Induction of c-Erb A-AP-1 Interactions and c-Erb A Transcriptional Activity in Myoblasts by RXR

CONSEQUENCES FOR MUSCLE DIFFERENTIATION*

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We have previously shown that c-Erb A and v-Erb A display a cell-specific activity in avian myoblasts. In this work, we have compared the molecular basis of thyroid hormone action in HeLa cells and in QM7 myoblasts. The transcriptional activity of c-Erb Aα and β through a palindromic thyroid hormone response element (TRE) was similar in both cell types. However, c-Erb A did not activate gene transcription through a direct repeat sequence (DR) 4 TRE in myoblasts in contrast to results obtained in HeLa cells. Moreover, whereas retinoic acid receptor-AP-1 interactions were functional in both cell types, thyroid hormone receptor (T3R)-AP-1 interactions were only functional in HeLa cells. Using electrophoretic mobility shift assays, functional tests, and Northern blot experiments, we observed that RXR isoforms are not expressed in proliferating myoblasts. Expression of RXRγ in these cells did not influence T3R transcriptional activity through a palindromic TRE but induced such an activity through a DR4 TRE. Moreover, it restored c-Erb A-AP-1 functionality in QM7 myoblasts and enhanced the myogenic influence of T3. We also observed that c-Jun overexpression in proliferating myoblasts. Expression of RXRγ in these cells did not influence T3R transcriptional activity through a palindromic TRE but induced such an activity through a DR4 TRE. Therefore, alternative mechanisms are involved in the induction of T3R transcriptional activity according to the cell status (proliferation: c-jun un; differentiation: RXR). In addition we provide the first evidence that RXR is required to allow inhibition of AP-1 activity by ligand-activated T3R. Lastly, we demonstrate the importance of RXR in the regulation of myoblast differentiation by T3.

Thyroid hormones exert critical effects on development, as well as a variety of metabolic pathways. They bind to thyroid hormone receptors (T3Rs),1 closely related to steroid hormone receptors, and control the expression of specific target genes in a ligand-dependent manner. There are two classes of T3Rs, TRα and TRβ, which are encoded on two separate genes. They bind to specific regulatory sequences (thyroid hormone response element (TRE)) usually found in the promoter area of T3 responsive genes. TREs are composed of hexamer half-sites (AGGTCA) with degeneracy in sequence and orientation. In the absence of T3, c-Erb A proteins generally repress basal transcription. In the presence of the hormone, they positively or negatively modulate transcription. In vitro, T3Rs bind to DNA as monomer, homodimer, and heterodimer with members of the nuclear receptor superfamily, such as retinoic acid receptors (RARs) (1, 2), RXR (3–5), vitamin D3 receptor (6), PPAR (7), or COUP-TF (8). It is generally assumed that the T3R-RXR heterodimer is a major transcription complex, at least through a DR4 TRE, whereas the T3R homodimer is probably not a significant transcription factor (9, 10).

In addition, as previously shown for ligand-activated glucocorticoid receptors (11) and retinoic acid receptors (12), liganded T3Rs repress AP-1 activity (13, 14). Conversely, stimulation of AP-1 activity by TPA or c-jun overexpression inhibits T3Rs transcriptional activity (14). It was proposed that a direct physical interaction between the AP-1 complex and T3Rs leads to a subsequent loss of activity through TRE or AP-1 responsive elements (14, 15). The involvement of a third partner in stabilization of the T3R-AP-1 complex was also postulated (13, 16). Therefore, although liganded c-Erb A proteins directly regulate T3 target gene expression, they repress transcription of AP-1-regulated genes. This dual pathway might regulate the expression of two different sets of genes respectively involved in cell proliferation and differentiation (13).

We have previously shown that T3 stimulation of quail myoblast differentiation was enhanced by T3Rα overexpression (17–19). In this work, we compared two T3-regulated mechanisms in QM7 myoblasts and in HeLa cells. We report that QM7 cells do not significantly express RXR, leading to a cell-specific activity of T3Rα. In contrast to its action in HeLa cells, c-Erb Aα and β do not inhibit AP-1 activity in QM7 cells, but RXR transduction influenced functionality of T3R-AP-1 interactions. Lastly, whereas RXR does not affect T3R transcriptional activity through a TREpal, it induces such an activity through a DR4 TRE. In agreement with these data, RXR expression potentiates the T3 stimulation of myoblast differentiation. These findings suggest a crucial role of RXR for the regulation of cell differentiation through interactions with T3R and AP-1 activity.

