region juxtaposed between exon 5 and exon 7 of the TRα gene from mouse liver and C2C12 cells.

The first interesting indication provided by the analysis was that 10 nucleotides substituting the sequence corresponding to exon 6 in the full-length transcript originate from microexon 6b, located between exons 6 and 7 (Fig. 3A). This microexon is located in the context of seven G-rich repeats, similar to that previously reported in the cardiac troponin T gene, acting as an intron splicing enhancer leading to the inclusion of a microexon in the corresponding transcript (17). This observation suggests that these G-rich repeats could be involved in the use of microexon 6b.

Another striking observation was the occurrence of two differences between the genomic DNA sequence and the TRα-ΔE6 sequence (Fig. 3C). Whereas a thymidine in the genomic sequence of microexon 6b did not appear in the transcript, an additional cytidine was inserted four nucleotides downstream. These changes were simultaneously observed in several sequence analyses performed in both mouse liver and C2C12 genomic sequences and TRα-ΔE6 transcripts, thus ruling out the possibility of an experimental artifact. Interestingly, both nucleotides changes occurred in a genomic region displaying a perfect palindromic structure possibly involved in the substitution (Fig. 3D). These data lead us to propose that an editing activity is involved in the synthesis of the TRα-ΔE6 transcript.

The TRα-ΔE6 Transcript Is Ubiquitously Expressed in Mouse Tissues

The expression pattern of the TRα-ΔE6 transcript was monitored in different mouse tissues in RT-PCR experiments using a specific primer hybridizing to microexon 6b (5′ΔE6). We observed that TRα-ΔE6 expression was detected in all tissues tested: spleen, liver, kidney, heart, lung, muscle, and white adipose tissue and was at the brink of detection in brain (Fig. 4).

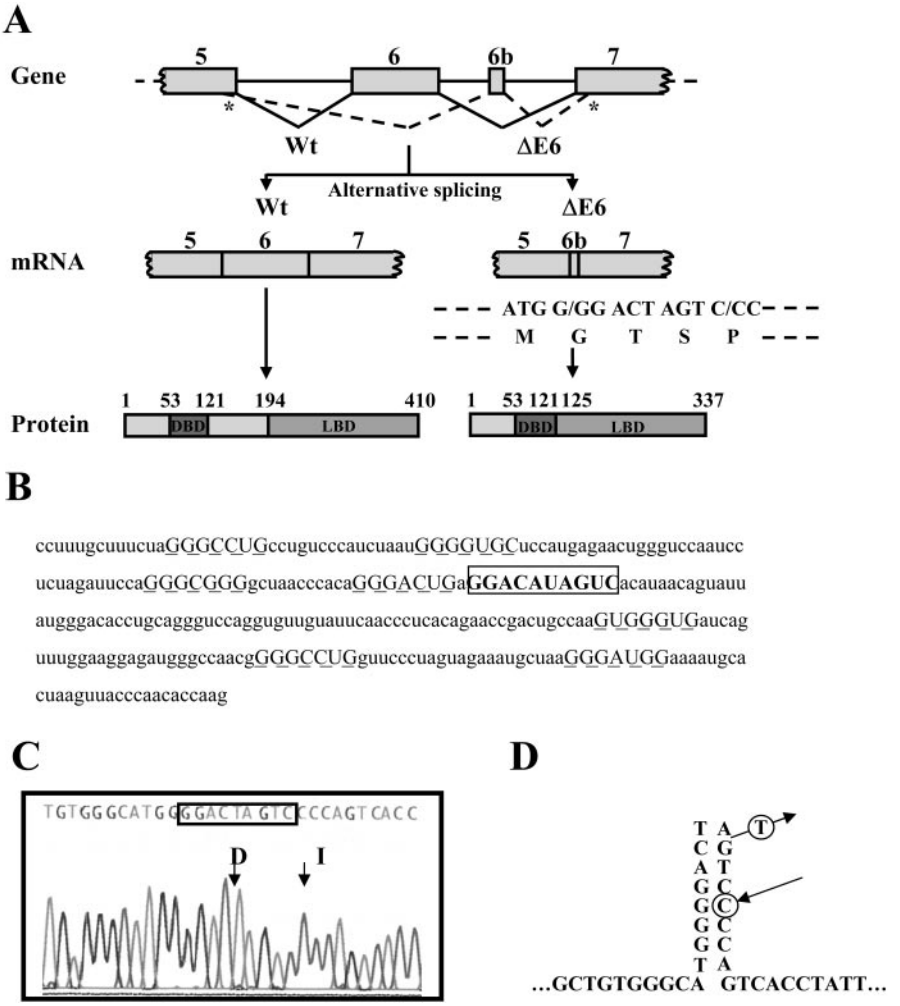

Fig. 3. The TRα-ΔE6 Transcript Is Generated by an Alternative Splicing Event Where a Microexon 6b Is Used Instead of Exon 6

A, Schematic representation of the splicing events Wt and ΔE6 generating TRα1 and TRα-ΔE6 transcripts, respectively. The variant transcript where a microexon 6b is used instead of exon 6 does not result in a frame shift introducing a premature stop codon. Predicted protein structure of TRα1 and TRα-ΔE6 is represented at the *bottom* of the figure (LBD, ligand-binding domain). *Numbers above the schematic structure* correspond to amino acids delimiting TRα domains. B, Sequence of the microexon 6b is indicated within the *box*, and the G-rich repeat is *underlined*. C, Schematic representation of the TRα-ΔE6 mRNA sequence around microexon 6b is where *arrows* indicate deletion (D) and insertion (I) compared with the genomic sequence. D, Representation of the perfect palindromic structure of the editing area. Wt, Wild type.

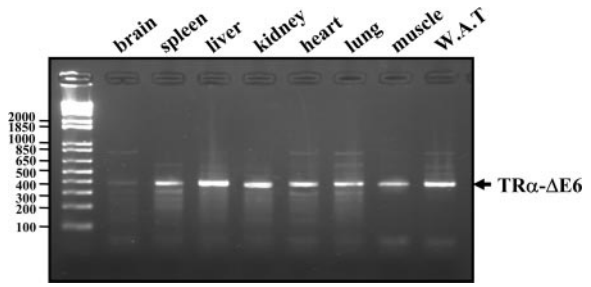

Fig. 4. TRα-ΔE6 Is Widely Expressed in Mouse Tissues

Expression of the TRα-ΔE6 transcript was analyzed by RT-PCR analysis as described in *Materials and Methods* using total RNA extracted from brain, spleen, liver, kidney, heart, lung, muscle, and white adipose tissue (W.A.T).

TRα-ΔE6 Encodes a TRα1 Protein without Hinge Domain

To test the ability of the TRα-ΔE6 transcript to encode the predicted protein, *in vitro* and *in vivo* translation experiments were performed with both TRα1 and TRα-ΔE6. First, we found that TRα-ΔE6 *in vitro* synthesis in rabbit reticulocyte lysate encodes only one protein with an apparent molecular mass of approximately 38 kDa in SDS-PAGE (Fig. 5A). Moreover, as expected, we found that this protein was not detected by an antibody raised against the hinge domain of TRα1 (TRα-144) (Fig. 5B). These observations confirmed that the alternative splicing does not result in a frame shift introducing a premature stop codon, but

