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Muriel Busson, Laetitia Daury, Pascal Seyer, Stéphanie Grandemange, Laurence Pessemesse, et al.. Avian MyoD and c-Jun Coordinately Induce Transcriptional Activity of the 3,5,3'-Triiodothyronine Nuclear Receptor c-ErbAalpha1 in Proliferating Myoblasts. Endocrinology, 2006, 147 (7), pp.3408-3418. 10.1210/en.2006-0101 . hal-02663290

HAL Id: hal-02663290
https://hal.inrae.fr/hal-02663290

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0013-7227/06/$15.00/0
Printed in U.S.A.

Endocrinology 147(7):3408–3418
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doi: 10.1210/en.2006-0101

Avian MyoD and c-Jun Coordinately Induce Transcriptional Activity of the 3,5,3′-Triiodothyronine Nuclear Receptor c-ErbAα1 in Proliferating Myoblasts

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Although physical interactions with other receptors have been reported, heterodimeric complexes of T_3 nuclear receptors (TR) with retinoid X receptors (RXRs) are considered as major regulators of T_3 target gene expression. However, despite the potent T_3 influence in proliferating myoblasts, RXR isoforms are not expressed during proliferation, raising the question of the nature of the complex involved in TRα transcriptional activity. We have previously established that c-Jun induces TRα1 transcriptional activity in proliferating myoblasts not expressing RXR. This regulation is specific to the muscle lineage, suggesting the involvement of a muscle-specific factor. In this study, we found that MyoD expression in HeLa cells stimulates TRα1 activity, an influence potentiated by c-Jun coexpression. Similarly, in the absence of RXR, MyoD or c-Jun overexpression in myoblasts induces TRα1 transcriptional activity through a direct repeat 4 or an inverted palindrome 6 thyroid hormone response element. The highest rate of activity was recorded when c-Jun and MyoD were coexpressed. Using c-Jun-negative dominants, we established that MyoD influence on TRα1 activity needs c-Jun functionality. Furthermore, we demonstrated that TRα1 and MyoD physically interact in the hinge region of the receptor and the transactivation and basic helix loop helix domains of MyoD. RXR expression (spontaneously occurring at the onset of myoblast differentiation) in proliferating myoblasts abrogates these interactions. These data suggest that in the absence of RXR, TRα1 transcriptional activity in myoblasts is mediated through a complex including MyoD and c-Jun. (*Endocrinology* 147: 3408–3418, 2006)

THYROID HORMONE IS considered as a major regulator of *in vivo* muscle development. It not only stimulates muscle growth by increasing the number and diameter of muscle fibers (1, 2), but it also regulates the transition between neonatal and adult myosin isoforms (3, 4) and the contractile features of adult muscle fibers (5). Despite this important influence, disruption of α- or β-genes encoding thyroid hormone receptors detected in this tissue (6) only slightly affects muscle development in mice (7). This surprising observation could, in particular, be explained by the fact that thyroid hormone receptors repress T_3 target gene expression when the hormone is absent; consequently, depletion of receptors may induce a less drastic phenotype (no stimulation of T_3 target genes) than absence of the hormone does (repression of T_3 target genes). However, given the lack of earlier *in vitro* studies, the mechanisms involved in the myogenic influence of T_3 have only been gradually elucidated over the last decade by studies using secondary cultures of embryonic quail myoblasts or the avian myoblast line QM7. We first established that T_3 accelerates irreversible myoblast withdrawal from the cell cycle and consequently potentiates myoblast differentiation (8). These data clearly suggest that the major myogenic influence of this hormone targets proliferating myoblasts. Study of the molecular events involved in this regulation indicates that, whereas T_3 stimulates MyoD and Myogenin expression in murine myoblasts (9), a major and more general mechanism occurring in several species is probably the repression of transcriptional activity in the c-Jun/c-Fos activator protein 1 (AP-1) complex, which is considered as a strong repressor of myoblast differentiation (10).

As our study of molecular mechanisms underlying the myogenic influence of T_3 in avian myoblasts progressed, we observed that, owing to the lack of retinoid X receptor (RXR) expression (11), T_3 nuclear receptor α1 (TRα1)/RXR complexes do not occur in proliferating myoblasts. Downes *et al.* (12) have reported similar data in murine C2C12 myoblasts and provided evidence that RXR isoform expression is only induced at the onset of terminal myoblast differentiation. In agreement with these results, TRα1 did not display any significant transcriptional activity in these cells through a direct repeat 4 thyroid response element (DR4-TRE) in transient transfection experiments using reporter genes, although RXR expression by transient or stable transfection restored its transcriptional activity in the presence of T_3. Because myogenic T_3 influence is induced during the myoblasts' prolif-

First Published Online March 23, 2006

* M.B. and L.D. have contributed an equal amount of work to this study.

Abbreviations: AP-1, Activator protein 1; bHLH, basic helix loop helix; CAT, chloramphenicol acetyltransferase; DR4, direct repeat 4; GST, glutathione-S-transferase; Ipal, inverted palindrome; pal, palindromic; RXR, retinoid X receptor; TRα1, T_3 nuclear receptor α1; TRE, thyroid hormone response element.

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eration period, these results raised the question of TRα transcriptional activity in proliferating cells. Interestingly, we found that c-Jun expression induced TRα1 activity through a DR4-TRE. But this surprising c-Jun influence did not occur in HeLa cells, thus suggesting that the functional interaction between TRα1 and the cellular oncogene requires the occurrence of a muscle-specific protein (muscle-specific factor, MSF) (11).

More recently, we found that TRα1 overexpression fully abrogates avian MyoD transcriptional activity, thus highlighting the occurrence of a functional interaction between TRα1 and the muscle-specific factor MyoD (13). Therefore, the purpose of this study was to test the possibility that, in proliferating myoblasts not expressing RXR, TRα transcriptional activity is at least partly induced through muscle-specific interactions with c-Jun and MyoD. In this study, we found that in myoblasts, MyoD induces TRα1 transcriptional activity through DR4 and inverted palindrome (Ipal)-TREs in a c-Jun-dependent manner. This functional interaction involves physical interactions and is abrogated by RXR expression.

Materials and Methods

Cell culture

Quail myoblasts of the QM7 cell line (14) were seeded at a plating density of 7000 cells/cm^2. They were grown in Earle's 199 medium supplemented with tryptose phosphate broth (0.2%), L-glutamine (2 mM), gentamycin (50 μg/ml), and fetal calf serum (10% vol/vol). Serum was T_3 depleted according to Samuels *et al.* (15). After hormonal depletion, serum T_4 and T_3 concentrations measured by RIA were below the limit of detection in the assay.

Plasmid and reporter genes

The DR4-tk-CAT plasmid upstream of the thymidine kinase promoter linked to the CAT gene was kindly provided by P. Chambon (LGME U184, Strasbourg, France). The gal4-tk-CAT reporter plasmid contains four response elements of the yeast transcription factor GAL4 upstream of the chloramphenicol acetyltransferase (CAT) coding sequence. The myogenin-CAT reporter plasmid contains the −131/+40 fragment of the chicken myogenin promoter upstream of the CAT coding sequence (16). The −73col CAT collagenase promoter has been described elsewhere (17). The expression vectors for chicken TRα1 and MyoD (pRSV TRα1 and pRSV CMD1) have already been described (18, 19). The RSV (Rous sarcoma virus)-β-galactosidase expression vector has been described by Cassar-Malek *et al.* (11). The expression vector for rat Gal4/TRα1 (pSVGal4α1) was provided by Dr. F. Flamant (ENS, Lyon, France).