EXPERIMENTAL PROCEDURES

Cell Cultures—Myoblasts of the QM7 cell line (20) were grown in Earle’s 199 Medium supplemented with 0.2% tryptose phosphate broth, penicillin (100 IU/ml), and 10% T3-depleted fetal calf serum. HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented...
with 10% fetal calf serum. Serum was T3-depleted according to Samuels et al. (21). After hormonal depletion, T3 levels measured by radioimmunoassay were always lower than the detection limit of the assay (80 pmoi/liter).

Plasmids and Reporter Genes—The (AP-1),tk-CAT plasmid carrying five AP-1 sites upstream of the thymidine kinase promoter linked to the CAT gene was kindly provided by P. Chambon (LGME-U184, Strasbourg, France). The collagenase promoter-CAT construct −73 col CAT previously described (22) was kindly provided by K. Vogt (The Scripps Research Institute, La Jolla, CA). The TREpal-glo-CAT plasmid carrying the synthetic palindromic TRE sequence (TREpal) upstream of the rabbit β-globin core promoter linked to the CAT gene (23) was constructed by A. Rascle (ENSLyon, France). The DR4-tk-CAT plasmid was obtained from P. Chambon (LGME-U184, Strasbourg, France), and the DR1-tk-CAT was from P. Balague (INSERM, U58, Montpellier, France). The expression vectors for chicken c-Erb A1 and RXR γ (pRS c-erb A1 and pRS RXR γ) had been described elsewhere (1, 24). The pRS chicken c-jun expression plasmid was obtained from P. K. Vogt (The Scripps Research Institute, La Jolla, CA).

Transient Transfections and CAT Assays—24 h before transfection, 0.3 × 10⁶ HeLa cells or QM7 cells per 100-mm dish were plated. Transfection of plasmid DNA into the cells was performed using a calcium phosphate co-precipitation procedure (25) as described previously (19). All transfections also included 1 μg of pCMV β-galactosidase as an internal control to normalize for transfection efficiencies. Cells were exposed to precipitates for 16–20 h. Then, they were refed for an additional 24 h, respectively, with either 0.5 or 10% depleted serum-containing medium and incubated with 50 ng/ml TPA and/or 10⁻⁸ M T3 when indicated. β-Galactosidase activity was measured as described previously (26). CAT enzymatic activity was measured at room temperature after following the kinetics of chloramphenicol acetylation with [3H]acetyl-CoA as a substrate (27). For each assay, the initial rate of the enzymatic reaction (v = −d[P]/dt) was determined. The results are expressed as the percentage of control values after β-galactosidase normalization.

Electrophoretic Mobility Shift Assay (EMSA)—Gel mobility shift assays were performed according to Graupner et al. (28), using whole cell extracts. HeLa cells or QM7 myoblasts were seeded at 0.3 × 10⁶ cell/100-mm dish 24 h before transfection and transfected with 10 μg of pRS poly(A), pRS c-erb A1, and/or pRS RXR γ by calcium phosphate co-precipitation, respectively. After 48 h, cells were scraped and pooled from two dishes, pelleted by centrifugation, and resuspended in 100 μl of buffer containing 10 mM Tris-HCl (pH 7.8), 400 mM KCl, 20% glycerol, and 2 mM dithiothreitol. Cell lysates were obtained after four freeze-thaw cycles and centrifuged at 10,000 × g of supernatant using bovine serum albumin as a standard. For EMSA, various combinations of 10 μg of whole cell extracts were used. For competition assays, 200 ng of cold oligonucleotide were preincubated with the reaction mixture before adding the probe. TREpal (GATCCTCGCATGCTCA) and DR4 (TCAGGTTGAGTTCA and DR4 (TCAGGTTGACCATGTTGTA) were used as probes. Cold DR5 (GGTAGGCGGTTAGGGTCA and DR5 (GGTACGTTGCTCA and TTACGAGGGTCA) were used for competition assays. Antibodies raised against c-Erb A1 (α-17) and RXR α, β, and γ isomers (4RX-1D12) were kindly provided by L. J. De Groot (University of Chicago, IL) and P. Chambon (LGME-U184, Strasbourg, France), respectively.