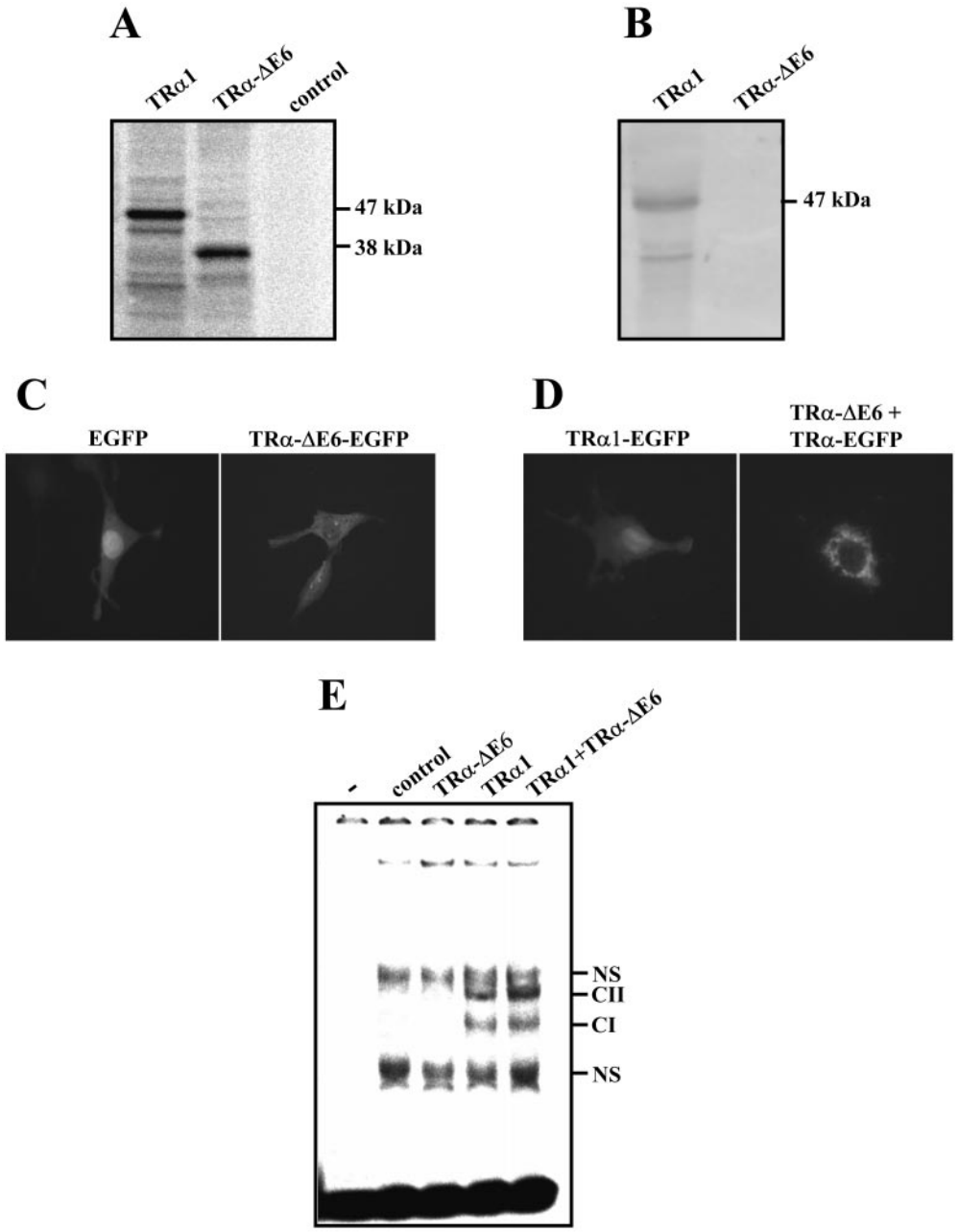

Fig. 5. TRα-ΔE6 Did Not Bind to DNA but Sequestered TRα1 in the Cytoplasm

A, Detection of TRα1 and TRα-ΔE6 proteins synthesized in rabbit reticulocyte lysate in the presence of labeled methionine. Unprogrammed reticulocyte lysate was used as control. B, After synthesis in rabbit reticulocyte lysate, only TRα1 is detected by Western blot using an antibody raised against the hinge domain of the full-length receptor (TRα-144). C, Subcellular localization of EGFP (control) or TRα-ΔE6 protein fused to EGFP in QM7 avian myoblasts. TRα-ΔE6 was predominantly localized in the cytoplasm. D, Subcellular localization of TRα1 protein fused to EGFP in QM7 avian myoblasts. Whereas TRα1 alone was predominantly localized in nucleus, coexpression of TRα-ΔE6 leads to a predominant cytoplasmic localization. (Magnification, ×400). E, Gel EMSAs experiment performed using a ^{32}P-labeled DR4 oligonucleotide as probe and cold mTRα1 and/or mTRα-ΔE6 synthesized *in vitro* using rabbit reticulocyte lysate. In each experiment 1 μl reticulocyte lysate was used. CI, TRα1 monomer; CII, TRα1 homodimer; NS, not specific.

gives rise to a TRα1 protein without amino acids 121–198 corresponding to the hinge domain. The hinge domain (D domain), located between the DNA-binding domain (DBD) and the ligand-binding domain, is involved in the nuclear localization of the receptor (18) and plays an important role in ligand and DNA binding activities (19–21).

To define the cellular localization of the TRα-ΔE6 protein, we performed transient transfection experiments in QM7 cells using vectors encoding TRα-ΔE6/

enhanced green fluorescent protein (EGFP) and TRα1-EGFP fusion proteins. As expected, TRα1 was predominantly expressed in the nucleus (Fig. 5D). In contrast, we observed that TRα-ΔE6 was predominantly expressed in the cytoplasm with minor nuclear staining (Fig. 5C). Interestingly, coexpression of TRα-ΔE6 with TRα1-EGFP induced a major cytoplasmic localization of the nuclear receptor (Fig. 5D). This observation indicates that the expression of the TRα variant induced a cytoplasmic sequestration of the full-length receptor.

To test the DNA binding activity of TRα-ΔE6, we performed gel mobility shift assays with *in vitro* synthesized proteins, using a direct repeat (DR) spaced by four nucleotides (DR4). As previously described (22), we found that TRα1 binds to a DR4 sequence as monomer and homodimer (Fig. 5E). In contrast, TRα-ΔE6 fails to bind this thyroid hormone response element and did not affect the binding pattern of TRα1 to a DR4 (Fig. 5E). This observation is in agreement with previous data indicating that efficient TRα1 DNA binding requires the D domain (20).

Functional Interactions of TRα-ΔE6 with TR, RXR, and RAR

To investigate the possibility that the TRα-ΔE6 protein could affect nuclear receptor activity, we performed transient transfection experiments in QM7 avian myoblasts using a CAT (chloramphenicol acetyl transferase) reporter plasmid driven by a palindromic T_3-responsive element (inverted repeat: IR0), or a DR spaced by one (DR1) or four nucleotides (DR4). In agreement with previous data, TRα1 expression induced a 3-fold stimulation of gene reporter expression through an IR0 in the presence of T_3 (306 ± 46%; $P < 0.05$) (Fig. 6A). Coexpression of TRα1 and RXRα induced a 2.4-fold stimulation of gene reporter expression through a DR4 thyroid hormone response element in the presence of T_3 (237 ± 33%, $P < 0.01$) (Fig. 6B). Though TRα-ΔE6 expression alone does not exert any significant influence by itself (Fig. 6, A and B), its coexpression abrogated TRα1 transcriptional activity in the T_3 presence through IR0 (306 ± 46% for TRα1 *vs.* 116 ± 6% for TRα1/TRα-ΔE6; $P < 0.05$) (Fig. 6A) or DR4 response elements (237 ± 33% for TRα1/RXRα *vs.* 124±16% for TRα1/RXRα/TRα-ΔE6; $P < 0.01$)(Fig. 6B). As previously described, liganded RXRα displayed a strong transcriptional activity through a DR1 response element (2118 ± 280%; $P < 0.01$) (Fig. 6C). Again, TRα-ΔE6 was devoid of activity by itself, whereas its coexpression with RXRα partially abrogated the ligand-dependent activity of this receptor (2118 ± 280% for RXRα *vs.* 1093 ± 121% for RXRα/TRα-ΔE6; $P < 0.05$) (Fig. 6C). In parallel experiments, we found that, in contrast, RAR activity is not affected by TRα-ΔE6 (Fig. 6D).

To identify the domain of TRα-ΔE6 involved in the inhibition of full-length TRα1 activity, we used TRα-ΔE6 mutants deleted of the DNA binding, activation function 2 (AF-2), or amino-terminal domains (Fig. 7A), in CAT assays experiments. Whereas deletion of the AF-2 domain did not alter the repressive influence of TRα-ΔE6 on the transcriptional activity of TRα1/RXRα through a DR4 (207 ± 23% for TRα1/RXRα *vs.* 118 ±