The expression vector for quail c-Jun (pDP18 c-Jun) and chicken RXRγ (pRSV RXRγ) have already been described (20, 21). The pDP18 c-Jun D176 expression vector (22) led to the expression of a quail c-Jun mutant deleted from almost all the transactivating domain. The pDP18 c-Jun eb1 expression vector (22) led to the expression of a quail c-Jun mutant in which the natural dimerization domain was replaced by the dimerization domain of the viral EB1 protein (c-Jun eb1 forms only homodimers).

pGEM-T TRα1, encoding the full-length thyroid hormone receptor TRα1, pGEM-T TRα1 (36–410), and pGEM-T TRα1 (152–410) have been described by Casas *et al.* (23). pGEM-T MyoD, pGEM-T JUN, and the mutants of TRα1 were generated by PCR from the full-length corresponding mRNA, inserted into the *Eco*RV site of pGEM-T plasmid (Promega, Charbonnieres, France).

Mutants of avian MyoD were generated with specific primers from the plasmid pRSV CMD1 and inserted in the *Sal*I/*Not*I sites of the pGEX4T2 (Pharmacia, Uppsala, Sweden) vector, in frame with the glutathione-S-transferase (GST) coding sequence.

Transient transfections and CAT assays

Transient transfections were performed using the calcium phosphate coprecipitation procedure as described by Cassar-Malek *et al.* (11). One microgram of pCMV β-galactosidase expression vector was cotransfected to provide an internal control of transfection efficiency. After cell exposure to precipitates for 24 h, the DNA-containing medium was replaced with fresh medium containing T_3 (10^{-8} M) when indicated, and the cells were grown for another 24 h. β-Galactosidase activity was measured as previously described (24). CAT enzymatic activity was measured by following the kinetics of chloramphenicol acetylation (11). For each assay, the initial rate of the enzymatic reaction was determined, and results were expressed as a percentage of control values after β-galactosidase normalization.

In vitro transcription and translation

In vitro transcription and translation were carried out with TNT-coupled reticulocyte lysate system (Promega) and *trans*-[^{35}S]methionine (ICN, Costa Mesa, CA), according to the manufacturer's protocol. The proteins were processed by SDS-PAGE, and quantification of the relative intensities was carried out with a phosphoimager (Molecular Dynamics, Sunnyvale, CA).

Western blots

Fifty micrograms of proteins of cell lysates were resolved by 10% SDS-PAGE minigels and blotted onto polyvinylidene difluoride membranes that were further incubated with anti-c-Jun (kindly provided by Dr. M. Castellazzi, Lyon, France), anti-MyoD (kindly provided by Dr. B. M. Paterson, Seattle, WA), anti-TRα1 RHTII (25), or anti-RXR (kindly provided by Prof. P. Chambon, Strasbourg, France). Signals were further detected by enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK) using a second horseradish peroxidase-conjugated antibody provided by the manufacturer.

Coimmunoprecipitation

Immunoprecipitation of proteins was performed as described by Casas *et al.* (26), with a few modifications, with *in vitro*-transcripted and -translated ^{35}S-labeled avian MyoD, RXR, c-Jun, TRα1, and mutants of avian MyoD and TRα1. Ten microliters of each protein were mixed with 1 μl of antibody raised against TRα1 or MyoD and 300 μl of binding buffer [50 mM HEPES (pH 7), 400 mM NaCl, 1% Nonidet P-40, and 1 μg/ml aprotinin] overnight at 4 C. Samples were incubated with 30 μl of protein G-Sepharose for 3 h at 4 C and washed three times with binding buffer and three times with TNE buffer [10 mM Tris (pH 7.5), 150 mM NaCl, and 1.2 mM EDTA]. Bound proteins were separated by SDS-PAGE, and the dried gel was exposed for autoradiography and analyzed with a phosphoimager (Molecular Dynamics).

A similar procedure was used for immunoprecipitation of cell extracts, using 400 μg cell lysate proteins and 2 μl of antibody raised against c-Jun. Revelation was performed as indicated for Western blotting with an antibody raised against TRα1. However, in HeLa cells, the second antibody used for enhanced chemiluminescence was rabbit True-Blot (Cliniscience Laboratories, Montrouge, France), not recognizing Igs.

GST pull-down experiments (27)

GST and GST fusion proteins were expressed in *Escherichia coli* DH5α, purified on glutathione-Sepharose beads (Amersham) and quantified by the Bradford method (Bio-Rad protein assay). Twenty micrograms of the GST fusion proteins were processed for SDS-PAGE and Coomassie stained to confirm the integrity of the full-length fusion proteins and to determine the amounts to use for the pull-down experiment.

For *in vitro* protein-protein interaction assays, comparable amounts of GST fusion proteins beads were incubated with 5 μl of *in vitro*-translated proteins in binding buffer [20 mM HEPES (pH 7.9), 50 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin] overnight at 4 C. After five washes in RIPA buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin), the bound proteins were separated by SDS-PAGE; the

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gel was then dried for 1 h at 70 C, exposed for autoradiography, and analyzed with a phosphoimager (Molecular Dynamics).

Statistical analysis

Statistical analyses were performed using the paired *t* test (28).

Results

c-Jun expression restores TRα1 transcriptional activity in proliferating myoblasts

We have previously shown that, in the absence of RXR, AP-1 activity does not inhibit TRα1 transcriptional activity. Furthermore, we reported that, in these circumstances, c-Jun expression is able to induce T_3-dependent transcriptional activity in the TRα1 through a DR4-TRE (11). To confirm and extend these data to other TREs, we performed transient transfection experiments using CAT reporter genes under the control of DR4-, palindromic (pal)-, or Ipal-TREs. Protein levels obtained after overexpression are shown in Fig. 1A. Whereas TRα by itself displayed a T_3-dependent transcriptional activity only through a pal-TRE (Fig. 1, C–E), coexpression of c-Jun restored positive TRα activity when reporter genes under the control of a DR4- or an Ipal6-TRE were used (respectively, 5- and 4-fold stimulation of CAT activity; $P < 0.001$ and $P < 0.005$; Fig. 1, D and E). However, it did not affect specific TRα activity through a pal-TRE (Fig. 1C). In parallel experiments, we found in myoblasts that RXRγ coexpression induced a positive TRα transcriptional activity only through a DR4-TRE (data not shown).

Influence of c-Jun and MyoD on TRα1 transcriptional activity

Taking into account the induction of TRα1 transcriptional activity through a DR4- or Ipal-TRE observed in the previous experiment, we tested the cell specificity of this functional interaction. To this end, transient transfection experiments were performed in HeLa cells and in QM7 myoblasts. Protein levels obtained after overexpression are shown in Fig. 2A.

As expected, TRα1 displayed significant transcriptional activity through an Ipal-TRE in HeLa cells, which naturally express RXR isoforms (3-fold increase; $P < 0.01$; Fig. 2B). However, c-Jun expression did not significantly change the extent of this transcriptional stimulation (3.1-fold increase compared with the control level). We tested the possibility that this c-Jun inefficiency resulted from the absence of a muscle-specific factor. Interestingly, we observed that avian MyoD expression on its own stimulated ligand-dependent transcriptional activity of TRα1 (2-fold increase; $P < 0.01$; Fig. 2B). In addition, the highest rate of activity was recorded when c-Jun and MyoD were simultaneously coexpressed with TRα (Fig. 2B; 2.5-fold increase compared with TRα alone; $P < 0.005$).