Statistical Analysis—Statistical analysis were performed using the paired t test (29).

RESULTS

T3Rα Transcriptional Activity in HeLa Cells and in QM7 Myoblasts—We assessed the transcriptional activity of T3Rα by transient transfections experiments using a TREpal-glo-CAT or a DR4-tk-CAT reporter gene together with the pRS c-Erb A1 expression vector.

In both cell types, co-transfection of the pRS c-Erb A1 expression vector induced a T3-dependent transcriptional activation of the TREpal-glo-CAT construct (up to 10-fold, p < 0.001; Fig. 1, A and B). Therefore T3Rα displayed a similar transcriptional activity in QM7 myoblasts and in HeLa cells through a TREpal.

Using the DR4-tk-CAT reporter gene, we observed striking differences. A significant induction of CAT activity by liganded c-Erb A1 was recorded in HeLa cells (3-fold induction, p < 0.005; Fig. 1C). In QM7 myoblasts, T3Rα significantly decreased basal CAT activity in the absence of T3 (p < 0.025). The deletion of the hormone abrogated this inhibition but did not induce any stimulation of CAT activity (Fig. 1D). Therefore, as expected, the liganded T3R was able to stimulate the expression of genes under the control of a DR4 TRE in HeLa cells. However, in QM7 myoblasts, T3Rα acts only as a transcriptional repressor of gene transcription through a DR4 TRE, and T3 abrogates this activity.

In Contrast to RAR α, T3Rα Inhibits AP-1 Activity in HeLa Cells but Not in QM7 Myoblasts—In order to compare T3Rα and RAR α interactions with the AP-1 complex in HeLa and QM7 cells, we performed transient transfection experiments in both cell types. The (AP-1)-tk-CAT reporter gene and pRS c-erb A1 or pRS RAR α expression vectors were simultaneously co-transfected in these cells. AP-1 activity was stimulated by TPA.

As expected, T3Rα and RAR α activated by their cognate ligands (10⁻⁸ M T3; 10⁻⁷ M RA) strongly inhibited the TPA-stimulated AP-1 activity in HeLa cells (in both cases: −80%, p < 0.005; Fig. 2A). However, liganded T3Rα did not repress the TPA-induced AP-1 activity in QM7 cells (Fig. 2B) or in secondary quail myoblasts (Fig. 2C). Similar results were obtained when AP-1 activity was stimulated by chicken c-jun overexpression using the −73 col-CAT reporter gene in QM7 cells (Fig. 2D).

However, in QM7 myoblasts the ligand-dependent repression by endogenous or exogenous RAR occurred as observed in HeLa cells (Fig. 2, A and B). Therefore, activated T3Rα and RAR α do not display a similar activity in myoblasts, whereas
no difference could be noted in HeLa cells. These results suggest that interactions of these receptors with the AP-1 complex might not be mediated through strictly identical pathways.

Stimulation of AP-1 Activity Differently Affects T3Rα Transcriptional Activity According to the Cell Type and the TRE—To further investigate T3R-AP-1 relationships in myoblasts, we studied the influence of TPA treatment on the transactivation of the TREpal-glo-CAT reporter by T3Rα. As previously reported in CV1 cells (14), activation of this reporter construct by liganded T3R was abrogated by TPA treatment in HeLa cells (90%, p < 0.001; Fig. 3A), whereas it was not affected in QM7 cells (Fig. 3B). However, using the same reporter gene, we observed that TPA abrogated RARα transcriptional activity in both cell type (data not shown).

More striking is the observation that in QM7 myoblasts, TPA induced a strong transcriptional activity of ligand-activated T3Rα (7-fold induction, p < 0.001) when using a DR4-tk-CAT gene reporter (Fig. 3D). Similar results were obtained using c-jun transfection (Fig. 3D). In contrast, using the same reporter gene, TPA treatment inhibited T3R transcriptional activity in HeLa cells, with an efficiency similar to that recorded using a TREpal-glo-CAT gene reporter (Fig. 3C, p < 0.005).

These data suggest that TPA influence upon T3R transcriptional activity depends on the TRE and on the cell type. In particular, an elevated AP-1 activity induces transcriptional functionality of liganded T3R through a DR4 TRE in QM7 myoblasts.