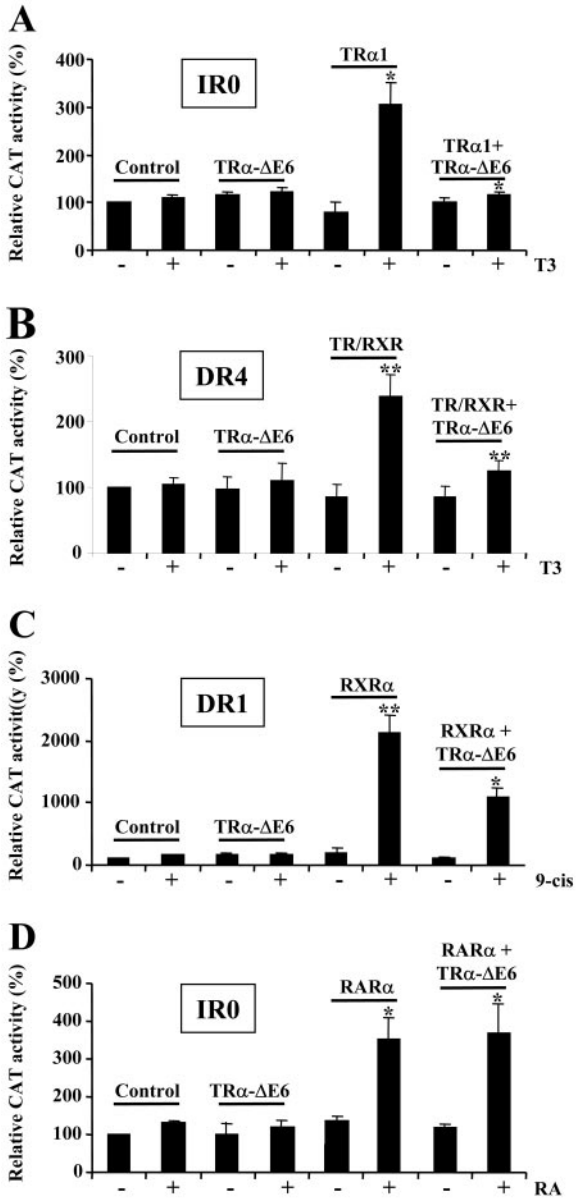

Fig. 6. Influence of TRα-ΔE6 on the Transcriptional Activity of Nuclear Receptors in QM7 Cells

A–D, Transient transfection assays were performed in QM7 cells cultured in a T_3-deprived medium using expression vectors and CAT reporter genes indicated in the figure. All results were normalized according to β-galactosidase activity and expressed as a percentage of the control value. Data are presented as the mean ± SEM of four separate experiments. When indicated, 10^{-8} M T_3, 5.10^{-8} M 9-*cis*-retinoic acid (9-cis), or 10^{-7} M all-*trans* retinoic acid (RA) were added to the culture medium. Statistical significant differences compared with control cells: *, $P < 0.05$; **, $P < 0.01$, Student's paired *t* test).

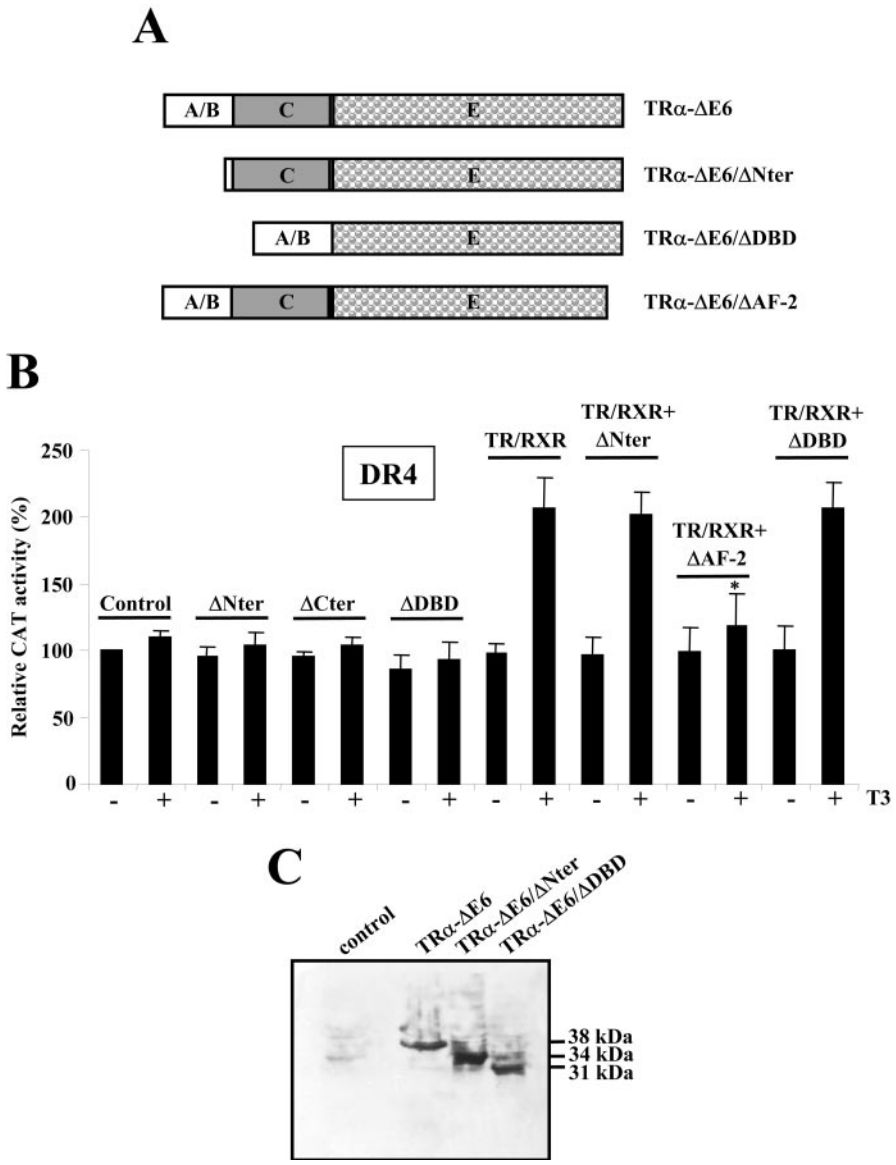

Fig. 7. Influence of TRα-ΔE6 Mutants on the Transcriptional Activity of TRα1 and RXRα through a DR4 Reporter Gene

A, Schematic representation of TRα-ΔE6 mutants deleted of DNA binding, AF-2, or amino-terminal domain. B, Transient transfection assays were performed in QM7 cells cultured in a T_3-deprived medium using a reporter gene and the expression vectors indicated in the figure. As previously demonstrated, proliferating QM7 myoblasts do not express RXR (15). In these cells, TRα1 activity through a DR4 TRE needs cotransfection with RXR isoform. All results were normalized according to β-galactosidase activity and expressed as a percentage of the control value. Data are presented as the mean ± SEM of four separate experiments. When indicated, 10^{-8} M T_3 was added to the culture medium. Statistically significant differences compared with TR/RXR overexpressing cells in the presence of T_3: *, $P < 0.05$; Student's paired *t* test. C, Detection of TRα-ΔE6, TRα-ΔE6/ΔNter, and TRα-ΔE6/ΔDBD proteins after transient transfection assays performed in QM7 cells. Western blot experiments were performed using an antiserum raised against carboxy-terminal domain of TRα1 (8). Proteins (50 μg) were loaded in each lane.

25% for TRα1/RXRα/TRα-ΔE6/ΔAF2; $P < 0.01$), we found that the two other mutations fully abrogated the ability of this TRα variant to repress TRα1/RXRα activity (Fig. 7B). Western blot experiments using an antiserum raised against the carboxy-terminal domain of TRα1 (8) ruled out the possibility that this lack of repressive activity could result from the absence of expression of these two mutants (Fig. 7C). These data indicated that the DNA-binding and amino-terminal domains of TRα-ΔE6 are involved in the inhibition of TRα1 activity.

TRα-ΔE6 Is Involved in the Regulation of Myoblast Differentiation

To study the function of TRα-ΔE6, initially cloned in C2C12 myoblasts, we stably overexpressed this protein in QM7 avian myoblasts using pIRES-TRα-ΔE6

vector. These cells were currently used in our team to study myoblast terminal differentiation. Control cells were obtained after transfection of the pIRES-EGFP empty vector. Experiments were carried out on polyclonal populations of transfected cells. TRα-ΔE6 overexpression was assessed by Northern blot and Western blot (Fig. 8, A and B).

In these experiments, we first assessed the influence of TRα-ΔE6 on myoblast withdrawal from the cell cycle, a major event allowing terminal differentiation to occur, by determining the frequency of cells expressing connectin, an early postmitotic marker (23), at the induction of differentiation occurring at cell confluence. Whereas in proliferation no connectin-positive myoblasts were detected in control cells transfected with the empty vector, TRα-ΔE6 overexpression increased the frequency of connectin-expressing cells (Fig. 8C) and induced a precocious appearance of small myotubes [fusion index (see *Materials and Methods*): 5.8 ± 1.3% *vs.* 0% for control myoblasts; $P < 0.05$) (Fig. 8D)]. In addition, this acceleration of myoblast withdrawal from the cell cycle was detected independently of T_3 presence (fusion index: 5.8 ± 1.3% *vs.* 5.8 ± 0.9% with T_3) (Fig. 8D).