Similar experiments were performed on QM7 myoblasts using an Ipal6-TRE. c-Jun or MyoD coexpression induced significant ligand-dependent TRα activity through this TRE, but again the highest activity was recorded in the presence of the three proteins (Fig. 2C). Moreover, this TRα1 transcriptional activity did not result from the induction of RXRα or -γ expression by c-Jun and/or MyoD expression (Fig. 2D).

To study these functional interactions further without any interference by endogenous nuclear receptors able to heterodimerize with TRα, we performed similar experiments using the Gal4 system (gal4-tk-CAT reporter and a chimeric T_3 receptor including the DNA-binding domain of Gal4 fused to the hinge region and the ligand-binding domain of TRα1). In these conditions, homodimers of the chimeric T_3 receptor stimulate transcription of the reporter gene, as shown in Fig. 3. Even using this experimental model, MyoD and c-Jun moderately but significantly stimulated Gal4-TRα1

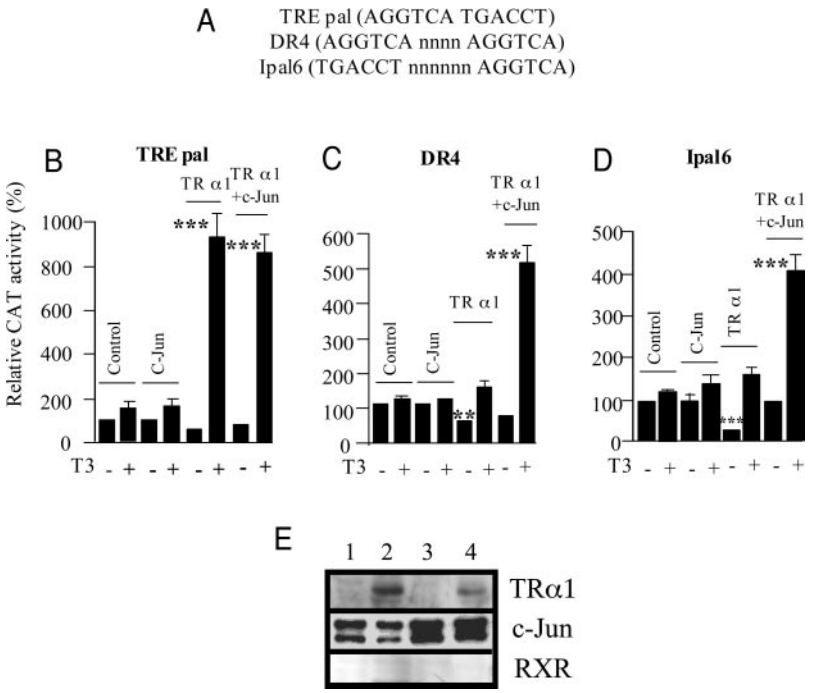

FIG. 1. Influence of c-Jun on TRα1 transcriptional activity in myoblasts. A, Sequences of the three TREs used in all experiments; B–E, QM7 myoblasts were transfected with 1 μg/dish of the reporter TRE pal-Glo-CAT (B), DR4-tk-CAT (C), or Ipal6-tk-CAT (D) and 2 μg of TRα1 and/or c-Jun expression vectors in the absence or presence of T_3 (10 nM). In all cases, the amount of transfected DNA was equalized by addition of the same empty vectors that were used for the cotransfected factors. All results are expressed as a percentage of CAT activity measured in basal conditions after β-galactosidase normalization. Data are presented as the mean ± SEM of five separate experiments. **, $P < 0.01$; ***, $P < 0.005$ when compared with control cells without T_3 stimulation. E, Western blot experiments indicating the levels of TRα1 and c-Jun reached after transient overexpression of each factor: 1, empty vectors; 2, TRα1; 3, c-Jun; and 4, TRα1 plus c-Jun. These transfections do not induce RXR expression.

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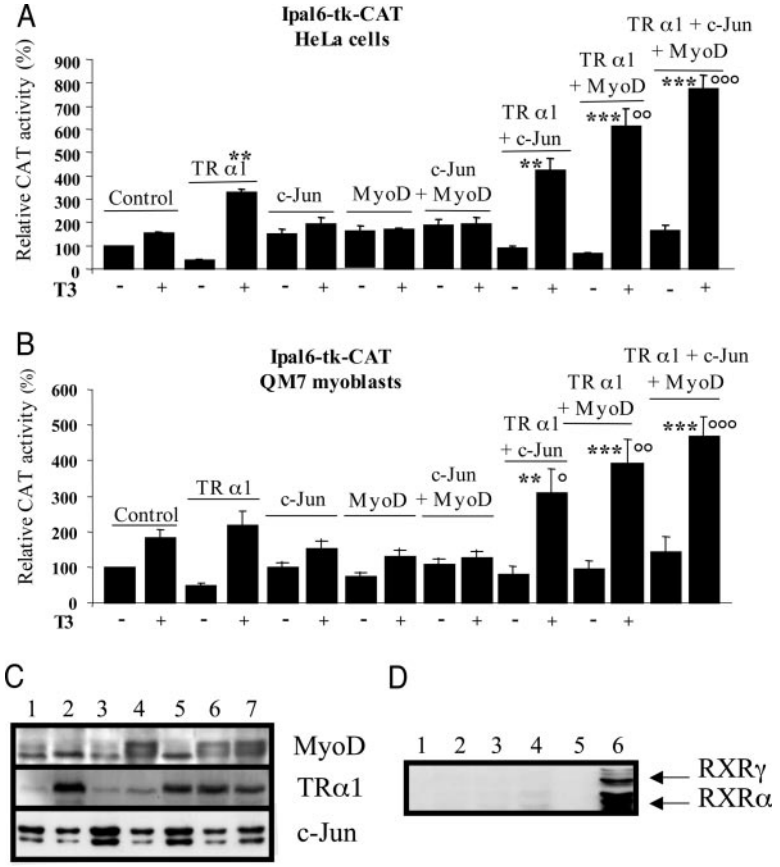

FIG. 2. c-Jun and MyoD potentiate TRα1 transcriptional activity in HeLa cells and in QM7 myoblasts but does not induce RXR expression. HeLa cells (A) or QM7 myoblasts (B) were transfected with 1 μg/dish of Ipal6-tk-CAT reporter gene and 2 μg TRα1, c-Jun, and/or MyoD expression vectors in the absence or presence of T_3 (10 nM). In all cases, the amount of transfected DNA was equalized by addition of the same empty vectors that were used for the cotransfected factors. All results are expressed as a percentage of CAT activity measured in basal conditions after β-galactosidase normalization. Data are presented as the mean ± SEM of four separate experiments. **, $P < 0.01$; ***, $P < 0.005$ when compared with control cells without T_3 stimulation. °, $P < 0.05$; °°, $P < 0.01$; °°°, $P < 0.005$ when compared with TRα1 overexpressing cells in the presence of T_3. ‡, $P < 0.05$ when compared with TRα1- plus c-Jun-overexpressing cells in the presence of T_3. Data obtained in TRα1- plus MyoD-overexpressing cells were not significantly different from that obtained in TRα1- plus c-Jun- or TRα1- plus c-Jun- plus MyoD-overexpressing cells ($P > 0.05$). C, Western blot experiments indicating the levels of TRα1, MyoD, and c-Jun reached after transient overexpression of each factor: 1, empty vectors; 2, TRα1; 3, c-Jun; 4, MyoD; 5, TRα1 plus c-Jun; 6, TRα1 plus MyoD; and 7, TRα1 plus c-Jun plus MyoD. D, Westerns blot experiments indicating that c-Jun and/or MyoD overexpression does not induce RXR expression: 1, empty vectors; 2, c-Jun; 3, MyoD; 4, c-Jun plus MyoD; 5, empty lane; and 6, *in vitro* synthesized RXRα and RXRγ proteins (reticulocyte lysate, vol/vol).

ligand-dependent activity ($P < 0.05$ and $P < 0.01$, respectively). In addition, coexpression of Gal4-TRα1, c-Jun, and MyoD induced a sharp rise in the transcriptional activity of the chimeric receptor (2.5-fold increase; $P < 0.001$; Fig. 3).