A Major T3R-RXR Heterodimer Is Detected in HeLa Cells but Not in QM7 Myoblasts—In EMSA experiments, we studied the c-Erb A1 binding pattern to TREs using QM7 or HeLa cells transiently transfected with the T3Rα expression vector.

When T3R overexpressing HeLa cell extracts were incubated with a direct repeat sequence (DR4) probe, three complexes displaying different binding intensities were detected with complex II on the brink of detection (Fig. 4A, lanes 1 and 3). Using a TREpal probe (Fig. 4B, lanes 3 and 5), no significant differences were observed in the binding ability of these three complexes. Using extracts of T3R-overexpressing QM7 myoblasts and a DR4 probe, only two fast mobility complexes (II and III) were observed (Fig. 4A, lanes 2 and 4). However, binding of complex III was strongly reduced when using a TREpal probe (Fig. 4B, lanes 2 and 4).

When a 5-fold protein excess of control HeLa cellular extract was mixed with T3R-expressing QM7 cellular extract, binding of the receptor as three complexes was observed (Fig. 5). Formation of complex I was associated with a decrease of complexes II and III (Fig. 5, lane 2). Preincubation with an antibody raised against c-Erb A1 confirmed that T3R was a component of these three complexes (Fig. 5, lane 6). In agreement with these last observation, an excess of cold DR4 was found to compete binding of complexes I, II, and III to the probe (Fig. 5, lane 5). Interestingly, binding of complex I was also efficiently competed by molar excess of cold DR5 and DR1 probes (Fig. 5, lanes 3 and 4). These data suggest that a T3R
partner able to bind to DR5 and DR1 responsive elements is expressed in HeLa but not in QM7 cells.

When RXRγ and T3Rα were co-expressed in QM7 myoblasts, a third complex was detected (Fig. 6, lane 7). It displayed the same mobility as complex I in QM7 expressing T3R extracts mixed with control extracts of HeLa cells (Fig. 6, lanes 7 and 8). In addition, preincubation of cell extracts with an antibody raised against all RXR isoforms suppressed the slow mobility signal (complex I) both in T3R-RXR-expressing myoblasts and in T3R-expressing HeLa cells (Fig. 6, lanes 4 and 5). In addition, binding of complexes II and III was not affected in these two cell types, thus demonstrating absence of RXR in these fast mobility complexes.

Because only two RXR isoforms (α and γ) are characterized in avian species (24, 30), expression of these receptors was assessed by Northern blot in proliferative QM7 myoblasts. Whereas the two transcripts were easily detected in 4.5- and 5.5-day-old quail embryo in agreement with previous data (24, 30), we failed to detect them in QM7 extracts (Fig. 7), in agreement with our EMSA data.

RXRγ Expression Differently Influences T3R Transcriptional Activity According to the Nature of the TRE in QM7 Myoblasts—The results lead us to study the influence of RXR on T3R transcriptional activity in QM7 myoblasts. In transient transfection experiments performed in HeLa cells, we observed that RXRγ expression did not affect T3Rα transcriptional activity when using a TREpal-glo-CAT reporter (Fig. 8A) but significantly enhanced this activity through a DR4-tk-CAT (Fig. 8C, p < 0.05).

QM7 myoblasts were transfected with pRS c-RXRγ and pSV2-neoR expression plasmids. Control myoblasts were obtained by co-transfecting pRS poly(A) vector with pSV2-neoR plasmid. Stable expression of RXRγ was tested using a DR1 CAT reporter gene (Fig. 8E). Using a TREpal-glo-CAT gene reporter, we observed that RXRγ expression did not significantly influence the liganded T3Rα transcriptional activity when using a TREpal-glo-CAT reporter (Fig. 8A) but restored a transcriptional activity of liganded c-ErbA through a DR4 TRE in QM7 myoblasts (about 4-fold induction, p < 0.005; Fig. 8D). Therefore, this set of data brings evidence that T3R could be fully active through a synthetic TREpal in the absence of RXR, whereas the T3R-RXR het-
erodimer is a major transcription complex on a DR4 TRE.