As previously reported (24), after induction of terminal differentiation, addition of T_3 to the culture medium induced a nearly 2-fold stimulation of myoblast differentiation in control cells, reflected by an increased

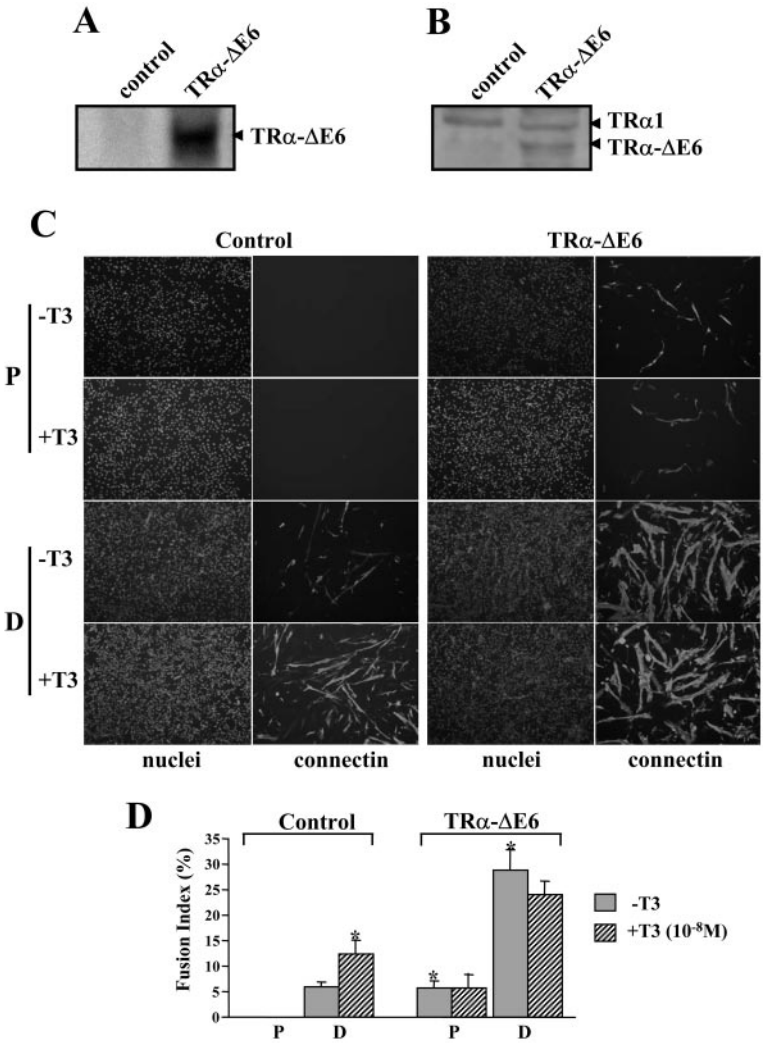

Fig. 8. Influence of TRα-ΔE6 Overexpression on QM7 Myoblast Differentiation

A, Northern blot analysis using total RNA extracted from the control or from TRα-ΔE6-overexpressing QM7. TRα-ΔE6 was detected after hybridization with TRα probe. B, Western blot analysis using total protein extracts from the control or from TRα-ΔE6-overexpressing QM7. Western blot experiments were performed using an antiserum raised against the carboxy-terminal domain of TRα1 (8). Proteins (50 μg) were loaded in each lane. C, Immunofluorescence staining with connectin antibody, during proliferation (P) and/or terminal differentiation (D) as indicated (magnification, ×100). Nuclei were stained with Hoeschst 33258. These results are representative of three separate experiments. D, Fusion index values are the mean ± SEM of three separate experiments. When indicated, 10^{-8} M T_3 was added to the culture medium. Statistically significant differences compared with control cells at the same stage: *, $P < 0.05$, Student's paired *t* test.

number of myotubes in the culture and a rise in the value of the fusion index (fusion index: 6 ± 0.8% *vs.* 12 ± 0.3% with T_3; $P < 0.05$) (Fig. 8, C and D). In agreement with its influence on myoblast withdrawal from the cell cycle, TRα-ΔE6 overexpression induced up to a 4-fold increase in the value of the fusion index relative to control cells, well reflected by the appearance of numerous thick myotubes (fusion index 29 ± 4.4% *vs.* 6 ± 0.8%; $P < 0.05$) (Fig. 8D). As for the induction of irreversible cell cycle arrest, this influence was independent of T_3 presence (fusion index 29 ± 4.4% *vs.* 24 ± 2.6%) (Fig. 8D).

In agreement with these data, we found that, in contrast to control cells, expression of the acetylcholine receptor α-subunit (AchRα), a valuable marker of myoblast differentiation (24), was detected in TRα-ΔE6 overexpressing myoblasts even during the proliferation period (Fig. 9A) (78 ± 25% for TRα-ΔE6 *vs.* 17 ± 11% for control; $P < 0.05$) (Fig. 9A). During differentiation, AchRα expression was strongly stimulated by TRα-ΔE6 overexpression in a T_3-independent manner (170 ± 21% for TRα-ΔE6 *vs.* 100% for control, without T_3; $P < 0.05$), whereas the hormone increased the level of this transcript in control cells. As previously shown in QM7 myoblasts, T_3 did not influence myogenin mRNA and protein levels (Fig. 9, A and B). However, TRα-ΔE6 overexpression induced a significant rise in these levels, independently of the T_3 presence (Fig. 9, A and B).

TRα-ΔE6 Impairs the Ability of TRα1 to Repress Chicken MyoD1 (CMD1) Transcriptional Activity

As myogenic factor expression is not a target of T_3 in QM7 myoblasts (25), these data raised the possibility that TRα-ΔE6 could exert its myogenic influence by interfering with a T_3-independent TRα mechanism. We have previously found that fundamental mechanisms involved in the regulation of myoblast differentiation by T_3 receptors occur through protein-protein interactions (15). In particular, we have recently shown that TRα1 could control the duration of myoblast proliferation by inhibiting CMD1 (avian MyoD) transcriptional activity (16). Because the functionality of this mechanism does not require T_3, we studied the influence of TRα-ΔE6 on this regulation.

In transient transfection experiments using a CAT reporter gene driven by a minimal myogenin promoter including two E boxes, CMD1 induced a 7-fold increase in CAT activity (773 ± 46%; $P < 0.01$), whereas TRα1 and TRα-ΔE6 were devoid of any transcriptional activity in the presence or absence of T_3 (Fig. 10A). We also confirmed that, in contrast to TRα1-ΔE6, coexpression of TRα1 significantly impaired CMD1 transcriptional activity, as reported by Daury *et al.* (16) (773 ± 46% for CMD1 *vs.* 420 ± 42% for CMD1/TRα1; $P < 0.05$) (Fig. 10A). However, coexpression of TRα1-ΔE6 abrogated the ability of the full-length receptor TRα1 to inhibit CMD1 activity (773 ± 46% for CMD1 *vs.* 713±44% for CMD1/TRα1/TRα-ΔE6) (Fig. 10A).

To test the linkage between the induction of myoblast differentiation by TRα-ΔE6 and its ability to inhibit TRα1-induced MyoD repression, we used the TRα-ΔE6 mutants previously tested for their capacity to repress TRα1 and RXRα transcriptional activity. As TRα-ΔE6, we found that deletion of the AF-2 domain alters the repressive activity of TRα1 on CMD1 transcriptional activity (638 ± 89% for CMD1/TRα1 *vs.* 1051 ± 192% for CMD1/TRα1 /TRα-ΔE6 *vs.* 1037 ± 186% for CMD1/TRα1 /TRα-ΔE6/ΔAF2; $P < 0.05$) (Fig. 10B). In contrast, the repression of CMD1 activity by TRα1 was not affected by the expression of mutants deleted from the DNA-binding or the amino-terminal domain (638 ± 89% for CMD1/TRα1 *vs.* 701 ± 114% for CMD1/TRα1/TRα-ΔE6/ΔNter *vs.* 679 ± 94% for CMD1/TRα1 /TRα-ΔE6/ΔDBD) (Fig. 10B). In transient transfection experiments using wild-type or mutants of TRα-ΔE6, we observed that, as previously shown, addition of T_3 to the culture medium induced a stimulation of myoblast differentiation in control cells (Fig. 10C). Moreover, TRα-ΔE6 overexpression induced a stronger increase of myoblast differentiation relative to control cells independently of T_3 presence. However, in good correlation with data from CAT assays, deletion of DNA binding or amino-terminal domains did not influence differentiation relatively to control cells, whereas overexpression of TRα-ΔE6/ΔAF-2 induced a stimulation of myoblast differentiation similar to that recorded for TRα-ΔE6 (Fig. 10C). These data demonstrated that the myogenic activity of TRα-ΔE6 is related to its ability to abrogate the repression of CMD1 activity by TRα1.