Furthermore, we have overexpressed two c-Jun mutants unable to functionally interact with MyoD instead of c-Jun. In c-Jun eb1, the natural dimerization domain has been replaced by a sequence allowing only homodimerization of the mutant (22); c-JunΔ176 is deleted from the transactivation domain of the protein. These two domains have been shown to be of crucial importance for interaction with MyoD (29). In addition, c-JunΔ176 could also act as a dominant negative

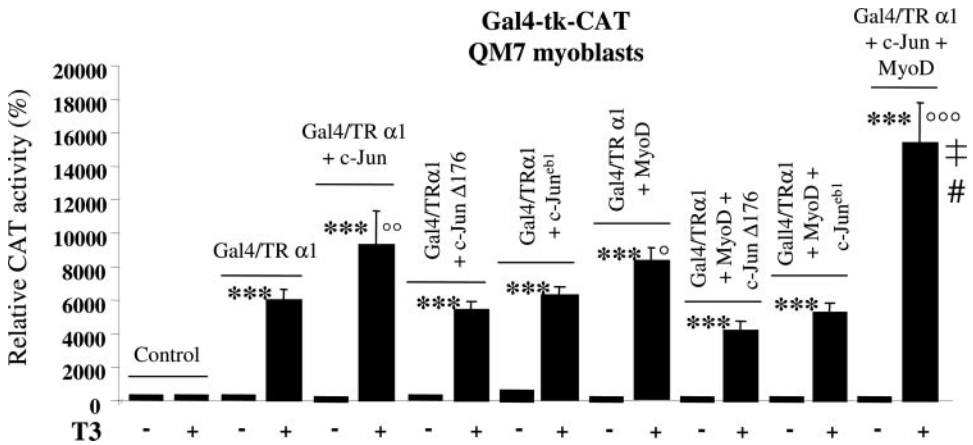

FIG. 3. c-Jun and MyoD potentiation of Gal4/TRα1 chimera receptor transcriptional activity in myoblasts needs the transactivation and dimerization domains of c-Jun. QM7 myoblasts were transfected with 1 μg/dish of Gal4-tk-CAT reporter gene and 2 μg Gal4/TRα1, MyoD, and/or c-Jun, c-Jun Δ176, c-Jun eb1 expression vectors in the absence or presence of T_3 (10 nM). In all cases, the amount of transfected DNA was equalized by addition of the same empty vectors that were used for the cotransfected factors. Results are expressed as percentage of CAT activity in basal conditions, after β-galactosidase normalization. Data are presented as the mean ± SEM of three separate experiments. ***, $P < 0.005$ compared with control cells without T_3 stimulation; °, $P < 0.05$; °°°, $P < 0.005$ compared with TRα1-overexpressing cells in the presence of T_3; ‡, $P > 0.01$ compared with TRα1- plus c-Jun-overexpressing cells in the presence of T_3; #, $P < 0.01$ compared with TRα1- plus MyoD-overexpressing cells in the presence of T_3.

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factor relatively to endogenous c-Jun. Interestingly, c-Jun mutants that can form only homodimers (c-Jun eb1), or which are deleted from the transactivation domain (c-JunΔ176), did not affect Gal4-TRα1 activity, unlike WT-c-Jun (Fig. 3). Furthermore, coexpression of these mutants fully abrogated the ability of MyoD to stimulate the transcriptional activity of the chimeric receptor (Fig. 3), thus suggesting that restoration (TRα) or stimulation (Gal4-TRα1) of receptor functionality results from interactions between the three partners. In addition, these data led us to conclude that the natural NH_2 terminus and DNA-binding domains of TRα1 are not involved in these functional interactions.

Physical interaction between MyoD and TRα1

Physical interactions between MyoD and c-Jun have already been identified and involve the same domains of the cellular oncoprotein as those shown to be crucial for the restoration of TRα transcriptional activity (transactivation and dimerization domains of the cellular oncogene) (29). We therefore tested the possibility of the occurrence of an additional physical interaction between MyoD and TRα leading to indirect recruitment of c-Jun by TRα.

In our first approach, we used coimmunoprecipitation experiments with *in vitro*-synthesized proteins. In these assays, MyoD and GFP were ^{35}S radiolabeled. Although devoid of any interaction with MyoD alone (Fig. 4A, lane 2), the antibody raised against the TRα-NH_2 terminus immunoprecipitated the myogenic factor only when TRα1 was present in the reaction mix (Fig. 4A, lane 3). Failure to immunoprecipitate GFP underlined the specificity of the interaction (Fig. 4, lane 1). This result was reassessed in GST pull-down experiments (Fig. 4B). As expected, the ^{35}S-radiolabeled T_3 receptor did not interact with GST alone (Fig. 4B, lane 2) but was retained by GST-MyoD beads (Fig. 4B, lane 4). The ^{35}S-radiolabeled GFP protein, used as a negative control, did not interact with GST or GST-MyoD (Fig. 4B, lanes 1 and 3). Similar experiments performed in the presence of increasing amounts of T_3 demonstrated that the interaction was independent of the ligand presence: T_3 addition (10^{-8} and 10^{-7} M) did not influence MyoD/TR interaction (Fig. 4C). Lastly, to test the occurrence of this interaction in living cells, we performed coimmunoprecipitation experiments in QM7 myoblasts. Because endogenous TRα1 is frequently at the brink of detection in these cells (Fig. 1E), we overexpressed this receptor by transient transfection, and we used antibodies raised against c-Jun to immunoprecipitate this protein. In agreement with our previous data, these experiments demonstrated that TRα1 interacts with c-Jun in MyoD-expressing