RXR Expression Restores the Functionality of T3R-AP-1 Interactions—The functionality of T3R-AP-1 interactions was also investigated in RXR γ-expressing myoblasts using the

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RXR isoforms are not expressed in proliferating QM7 myoblasts. RXR α (5 kilobases) and γ (2.5 kilobases) RNAs were detected by Northern blot analysis using homologous probe in chicken embryos but not in QM7 cells. Lane 1, total RNA from stage 25 chicken embryos (4.5 days in ovo). Lane 2, total RNA from stage 27 chicken embryos (5.5 days in ovo). Lane 3, total RNA from QM7 cells 48 h after seeding (T3 depleted culture medium). Lane 4, total RNA from QM7 cells 48 h after seeding (0.6 nM T3 supplemented medium). 15 and 30 μg were loaded in lanes 1 and 2 and in lanes 3 and 4, respectively. (AP-1)γ-tk-CAT reporter plasmid. In absence of T3, stimulation of AP-1 activity by TPA was not affected in control or RXR-expressing myoblasts. A significant inhibition of CAT activity was recorded in RXR γ-expressing myoblasts in comparison with control myoblasts after 10^{-8} μM T3 stimulation (Fig. 9A; p < 0.025; RXR γ versus control myoblasts). Therefore, in contrast to control cells, T3-activated endogenous c-Erb A receptors inhibited AP-1 activity in RXR γ-expressing cells. In addition, T3Rα overexpression induced a strong inhibition of the TPA-stimulated AP-1 activity in RXR γ-expressing myoblasts after 10^{-8} μM T3 addition (Fig. 9A; p < 0.01, RXR γ versus control myoblasts). Similar results were recorded when expression of RXR γ or RXR α was performed in transient transfection experiments (data not shown). Furthermore, in control experiments, PPARα, RAR α, or COUP-Tf I expression failed to restore functionality of T3R-AP-1 interactions (data not shown). These data demonstrated that RXR expression is specifically required for inhibition of AP-1 activity by T3R.

Conversely, we demonstrated that RXR γ expression restored the inhibition of T3R transcriptional activity by AP-1 in QM7 myoblasts, whatever the TRE. Using a TRE_pal probe, TPA stimulation of AP-1 activity strongly inhibited CAT induction by liganded T3R in RXR γ-expressing myoblasts (−85%, p < 0.005; Fig. 9B). Similar data were obtained using a DR4 TRE (−55%, p < 0.025; Fig. 9C), but a significant induction of CAT activity remained (3-fold induction, p < 0.01; Fig. 9C), suggesting that RXR expression partly preserved the transcriptional T3R activity through a DR4 TRE, even when AP-1 activity was elevated.

**PHYSIOLOGICAL CONSEQUENCES**—Because c-Erb A-AP-1 interactions and T3R transcriptional activity are probably involved in the myogenic influence of T3 (17–19), we studied the influence of RXR γ expression on myoblast differentiation. QM7 myoblasts stably expressing pRS poly(A) or pRS cRXR γ were infected with the avian retrovirus CASBA 9 enabling expression of T3Rα as described previously (19). Myoblast differentiation was studied by cyto-immunofluorescence experiments assessing myoblast fusion and connectin (a muscle-specific protein) expression. Whereas RXR γ expression did not affect differentiation by itself (Fig. 10, E versus A), it strongly potentiated the myogenic influence of T3 in control (Fig. 10, F versus B) or in T3R-overexpressing cells (Fig. 10, H versus D). Therefore, by altering the T3R-AP-1 pathway and T3R transcriptional activity, RXR expression modulates the stimulation of cell differentiation induced by T3.