DISCUSSION

We cloned and characterized a new transcript generated by the TRα locus, TRα-ΔE6, resulting from an alternative splicing with microexon 6b instead of exon 6 and from RNA editing. This transcript, first identified in mouse liver and C2C12 cells, was found in all mouse tissues tested, indicating that this isoform is ubiquitously expressed.

Analysis of the genomic context leading to the generation of TRα-ΔE6 through use of a microexon points out striking similarities with that previously described for the chicken cardiac troponin T gene, giving rise to a transcript displaying a sequence corresponding to a microexon of six nucleotides (17). As in the TRα locus for microexon 6b, this microexon is surrounded by short G-rich repeats acting as intron-splicing enhancers leading to its inclusion for mRNA synthesis (17). A reasonable speculation leads us to propose that this mechanism is probably involved in the skipping of exon 6 in the case of TRα-ΔE6.

In addition, we found that two nucleotides differed when we compared the genomic sequence of microexon 6b and the corresponding sequence in mRNA. These differences were systematically ob-

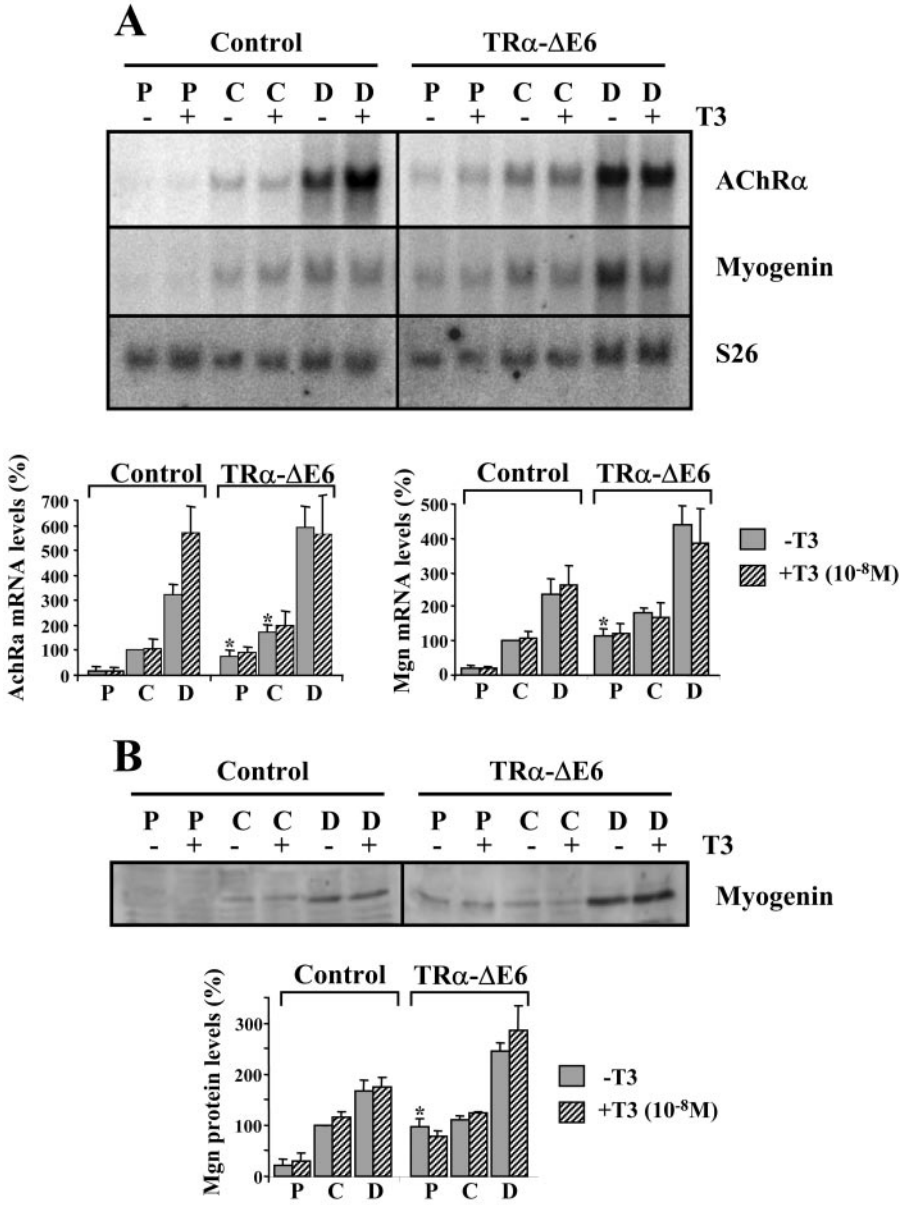

Fig. 9. Influence of TRα-ΔE6 Overexpression on Markers of Myoblast Differentiation

A, Total RNAs (10 μg) isolated from control or TRα-ΔE6-overexpressing myoblasts at the indicated stage [48 h proliferation (P); confluence (C); 3 d of differentiation (D)] were analyzed by Northern blot for AchRα and myogenin mRNA. Quantification was performed using a STORM PhosphorImager (Molecular Dynamics) and normalized in relation to S26 levels for mRNAs. Results are expressed as a percentage of the control value recorded in confluent myoblasts. B, Proteins (50 μg) isolated from control or TRα-ΔE6-overexpressing myoblasts at the indicated stage were analyzed by Western blot using an antiserum raised against myogenin. Quantification was performed using a STORM PhosphorImager. Results are expressed as a percentage of the control value recorded in confluent myoblasts. Data are the mean ± SEM of three separate experiments. When indicated, 10^{-8} M T_3 was added to the culture medium. Statistically significant differences compared with control cells at the same stage: *, $P < 0.05$, Student's paired *t* test.

served after several separate experiments, in C2C12 cells as well as in mouse liver, thus ruling out the possibility of artifacts linked to polymerase amplification fidelity or sequencing errors. Generally, editing processes lead to the substitution of a cytidine by a thymidine in the generated transcripts. Surprisingly, the editing process leading to the TRα-ΔE6 transcript involves both deletion of a thymidine and addition of a cytidine downstream. Although the mechanism involved in this process remains unclear, one possibility is that the perfect palindromic structure of the editing area could be an important feature for this microedition event. Although the exact mechanisms involving alternative splicing cou-