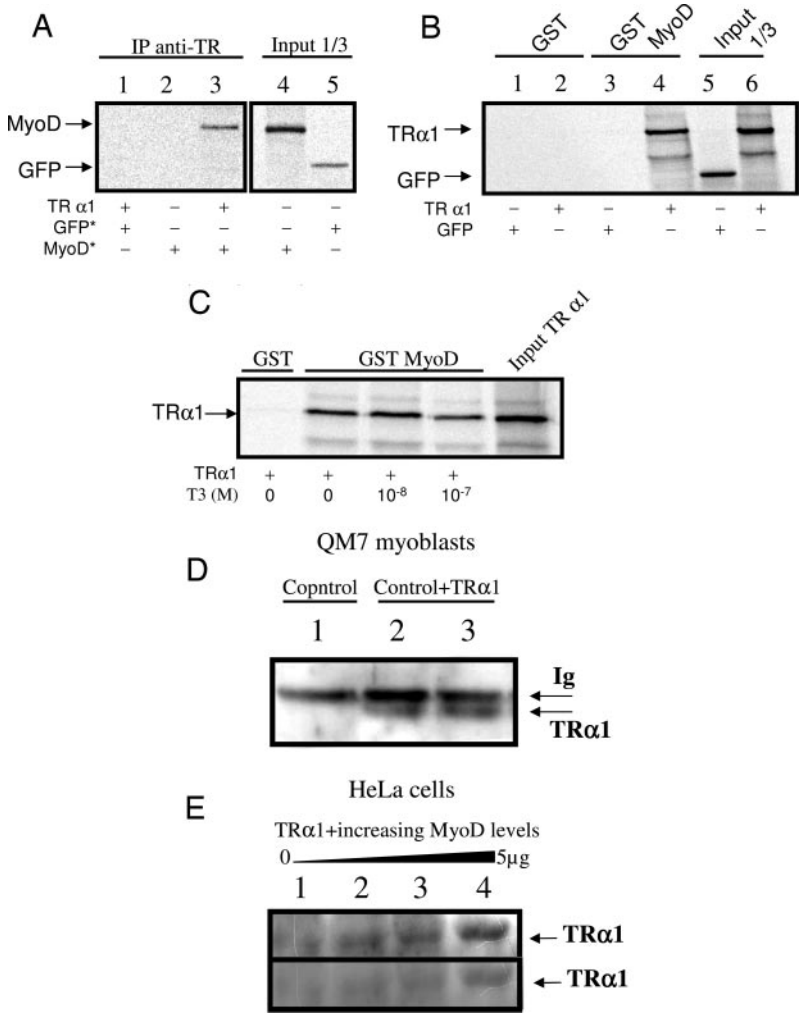

FIG. 4. TR and MyoD proteins physically interact directly regardless of the presence of T_3. A, Coimmunoprecipitation experiment using an antibody raised against TR and radiolabeled *in vitro*-synthesized [^{35}S]GFP and [^{35}S]MyoD proteins and *in vitro*-synthesized cold TR. B, GST pull-down assay using *in vitro*-synthesized [^{35}S]TRα1 or [^{35}S]GFP (negative control) proteins and GST-MyoD or GST beads. The input corresponds to one third of the amount of *in vitro*-translated products loaded in each lane. C, GST pull-down assay using *in vitro*-synthesized [^{35}S]TRα1 proteins and GST-MyoD or GST beads in the presence of different amounts of T_3. The input corresponds to one third of the amount of *in vitro*-translated TR loaded in each lane. All these results are representative of at least three separate experiments for each interaction. D, In QM7 myoblasts, overexpressed TRα1 coimmunoprecipitates with endogenous c-Jun in QM7 myoblasts. Immunoprecipitation was performed with a c-Jun antibody. Western blot was performed with a TRα antibody. 1, Control cells; 2 and 3, overexpression of TRα1. E, In HeLa cells, overexpressed TRα1 coimmunoprecipitates with endogenous c-Jun only in the presence of MyoD. Immunoprecipitation was performed with a c-Jun antibody. Western blot was performed with a TRα antibody. 1, TRα1 overexpression (5 μg expression vector); 2, TRα1 (5 μg) and MyoD overexpression (1 μg expression vector); 3, TRα1 (5 μg) and MyoD overexpression (2 μg expression vector); 4, TRα1 (5 μg) and MyoD overexpression (5 μg expression vector). In contrast to D, the second antibody (coupled to peroxidase) does not recognize Ig (see *Materials and Methods*). Two exposure times of the same blot are shown.

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cells (Fig. 4D). Moreover, using the same approach in HeLa cells, which do not express MyoD, we found that an antibody raised against c-Jun immunoprecipitates TRα1 only after MyoD expression (Fig. 4E). This last result confirms that the TRα1/c-Jun physical interaction needs MyoD recruitment by the T_3 receptor.

Characterization of TRα domains involved in this interaction

With the aim of characterizing the TRα domains involved in the interaction with MyoD, we used several deletion mutants of the receptor (Fig. 5A) in GST pull-down experiments. All TRα1 mutants sharing the DNA-binding domain and the hinge region strongly interacted with GST-MyoD (Fig. 5C). Results obtained with the 1–408, 36–408, 36–194, and 152–408 mutants demonstrated that the 36–152 amino acid sequence plays an important role in TRα1 interaction with the myogenic factor; this is supported by the use of the 1–194 mutant (Fig. 5C). In addition, the carboxyl-terminal part of the receptor did not significantly interact with MyoD (152–408 and 194–408 mutants, Fig. 5C).

When compared with the functional study using the chimeric Gal4-TR, demonstrating that the natural DNA-binding domain of TRα1 is not needed for the interaction with MyoD (Fig. 3), these data indicate that the 121–152 hinge region of the receptor is of crucial importance for binding to the myogenic factor.

Characterization of MyoD domains involved in this interaction

Using a similar experimental approach, we attempted to characterize the MyoD domains involved in the interaction with TRα1 in GST pull-down experiments. To this end, we used several mutants of the myogenic factor fused to GST (Fig. 6A). We observed first that only one mutant did not interact with TRα1 (Fig. 6B, 162–318 mutant), thus ruling out involvement by the carboxyl-terminal part of MyoD. Assessment of the results obtained with 1–162 and 52–318 mutants led us to conclude that the 52–162 domain interacts with TRα1 (Fig. 6B). More specifically, using the 102–162 deletion mutant, we found that the basic helix loop helix (bHLH) domain of MyoD is involved in the interaction with TRα1 (Fig. 6B). However, a second interaction sequence was also observed in the transactivation domain (1–52 sequence, Fig. 6B) of the myogenic factor.

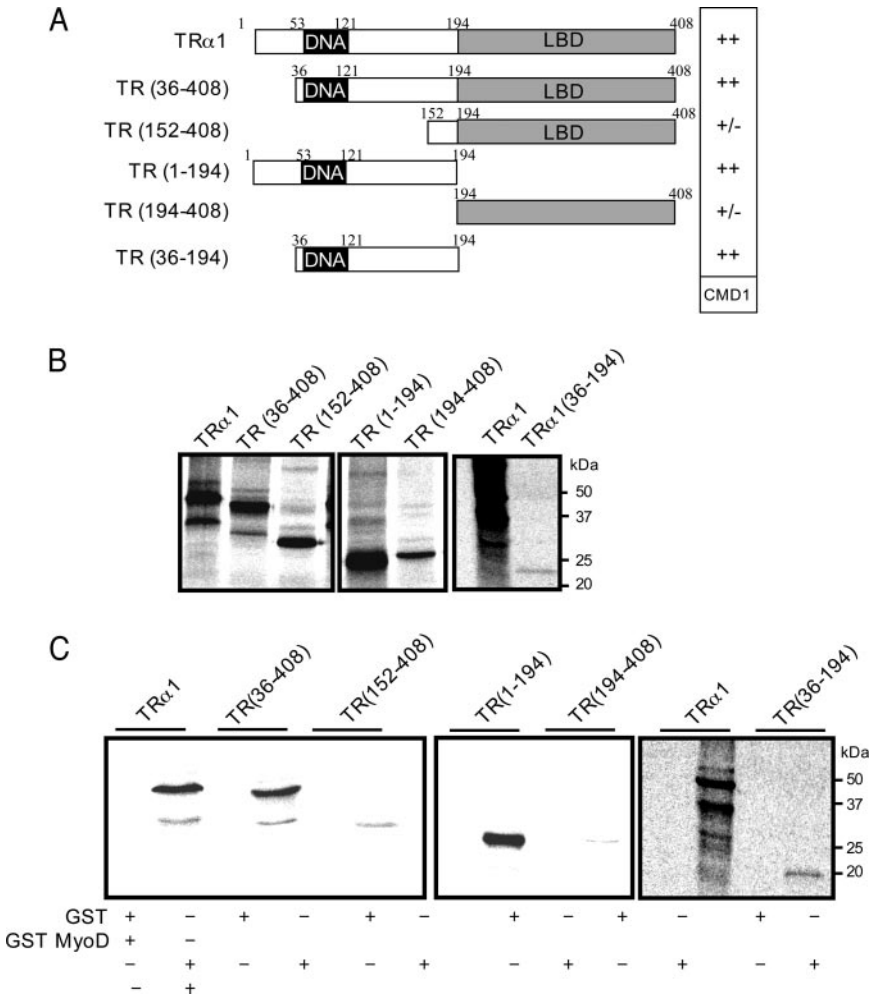

FIG. 5. The 36–152 amino acid sequence of TR induces interaction with MyoD. A, Schematic diagram of TRα1 mutants used in this experiment. Interaction efficiency of TRα1 wild-type or mutants with MyoD is shown on the *right* of the diagram. B, SDS-PAGE showing the synthesis efficiency of each ^{35}S-labeled TRα1 mutant. Note that translation efficiency was very poor for the 36–194 mutant. C, GST pull-down assay using *in vitro*-synthesized ^{35}S-labeled TRα1 mutants with GST-MyoD or GST beads. Because of the lower amounts of the 36–194 mutant, the time of exposure was longer than for other mutants. These results are representative of three separate experiments.

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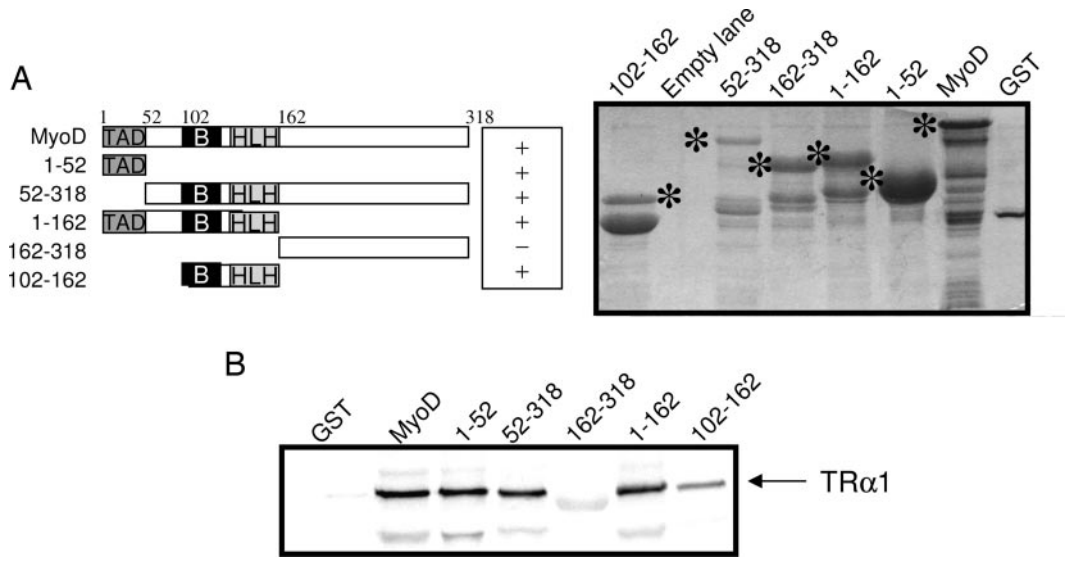

FIG. 6. The transactivation and bHLH domains of MyoD induce interaction with TR. A, *Left*, schematic diagram of all MyoD mutants with a summary of the interaction efficiency between TR and MyoD wild-type or mutants obtained in B (TAD, transactivation domain; BHLH, bHLH domain); *right*, synthesis efficiency of each GST-MyoD mutant was assessed by Coomassie-stained SDS-PAGE. *, GST-MyoD signal. Signal intensity was used to load a similar amount of each mutant in GST pull-down experiments. B, GST pull-down assay using *in vitro*-synthesized ^{35}S-labeled TRα1 with GST-MyoD mutants or GST beads. These results are representative of four separate experiments.

RXRγ abrogates TRα1/c-Jun/MyoD functional interactions

To study the influence of the induction of RXR expression occurring at the onset of myoblast differentiation, we tested its influence on the ability of TRα1 to interact functionally with MyoD and c-Jun.

This study was performed in transient transfection experiments with the Gal4 system previously used to avoid interferences by endogenous nuclear receptors able to heterodimerize with TRα. As expected, RXRγ expression did not influence basal Gal4-tk-CAT reporter expression (Fig. 7A) and did not affect Gal4-TRα1 transcriptional activity independently of the presence of T_3 (Fig. 7B). More interesting is the observation that in the presence of RXRγ, c-Jun (58-fold stimulation of reporter activity), MyoD (54-fold stimulation), and c-Jun plus MyoD (62-fold stimulation) did not stimulate Gal4-TRα1 transcriptional activity (60-fold stimulation; not significant; Fig. 7B).

Discussion

Numerous studies have been performed to define the importance of RXR for TRα transcriptional activity. Sugawara *et al.* (30) reported that TRα/RXR are quantitatively predominant in all transcriptional complexes including TRs. In addition, several *in vitro* experiments suggest that T_3 dissociates TRα homodimers from DR4-TREs but does not affect this binding to a pal-TRE or the binding of TRα/RXR complexes to DR4-TREs (31–33). These observations gave rise to the idea that TRα/RXR is an important T_3-dependent transcription complex, at least through a DR4-TRE (34, 35). More generally, TR/RXR are considered as major complexes for the control of gene expression by the thyroid hormone. However, RXR expression is obviously different from one cell type to another; for instance, TR/RXR complexes are not detectable in ES cells because of the very low expression levels of RXR (36). Moreover, two separate studies have reported that RXR isoforms are not expressed in mice or avian proliferating myoblasts (11, 12). In the light of the observation that T_3 stimulates myogenic differentiation by acting on proliferating myoblasts and inducing their withdrawal from the cell cycle (8, 37), these data clearly suggested that T_3 transcriptional activity in myoblasts is mediated through TR complexes that do not include RXR.

TR/c-Jun/MyoD functional interactions in myoblasts

c-Jun is generally considered as a potent repressor of TR transcriptional activity through its involvement in the c-Jun/c-Fos AP-1 complex (38, 39). However, we have previously reported that, in the absence of RXR, AP-1 activity (c-Jun/c-Fos) does not repress TRα1 transcriptional activity in myoblasts. Instead of this, we found that, in these circumstances, c-Jun expression induces positive TRα1 activity through a DR4-TRE (11), a result extended in the present study to the Ipal-TRE. In addition, as observed for RXR (data not shown), this influence is not detected using a reporter gene controlled by a pal-TRE. Overall, this set of data suggests that, in proliferating myoblasts, transcriptional activity of TRα1 is mediated by homodimers or TRα/retinoic acid receptor complexes (pal-TRE) or by functional interactions with c-Jun (DR4- and Ipal-TREs).