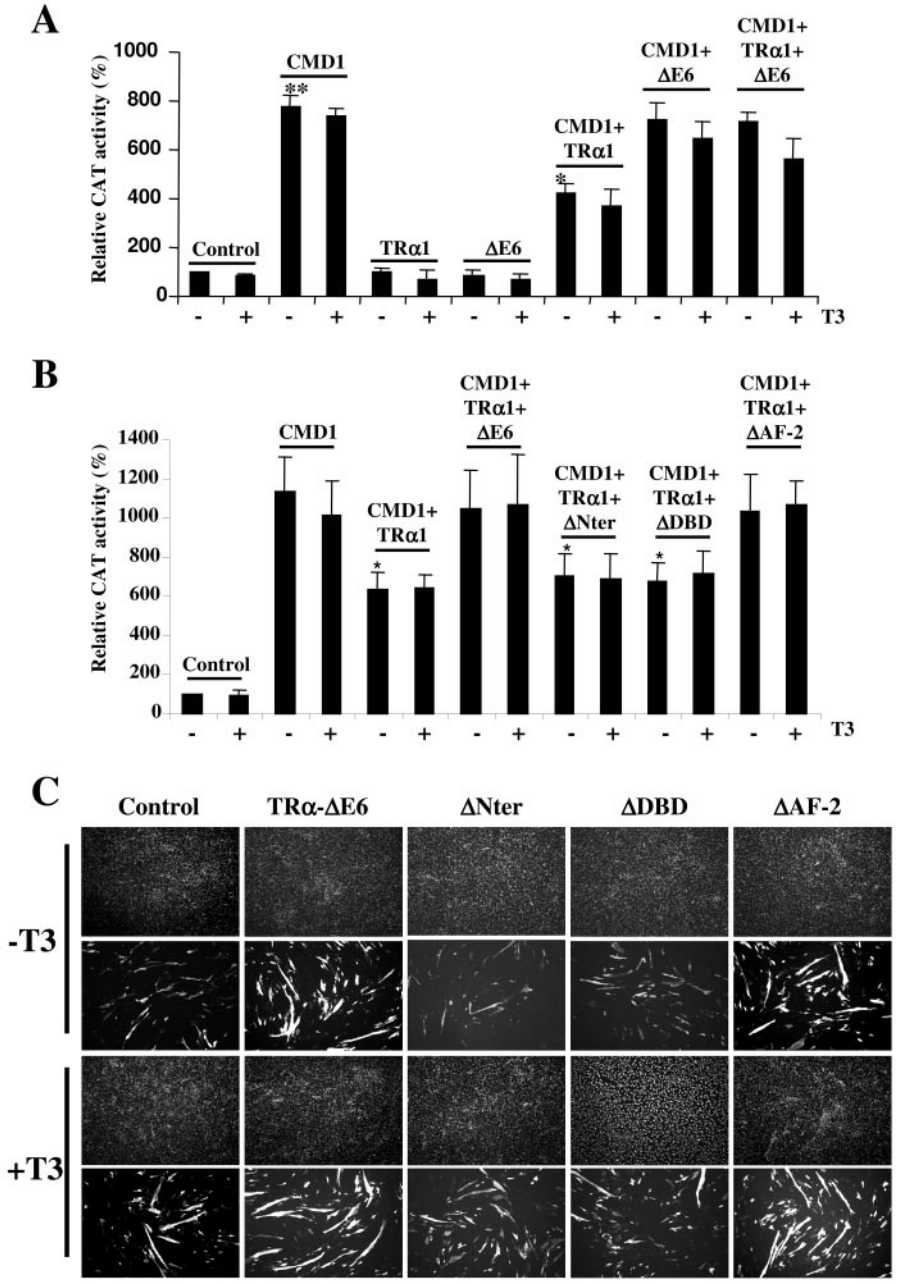

Fig. 10. The Myogenic Activity of TRα-ΔE6 Is Related to Its Capacity to Abrogate TRα1/CMD1 Functional Interaction

A and B, Transient transfection assays were performed in T_3-deprived QM7 cells using a CAT reporter gene driven by a minimal myogenin promoter harboring two E boxes and expression vectors indicated in the figure. All results were normalized according to β-galactosidase activity and expressed as a percentage of the control value. Data are presented as the mean ± SEM of four separate experiments. When indicated, 10^{-8} M T_3 was added to the culture medium. Statistically significant differences compared with control cells: **, $P < 0.01$; statistically significant differences compared with CMD1 overexpressing cells: *, $P < 0.05$, Student's paired *t* test. A, TRα-ΔE6 restored CMD1 transcriptional activity by abrogating the TRα1/CMD1 functional interaction. B, The mutant deleted of AF-2 domain, which has kept the ability to abrogate the repression of MyoD activity by TRα1, stimulates differentiation as well as TRα-ΔE6. C, Immunofluorescence staining with connectin antibody, during terminal differentiation (magnification, ×100). Nuclei were stained with Hoeschst 33258. When indicated, 10^{-8} M T_3 was added to the culture medium. These results are representative of three separate experiments.

pled to RNA editing is far from proven, a TRα protein without a hinge domain is generated.

Previous studies have demonstrated that the hinge domain is required in ligand binding activity. In particular, Lin *et al.* (21) and Miyamoto *et al.* (20) demonstrated that deletion of the D domain fully abrogated the ligand binding activity of TRα1 and TRβ1. Moreover, a naturally occurring point mutation in the hinge

domain of TRβ1was reported to impair T_3 binding activity (26, 27). Last, structural study of TRα1 indicated that α-helix H1 occurring in the hinge domain is required for hormone binding (28). All these data support the importance of the hinge region in hormone binding and strongly suggest that the absence of hinge domain impairs TRα-ΔE6 ligand binding.

The hinge domain is also important for TRα1 DNA binding. Thus, Miyamoto *et al.* (20) showed that deletion of this domain fully abrogated the DNA binding activity of TRα1, in agreement with structural studies indicating that residues of the hinge domain form a turn after the second helix of the DBD (A box) (29), which participates in formation of heterodimers TR/RXR, and an extended carboxy-terminal α-helix, which binds in the minor groove of the DNA to allow binding (30). In line with these data, we found in gel mobility shift assay using *in vitro* synthesized proteins, that TRα-ΔE6 did not bind to DNA. Moreover, the TRα variant did not affect the binding pattern of TRα1 on a DR4.

In line with the previous observation that the hinge domain is also involved in the nuclear localization of the receptor (18), we found that, in contrast to the full-length receptor TRα1 predominantly expressed in the nucleus, TRα-ΔE6 displayed a cytoplasmic localization. Interestingly, we also observed that the full-length receptor TRα1 could be sequestered in the cytoplasm by TRα-ΔE6.

We found that TRα-ΔE6 represses the activity of TRα1 and RXRα. Although the mechanism mediating this inhibitory activity is still unclear, the cytoplasmic sequestration of TRα1 by TRα-ΔE6 could be a first element. Another possibility could be that TRα1-ΔE6 exerts its influence by sequestration of coactivators normally recruited by TRα1 or RXR. In this hypothesis, the observation that RAR activity was unaffected by TRα-ΔE6 could suggest that the main target of TRα mutant is a coactivator that interacts with TRα1 and RXR, but not with RAR. Moreover, interaction between TRα1-ΔE6 and the target factor may take place in the cytoplasm because TRα-ΔE6 is predominantly localized in this compartment, resulting in coactivator sequestration outside the nucleus. Similar hypotheses have been raised by Chassande *et al.* (13) for the TRΔα1 isoform, which also antagonizes the transcriptional activation induced by TRα1 and RXR but not by RAR, without any interaction with these receptors. However, identification of this factor will be necessary for further insight into the mechanisms of inhibition by TRα-ΔE6.

To identify the domains of TRα-ΔE6 involved in the inhibition of full-length TRα1 activity, we used TRα-ΔE6 mutants deleted of the DNA binding, AF-2, or amino-terminal domains in CAT assay experiments. We found that deletion of the AF-2 domain did not abrogate the ability of TRα-ΔE6 to repress TRα1 and RXRα transcriptional activity through a DR4. In contrast, in the absence of the DNA-binding or amino-terminal domains, TRα-ΔE6 failed to block this transcriptional activity. These data demonstrated that the DNA-binding and amino-terminal domains are involved in the repressing activity of the TRα variant.

To study the biological function of TRα-ΔE6, we stably overexpressed the protein in QM7 myoblasts. We found that TRα-ΔE6 overexpression accelerated myoblast withdrawal from the cell cycle and terminal differentiation, as reflected by fusion index values, and expression of muscle-specific proteins. One striking feature of this influence is that it occurred independently of T_3 presence, in agreement with the absence of a hinge domain in the TRα variant leading to an impairment to bind T_3 (19, 20). In addition, this result rules out the possibility that the mechanism involved in this myogenic influence includes inhibition of the T_3-dependent TRα1 transcriptional activity demonstrated in this study. More recently, we have also characterized a new mechanism involving TRα1 to maintain an optimal duration of the proliferation period, through T_3-independent inhibition of MyoD transcriptional activity, a major actor involved in the induction of myoblast differentiation (16). Interestingly, in this study, we found that TRα-ΔE6 strongly inhibits this functional interaction, in agreement with an anticipated myoblast withdrawal from the cell cycle resulting from a restoration of MyoD transcriptional activity. Because MyoD is involved in a positive autoregulatory loop controlling its own transcription rate as well as myogenin expression (31), this possibility is also well supported by the observation indicating that myogenin mRNA levels are increased by TRα-ΔE6 overexpression.

In this study, we also found a correlation between the capacity of TRα-ΔE6 mutants to abrogate the ability of TRα1 to inhibit MyoD activity and their capacity to stimulate myoblast differentiation. Indeed, the mutant deleted of AF-2 domain, which has kept the ability to abrogate the repression of MyoD activity by TRα1, stimulates differentiation as well as TRα-ΔE6. In contrast, the two other mutants deleted of DNA binding or amino-terminal domains, which do not repress the TRα1/MyoD functional interaction, did not influence myoblast differentiation. These data demonstrated that the ability of TRα-ΔE6 to stimulate differentiation is related to its capacity to repress TRα1.

In conclusion, the activity of truncated proteins as potent inhibitors of their full-length counterparts has been established for several transcription factors. The inhibition of TRα1 activity by TRα-ΔE6 is a novel example of this kind of regulation. In particular, by inhibiting the TRα1/CMD1 interaction, TRα1-ΔE6 could help to restore MyoD activity and enables the induction of terminal differentiation. If such a mechanism occurs *in viv*o, this balance between TRα1 and TRα-ΔE6 could be critical for correct muscle development by controlling the duration of myoblast proliferation. Lastly, the observation that TRα-ΔE6 was found to be expressed in numerous tissues such as brain, liver, or heart suggests that this function is not restricted to muscle.