As expected (38, 39), we found in our initial study that, in nonmyogenic cells expressing RXR (HeLa cells), c-Jun expression efficiently represses TRα1 transcriptional activity through pal- and DR4-TREs (11). In the present study, we report that this repression is not efficient through an Ipal-TRE, a regulatory sequence not previously used to study TRα/AP-1 antagonism. Interestingly, it appears that using this particular TRE, which is insensitive to the functional TRα/AP-1 antagonism, c-Jun does not stimulate TRα1 transcriptional activity in HeLa cells. This failure could result from the absence of a muscle-specific partner. This possibility

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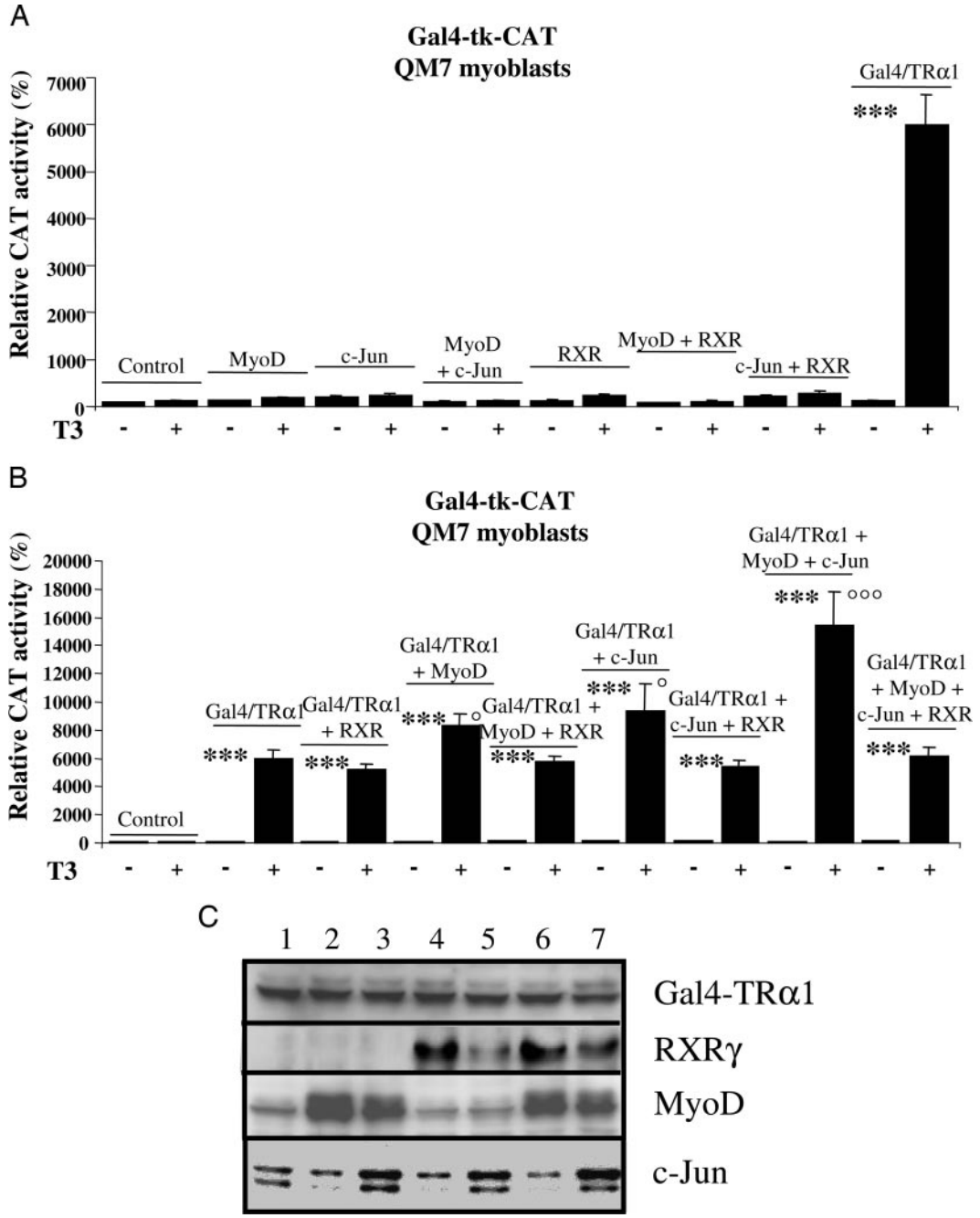

FIG. 7. RXRγ expression abrogates the functionality of Gal4/TRα1/c-Jun/MyoD interaction in myoblasts. A, QM7 myoblasts were transfected with 1 μg/dish of Gal4-tk-CAT reporter gene and 2 μg RXRγ and/or MyoD or c-Jun expression vectors. In all cases, the amount of transfected DNA was equalized by addition of the same empty vectors that were used for the cotransfected factors. Results are expressed as a percentage of CAT activity measured in basal conditions after β-galactosidase normalization. Data are presented as the mean ± SEM of three separate experiments. ***, $P < 0.005$ when compared with control cells without T_3 stimulation. B, QM7 myoblasts were transfected with 1 μg/dish of Gal4-tk-CAT reporter gene and 2 μg of Gal4/TRα1, MyoD, c-Jun, and/or RXRγ expression vectors. Results are expressed as a percentage of CAT activity measured in basal conditions after β-galactosidase normalization. Data are presented as the mean ± SEM of three separate experiments. ***, $P < 0.005$ when compared with control cells without T_3 stimulation; °, $P < 0.05$; °°°, $P < 0.005$ when compared with TRα1-overexpressing cells in the presence of T_3. C, Western blot experiments indicating the levels of RXRγ, Gal4-TRα1, MyoD, and c-Jun reached after transient overexpression of each factor: 1, Gal4-TRα plus c-Jun; 2, Gal4-TRα plus MyoD; 3, Gal4-TRα plus c-Jun plus MyoD; 4, Gal4-TRα plus RXRγ; 5, Gal4-TRα plus c-Jun plus RXRγ; 6, Gal4-TRα plus MyoD plus RXRγ; and 7, Gal4-TRα plus c-Jun plus MyoD plus RXRγ.

is well supported by the observation that MyoD expression stimulates TRα transcriptional activity in these cells and that the highest rate of activity is recorded when TRα, c-Jun, and MyoD are expressed simultaneously. Furthermore, using the Gal4 system working only with Gal4-TR homodimers in myoblasts, we observed that c-Jun or MyoD stimulates the activity of the chimeric receptor and that the greatest stimulation occurred again in the presence of c-Jun and MyoD.

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However, we also observed that this influence of c-Jun and MyoD was not related to the induction of RXR expression. In addition, after having established that c-Jun mutants lacking the transactivation or the natural dimerization domains are unable to stimulate TRα activity, we found that they fully abrogate the influence of MyoD, thus suggesting that functionality of the interaction requires the presence of both c-Jun and MyoD.

TR/MyoD/c-Jun physical interactions

Several arguments stress the occurrence of a common physical interaction between TRα, MyoD, and c-Jun. The myogenic factor has already been shown to interact physically with RXR, a member of the nuclear receptor superfamily belonging to the same subclass as TR. In the present study, coimmunoprecipitation and GST pull-down experiments clearly support the existence of direct physical interactions between TRα and MyoD and have identified the interacting sequences of each partner. Despite the fact that, with one exception (40), all teams working on TR/AP-1 interactions have failed to detect physical interactions between TR and c-Jun, MyoD and c-Jun do physically interact (29). Furthermore, in coimmunoprecipitation experiments, we report the occurrence of a TRα/c-Jun physical interaction in myoblasts. However, in HeLa cells that do not express MyoD, this interaction does not occur but is induced by expression of different amounts of MyoD. This set of results led us to propose that interaction of TRα with MyoD enables the recruitment of c-Jun in a complex inducing TRα activity through DR4- and Ipal-TREs.

Interestingly, like TRα, c-Jun represses MyoD transcriptional activity (29). Furthermore, the transactivation and dimerization domains of c-Jun play a crucial role in MyoD/c-Jun interaction. In the present study, we have established that the same c-Jun domains are essential for the stimulation of TRα activity by the cellular oncoprotein and by MyoD. All these data could be summarized as follows: 1) TRα and c-Jun similarly repress MyoD activity; 2) c-Jun and MyoD similarly stimulate TRα activity; 3) c-Jun stimulates TRα activity only in the presence of MyoD, and MyoD stimulatory activity is abrogated by mutated or deleted forms of c-Jun unable to interact with the myogenic factor; 4) identical c-Jun domains are involved in the repression of MyoD transcriptional activity and in the stimulation of TRα activity; and 5) TRα physically interacts with MyoD and MyoD physically interacts with c-Jun. This set of results led us to propose that interaction of TRα with MyoD enables the recruitment of c-Jun in a complex inducing TRα activity through DR4- and Ipal-TREs and repressing MyoD activity through an E box (Fig. 8). This possibility is well supported by the observation that, in HeLa cells, TRα is immunoprecipitated by a c-Jun antibody only when MyoD is expressed.