MATERIALS AND METHODS

Tissue and Cell RNA Extraction and RT-PCR Analysis

Total cellular RNAs from tissues and C2C12 cells were isolated as previously described (32). For analysis of TRα-ΔE6 expression by RT-PCR, total RNA was reverse transcribed using random hexamer primers and AMV reverse transcriptase (Promega Corp., Madison, WI). The resulting cDNA product was subsequently PCR-amplified (30 cycles of 45 sec at 94 C; 45 sec at 58 C; 45 sec at 72 C) with the following primers: 5′-cea156 (TGT GTC GTG TGT GGG GAC AA) and 3′-cea927 (ACT TCC GTG TCA TCC AGG TT). Half-nested PCR was then performed using the internal primers 5′-ceaΔE6 (GTG GGC ATG GGG ACT AGT) and 3′-cea927 in similar conditions.

Cloning and Construction of Recombinant Plasmids

The mouse TRα1 cDNA containing the entire open reading frame and the mouse TRα-ΔE6 cDNA containing the shorter fragment, without exon 6, were produced by RT-PCR amplification of total RNAs collected from mouse liver or C2C12 cells using the following primers: 5′cea-4 (GTG AAT GGA ACA GAA GCC AAG CAA GGT) and 3′cea1250 (GCC GCC TGA GGC TTT AGA CTT CCT GAT). The following primers were also used to characterize mTRα-ΔE6: 5′-cea107 (AGC AGC ATG TCA GGG TAT ATC CCT), 5′-cea444 (AGG CGA AAG GAG GAG ATG ATT CGC TCA CT) and 5′-cea733 (GAA GAC CAG ATC ATC CTC CT). The amplified cDNAs were cloned with Promega pGEM-T easy vector. The PCR fragments were released by *Eco*RI digestion and inserted into the *Eco*RI site of the pSG5 vector, resulting in pSG5-mTRα1 and pSG5-mTRα-ΔE6 constructs. For stable transfection, mTRα-ΔE6 was released by *Eco*RI digestion and inserted into the *Eco*RI site of the pIRES-EGFP vector, resulting in the pIRES-mTRα-ΔE6 construct. To study the subcellular localization of TRα1 and TRα-ΔE6 proteins, we fused the cDNA sequence of the proteins to the EGFP gene. The recombinant DNA molecules were generated by PCR and inserted in the *Eco*RI site of the pSG5 vector (pSG5-TRα1/EGFP, pSG5-TRα-ΔE6/EGFP). The TRα-ΔE6 mutants were generated by PCR using the following primer: 1) TRα-ΔE6/ΔNter: 5′-cea ΔNter (AGC AGC ATG TCA GGG TAT ATC CCT) and 3′-cea1250 (GCC GCC TGA GGC TTT AGA CTT CCT GAT); 2) TRα-ΔE6/ΔDBD: 5′-cea-4 (GTG AAT GGA ACA GAA GCC AAG CAA GGT) and 3′-cea Δ DBD (AGT CCC CAT GCC CTG CTC GTC TTT GTC AGG TAA CT) for the first fragment, 5′-cea ΔDBD (AAAGAC GAG CAG GGC ATG GGG ACT AGT CCC CAG TC) and 3′-cea1250 for the second fragment; 3) TRα-ΔE6/ΔAF-2: 5′-cea-4 and 3′-cea ΔAF-2 (GCT TTA GAG CGG GGG GAA GAG TTC). For TRα-ΔE6/ΔDBD, a second step of PCR using the two mutated fragments was performed. The amplified cDNAs were cloned with Promega pGEM-T easy vector. The PCR fragments were released by *Eco*RI digestion and inserted into the *Eco*RI site of the pSG5 vector, resulting in pSG5-TRα-ΔE6/ΔNter, pSG5-TRα-ΔE6/ΔDBD, and pSG5-TRα-ΔE6/ΔAF-2 constructs.

DNA Sequencing and Identification of a TRα Microexon

Sequencing of the mouse TRα-ΔE6 cDNA from liver and C2C12 cells was performed with the Big Dye terminator kit from PerkinElmer Life Sciences (Norwalk, CT) following the supplier's protocol. Genomic cDNA clones containing the microexon 6 were produced by amplification of mouse liver and C2C12 cell DNA (30 cycles of 45 sec at 94 C, 45 sec at 58 C, 1 min at 72 C) using the following primers: 5′-ceaE6 (ATG ATT CGA AGC GGG TGG CCA AA) and 3′-ceaE7 (CTC GGA GAA CAT GGG CAG TTT T). The genomic cDNA fragment was sequenced as previously described.

Cell Cultures and Transcriptional Activation Assays

Quail myoblasts of the QM7 cell line (33) were seeded at a plating density of 7000 cells/cm^2. They were grown in Earle's 199 medium supplemented with 0.2% tryptose phosphate broth, penicillin (100 IU/ml), and 10% of T_3-depleted fetal calf serum. The serum was T_3 depleted as previously described (34). Terminal differentiation was induced at cell confluence by lowering the medium's fetal calf serum concentration to 0.5%.

Transient transfection assays were performed in QM7 cells using expression plasmids, CAT reporter, and pRSV-β-galactosidase expression vector, as described in Cassar-Malek *et al.* (15). The following expression vectors were used: pSG5, pSG5-TRα1, pSG5-TRα-ΔE6, pSG5-RXRα, pSG5-RARα, pSG5-TRα-ΔE6/ΔNter, pSG5-TRα-ΔE6/ΔDBD, pSG5-TRα-ΔE6/ΔAF-2, and pRSV-CMD1. When indicated, 10^{-8} M T_3, 5.10^{-8} M 9-*cis*-retinoic acid or 10^{-7} M all-*trans* retinoic acid was added to the culture medium. After normalization according to β-galactosidase activity, all results were expressed as a percentage of the control value. Data are presented as the mean ± SEM of four separate experiments.

Cytoimmunofluorescence Studies

Myoblast differentiation was assessed by morphological changes and accumulation of connectin, a muscle-specific marker expressed just after myoblast withdrawal from the cell cycle (23). After methanol fixation and appropriate washings, cells were stained with a monoclonal antibody raised against connectin (kindly provided by Dr. F. Pons, Institut National de la Santé et de la Recherche Médicale, Montpellier, France) and a fluorescein-conjugated antibody raised against mouse Igs. Nuclei were stained with Hoechst 33258 (1 μg/ml). To quantify terminal differentiation, the fusion index was calculated by counting the total number of nuclei and the number of nuclei incorporated in myotubes (fusion index = number of nuclei in myotubes × 100/total number of nuclei).

The intracellular localization of mouse TRα1 or TRα-ΔE6 was assessed in transient transfection experiments using QM7 cells and the fusion protein TRα1/EGFP or TRα-ΔE6/EGFP.

Preparation of Protein Extracts and *in Vitro* Protein Synthesis

QM7 cells plated in coated dishes (7000 cells/cm^2) were harvested in 1.5 ml of PBS and then centrifuged for 5 min at 12,000 × *g*. The pellet was resuspended and lysed in 100 μl Tris-Nonidet P-40 (0.7% Nonidet P-40; 10 mM Tris, pH 7.8). Proteins were synthesized *in vitro* using the rabbit reticulocyte lysate Transcription/Translation kit TNT (Promega) according to the manufacturer's instructions. Reactions were performed using the pGEM-mTRα1 and pGEM-mTRα-ΔE6 vectors.

Gel EMSAs

Gel EMSA experiments were performed according to Wrutniak *et al.* (8) using a ^{32}P-labeled DR4 oligonucleotide as probe (TC**AGGTCA**CAGG**AGGTCA**) and cold mTRα1 and/or mTRα-ΔE6 synthesized *in vitro* using the rabbit reticulocyte lysate. TR binds to specific target sequences termed thyroid-response element (TRE). TRE consists of two half-sites. *Bold* letters indicate the two half-sites (5′-AGGTCA-3′ consensus sequence).

Western Blot Analysis

Total cellular extracts (50 μg) were electrophoresed onto 10% SDS-PAGE gels and blotted onto polyvinylidine difluo-

ride membranes. The presence of TRα-ΔE6 was assessed using RHTII antiserum (8). To detect the full-length receptor TRα1, we also used a polyclonal antibody raised against the hinge domain of TRα1 (TRα-144, Interchim, Montluçon, France). The presence of myogenin was detected using a polyclonal antibody raised against chicken myogenin (kindly provided by Dr. B. M. Paterson, Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD) (35). Signals were revealed using a chemifluorescence detection procedure (enhanced chemifluorescence kit, Amersham Biosciences, Orsay, France) and analyzed with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Northern Blot Analysis

Total RNA (20 μg) was loaded in each lane of 1% formaldehyde-agarose gel. After migration, RNAs were transferred onto a nylon membrane. Membranes were hybridized with specific cDNA probes labeled using the megaprime DNA labeling system (Amersham Biosciences). A S26 ribosomal cDNA probe was used to provide an invariant control as previously described (36). Quantification was performed using a STORM PhosphorImager (Molecular Dynamics).

Statistical analyses were performed using Student's paired *t* test.

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REFERENCES

1. Lazar MA 1993 Thyroid hormone receptors: multiple forms, multiple possibilities. Endocr Rev 14:184–193
2. Brent GA, Moore DD, Larsen PR 1991 Thyroid hormone regulation of gene expression. Annu Rev Physiol 53: 17–35
3. Sap J, Munoz A, Damm K, Sap J, Munoz A, Damm K, Goldberg Y, Ghysdael J, Leutz A, Beug H, Vennstrom B 1986 The c-erb-A protein is a high-affinity receptor for thyroid hormone. Nature 324:635–640
4. Weinberger C, Thompson EB, Ong S, Lebo R, Gruol DJ, Evans R 1986 The c-erb A gene encodes a thyroid hormone receptor. Nature 324:641–646
5. Hodin RA, Lazar MA, Wintman BI, Darling DS, Koenig RJ, Larsen PR, Moore DD, Chin WW 1989 Identification of a thyroid hormone receptor that is pituitary-specific. Science 244:76–79
6. Koenig R, Warne RL, Brent GA, Harney IW, Larsen PR, Moore DD 1988 Isolation of cDNA clone encoding a biologically active thyroid hormone receptor. Proc Natl Acad Sci USA 85:531–535
7. Williams GR 2000 Cloning and characterization of two novel thyroid hormone receptor β isoforms. Mol Cell Biol 20:8329–8342
8. Wrutniak C, Cassar-Malek I, Marchal S, Rascle A, Heusser S, Keller JM, Flechon J, Dauca M, Samarut J, Ghysdael J, Cabello G 1995 A 43-kDa protein related to c-Erb A α 1 is located in the mitochondrial matrix of rat liver. J Biol Chem 270:16347–16354
9. Forrest D, Sjoberg M, Vennstrom B 1990 Contrasting developmental and tissue-specific expression of α and β thyroid hormone receptor genes. EMBO J 9:1519–1528
10. Casas F, Rochard P, Rodier A, Cassar-Malek I, Marchal-Victorion S, Wiesner RJ, Cabello G, Wrutniak C 1999 A variant form of the nuclear triiodothyronine receptor c-ErbAα1 plays a direct role in regulation of mitochondrial RNA synthesis. Mol Cell Biol 19:7913–7924
11. Lazar MA, Hodin RA, Darling DS, Chin WW 1988 Identification of a rat c-erb A?-related protein which binds deoxyribonucleic acid but does not bind thyroid hormone. Mol Endocrinol 2:893–901
12. Mitsuhashi T, Tennyson GE, Nikodem VM 1988 Alternative splicing generates messages encoding rat c-erb A proteins that does not bind thyroid hormone. Proc Natl Acad Sci USA 85:5804–5808
13. Chassande O, Fraichard A, Gauthier K, Flamant F, Legrand C, Savatier P, Laudet V, Samarut J 1997 Identification of transcripts initiated from an internal promoter in the c-erbA α locus that encode inhibitors of retinoic acid receptor-α and triiodothyronine receptor activities. Mol Endocrinol 11:1278–1290
14. Flamant F, Samarut J 2003 Thyroid hormone receptors: lessons from knockout and knock-in mutant mice. Trends Endocrinol Metab 14:85–90
15. Cassar-Malek I, Marchal S, Rochard P, Casas F, Wrutniak C, Samarut J, Cabello G 1996 Induction of c-Erb A-AP-1 interactions and c-Erb A transcriptional activity in myoblasts by RXR. Consequences for muscle differentiation. J Biol Chem 271:11392–11399
16. Daury L, Busson M, Casas F, Cassar-Malek I, Wrutniak-Cabello C, Cabello G 2001 The triiodothyronine nuclear receptor c-ErbAα1 inhibits avian MyoD transcriptional activity in myoblasts. FEBS Lett 508:236–240
17. Carlo T, Sierra R, Berget SM 2000 A 5′ splice site-proximal enhancer binds SF1 and activates exon bridging of a microexon. Mol Cell Biol 20:3988–3995
18. Dang CV, Lee WM 1989 Nuclear and nucleolar targeting sequences of c-erb-A, c-myb, N-myc, p53, HSP70, and HIV tat proteins. J Biol Chem 264:18019–18023
19. Horowitz ZD, Yang C, Forman BM, Casanova J, Samuels HH 1989 Characterization of the domain structure of chick c-erb A by deletion mutation: *in vitro* translation and cell transfection studies. Mol Endocrinol 3:148–156
20. Miyamoto T, Kakizawa T, Ichikawa K, Nishio S, Takeda T, Suzuki S, Kaneko A, Kumagai M, Mori J, Yamashita K, Sakuma T, Hashizume K 2001 The role of hinge domain in heterodimerization and specific DNA recognition by nuclear receptors. Mol Cell Endocrinol 181:229–238
21. Lin KH, Parkinson C, McPhie P, Cheng SY 1991 An essential role of domain D in the hormone binding activity of the human thyroid hormone nuclear receptor. Mol Endocrinol 5:485–492
22. Umesono K, Murakami KK, Thompson CC, Evans RM 1991 Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin-D3 receptors. Cell 65:1255–1266
23. Hill C, Weber K 1986 Monoclonal antibodies distinguish titins from heart and skeletal muscle. J Cell Biol 102: 1099–1108
24. Marchal S, Cassar-Malek I, Pons F, Wrutniak C, Cabello G 1993 Triiodothyronine influences quail myoblast proliferation and differentiation. Biol Cell 78:191–197
25. Marchal S, Cassar-Malek I, Magaud JP, Rouault JP, Wrutniak C, Cabello G 1995 Stimulation of avian myoblast differentiation by triiodothyronine: possible involvement of the cAMP pathway. Exp Cell Res 220:1–10
26. Behr M, Loos U 1992 A point mutation (Ala229 to Thr) in the hinge domain of the c-erbA β thyroid hormone re-

ceptor gene in a family with generalized thyroid hormone resistance. Mol Endocrinol 6:1119–1126
27. Yagi H, Pohlenz J, Hayashi Y, Sakurai A, Refetoff S 1997 Resistance to thyroid hormone caused by two mutant thyroid hormone receptors β, R243Q and R243W, with marked impairment of function that cannot be explained by altered *in vitro* 3,5,3′-triiodothyroinine binding affinity. J Clin Endocrinol Metab 82:1608–1614
28. Wagner RL, Apriletti JW, McGrath ME, West BL, Baxter JD, Fletterick RJ 1995 A structural role for hormone in the thyroid hormone receptor. Nature 378:690–697
29. Kurokawa R, Yu VC, Naar A, Kyakumoto S, Han Z, Silverman S, Rosenfeld MG, Glass CK 1993 Differential orientations of the DNA binding domain and C-terminal dimerization interface regulate binding site selection by nuclear receptor heterodimers. Genes Dev 7:1423–1435
30. Rastinejad F, Perlmann T, Evans RM, Sigler PB 1995 Structural determinants of nuclear receptor assembly on DNA direct repeats. Nature 375:203–211
31. Edmondson DG, Cheng TC, Cserjesi P, Chakraborty T, Olson EN 1992 Analysis of the myogenin promoter reveals an indirect pathway for positive autoregulation mediated by the muscle-specific enhancer factor MEF-2. Mol Cell Biol 12:3665–3677
32. Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
33. Antin PB, Ordhal CP 1991 Isolation and characterization of an avian myogenic cell line. Dev Biol 143:111–121
34. Samuels HH, Stanley F, Casanova J 1979 Depletion of L-3,5,3′-triiodothyronine and L-thyroxine in euthyroid calf serum for use in cell culture studies of the action of thyroid hormone. Endocrinology 105:80–85
35. Yablonka-Reuveni Z, Paterson BM 2001 MyoD and myogenin expression patterns in cultures of fetal and adult chicken myoblasts. J Histochem Cytochem 49:455–462
36. Vincent S, Marty L, Fort P 1993 S26 ribosomal protein RNA: an invariant control for gene regulation experiments in eucaryotic cells and tissues. Nucleic Acids Res 21:1498

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