RXR expression abrogates the functionality of TRα/MyoD/c-Jun interaction

Because RXR expression occurs at the onset of myoblast differentiation (12), we studied the influence of RXR on the functionality of TRα/MyoD/c-Jun interaction. Using the Gal4 system in which transcription can be only stimulated by

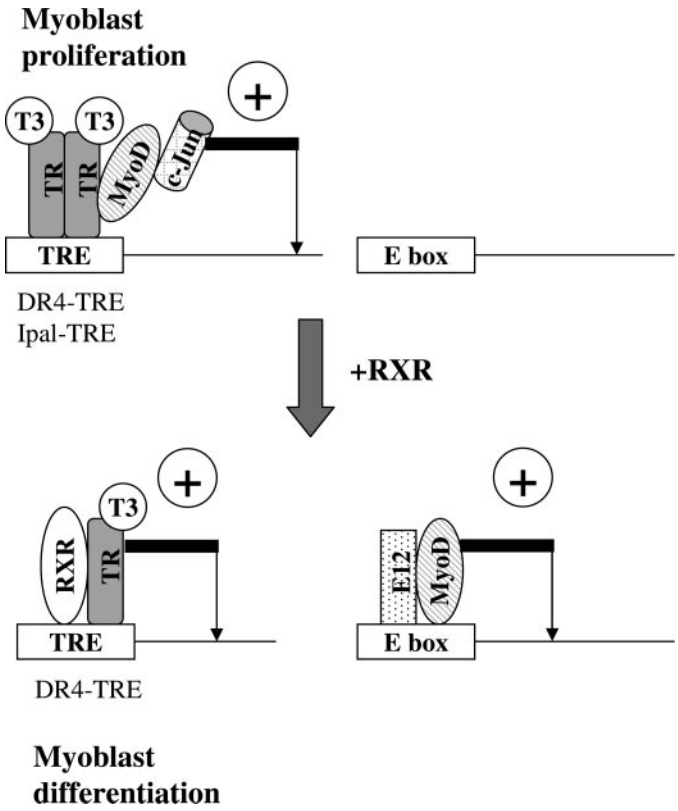

FIG. 8. Hypothesis concerning the influence of a TR/MyoD/c-Jun complex on TR and MyoD transcriptional activity. RXR isoforms are not expressed in proliferating myoblasts (12). During this period, TR physically interacts with MyoD, enabling the recruitment of c-Jun in a transcriptional complex active on TRE sequences (Ipal- or DR4-TREs) but inactive on E boxes (sequences recognized by the myogenic factor MyoD). This interaction induces TR transcriptional activity but represses the activity of MyoD and therefore could delay the onset of myoblast differentiation. During terminal differentiation, TR/RXR complexes replace the TR/MyoD/c-Jun complex with different consequences for the regulation of T_3 target gene expression because of the nature of the TRE in the promoter of the gene. Disruption of the TR/MyoD/c-Jun complex allows MyoD to interact with its heterodimerization partner E12, to bind to its cognate response element (E box), and to stimulate the expression of genes involved in myogenic differentiation.

homodimeric binding of the chimeric Gal4-T_3 receptor, we established that RXR abrogates the ability of c-Jun, MyoD, or c-Jun plus MyoD to enhance TRα1 transcriptional activity. In this model, RXR cannot bind to DNA in heterodimeric complexes with Gal4-TR, thus suggesting that this influence probably results from recruitment of MyoD by RXR in a previously identified RXR/MyoD complex (41). However, on natural TREs, TR/MyoD/c-Jun complexes could be replaced by the major TR/RXR complex, as suggested by restoration of this heterodimeric complex in electrophoretic mobility shift assay experiments after RXR overexpression in myoblasts (11). Therefore, this possibility raises the question of the control of T_3 target genes driven by an Ipal-TRE, which is not sensitive to TR/RXR complexes (data not shown). Interestingly, if physiologically relevant, this event could lead to differential stimulation of gene expression by T_3 in proliferating (functionality of all TREs) and differentiating (Ipal-TRE not activated) myoblasts, inducing a duality in the

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regulation of myoblast differentiation by TRs. Such duality is already apparent in the observation that TRα1 inhibits MyoD activity in proliferating myoblasts (13), thus repressing terminal differentiation, but represses AP-1 activity (a major inhibitor of myogenic differentiation) at the onset of terminal differentiation. Interestingly, we have reported a similar duality of c-Jun influence during myoblast proliferation (repression of myoblast withdrawal from the cell cycle) and differentiation (stimulation of myogenin expression and myoblast differentiation) (10). These data are in agreement with a recent work in progress of our team using microarray analysis, clearly suggesting that T_3 target genes significantly differ in proliferating myoblasts when compared with differentiating cells (in particular, genes involved in withdrawal from the cell cycle *vs.* genes involved in differentiation).

In conclusion, although their exact physiological relevance remains to be more clearly established, the present data indicate that RXR is probably an important, but not essential, partner involved in the transcriptional activity of the T_3 receptor. Instead of this, they highlight the possible existence of cell-specific complexes able to support TR transcriptional activity, thus introducing an additional level of regulation resulting in fine tuning of T_3 influence during cell proliferation and differentiation, at least in the myogenic lineage (Fig. 8). In particular, we propose that in proliferating myoblasts, TR transcriptional activity is induced through a TR/MyoD/c-Jun complex, thus reducing the amounts of the myogenic factors able to bind to their specific response elements and delaying the onset of myoblast differentiation. Thereafter, RXR expression occurring at the induction of terminal differentiation could disrupt this complex, thus increasing the amounts of MyoD able to bind to its response element and to stimulate terminal differentiation. Such a mechanism could partly explain the prominent influence of T_3 on muscle development by increasing the duration of the proliferative period of myoblasts and stimulating terminal differentiation.

Acknowledgments

We are grateful to Dr. Samarut, Dr. Flamant, Dr. Rouault (Lyon, France), Professor P. Chambon (LGME-U184, Strasbourg, France), Dr. P. K. Vogt (The Scripps Institute, La Jolla, CA), Dr. P. B. Antin and Dr. C. P. Ordhal (San Francisco, CA), Dr. D. Montarras (Institut Pasteur, Paris, France), Dr. C. Deschene (Institut National de la Santé et de la Recherche Médicale, Nice, France), and Dr. M. Castellazzi (ENS Lyon) for the gift of pRSV cErbAα1 and pRSV CMD1, pSV Gal4α1 and Gal4-tk-CAT, BTG1, DR4-tk-CAT reporter construct, −73colCAT reporter gene and pRS chicken c-Jun expression plasmid, the myoblast QM7 line, pEMSV hMyf5 and pRSV Myogenin, c-Jun eb1, and c-JunΔ176, respectively.

Received January 27, 2006. Accepted March 16, 2006.

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This work was supported by the Institut National de Recherche Agronomique and grants from Association Française pour la recherche contre les Myopathies and Association pour la Recherche contre le Cancer.

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Disclosure of potential conflicts of interest: M.B., L.D., P.S., S.G., L.P., F.C., C.W-C., and G.C. have nothing to declare.

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