**DISCUSSION**

T3R-RXR Heterodimeric Binding Is Detected on a TRE Sequence in HeLa Cells but Not in Avian Myoblasts—We have
shown that in both cell types, T3Rα binds to a palindromic TRE or a DR4 motif as two fast migrating complexes (II and III). We have detected a third slow migrating complex in HeLa cells (complex I). Moreover, addition of HeLa extracts to T3R overexpressing QM7 extracts induced formation of an additional complex with a mobility similar to that of complex I in myoblasts. Because an excess of cold DR1 or DR5 probes efficiently competed complex I binding to a TREpal, RXR, PPAR, or COUP-TF I and II, which are able to bind to these response elements (31–39), could be possible partners of T3Rα in HeLa cells. In EMSA experiments, using 4RX-1D12 antibody reacting against all RXR isoforms (40), we identified RXR as the partner of T3R in complex I of HeLa cells. Furthermore, RXR expression induced formation of an additional complex in QM7 myoblasts with the same mobility as complex I. These results were in line with some previous data suggesting that RXR isoforms are not expressed in proliferating myoblasts (41). The present study also clearly indicates that RXR isoforms are weakly or not expressed in proliferative quail myoblasts: (i) transcriptional activity of 9-cis-RA from a DR1-tk-CAT reporter gene is not significant (Fig. 7); (ii) we failed to detect any T3R-RXR heterodimers in avian myoblasts (Fig. 6); and (iii) we failed to detect RXR mRNAs in our cell extracts.

**QM7 Myoblasts Provide a Useful Experimental Model to Study the Influence of RXR upon T3R Activity**—Numerous studies have been performed in order to define the exact influence of RXR on T3R activity. Whereas T3 induces dissociation of the T3R homodimer from a direct repeat TRE, the hormone does not affect this binding to a TREpal or the binding of a T3R-RXR complex to a DR4TRE (9, 10, 42). In addition, several studies strongly suggest that T3R-RXR is the major T3 dependent-transcription complex through a direct repeat TRE (43–45). Interestingly, our study clearly suggests that RXR is not required to induce a T3R transcriptional activity through a TREpal; its efficiency is not affected by the absence of RXR, and conversely RXR expression does not influence T3R activity. Therefore, the T3R homodimer could be a fully active transcrib-
tion complex on this particular TRE, in disagreement with previous data (4, 37, 46, 47). Alternatively, such transcriptional activity could be induced by a T3R complex including a partner other than RXR.

However, T3-activated c-Erb A is devoid of transcriptional activity through a DR4 TRE in the absence of RXR: (i) T3R represses the basal expression level in absence of T3; (ii) the addition of the hormone abrogates this inhibition but does not stimulate transcription; (iii) in cells expressing RXR such as HeLa cells, liganded T3R displays a significant transcriptional activity through a DR4 TRE; and (iv) a similar activity is restored in myoblasts after RXR expression. Therefore these data clearly indicate that the T3R-RXR heterodimer is a major transcription complex on a DR4 TRE.

We report a striking exception to this rule. In QM7 cells, TPA stimulation or c-Jun overexpression induces a strong transcriptional activity to liganded T3R through a DR4 TRE. Dissipation by T3 of the c-Erb A homodimer binding to a DR4 TRE (10, 42) probably explains the inability of T3R to increase gene transcription by itself. Therefore, it could be proposed that c-Jun acts by stabilizing homodimer binding to DNA. In addition, according to the hypothesis of Pfahl (16), J un could function as a bridging molecule between c-Erb A and the transcriptional machinery. However, because we have not observed a similar T3R-J un interaction in HeLa cells, a muscle-specific protein could be involved in this bridging, as already proposed (Ref. 16 and Fig. 11).

Furthermore, we have obtained original data establishing a major role of RXR in of T3R-AP-1 functionality. In contrast to RAR, T3R does not repress AP-1 activity in quail myoblasts. In addition, TPA stimulation of endogenous AP-1 activity does not inhibit the ligand-dependent transcriptional activity of T3R in these cells. Interestingly, in contrast to COUP-TF I, PPAR α, or RAR α, RXR γ expression strongly enhances the stimulation of QM7 myoblast differentiation induced by T3 in control or c-Erb A α1 overexpressing cells. Connectin expression was assessed by cytoimmunofluorescence 2 days after the induction of differentiation with an antibody raised against connectin and a fluorescein-conjugated antibody raised against mouse immunoglobulins (×100). When indicated, 0.6 nM T3 was added in the culture medium. A, control cells. B, control cells + T3. C, T3R-expressing cells. D, T3R-expressing cells + T3. E, RXR γ expressing cells. F, RXR γ expressing cells + T3. G, T3R + RXR γ expressing cells. H, T3R + RXR γ expressing cells + T3. These microphotographs are representative of three independent experiments.

**Fig. 10.** RXR γ expression strongly enhances the stimulation of QM7 myoblast differentiation induced by T3 in control or c-Erb A α1 overexpressing cells. Connectin expression was assessed by cytoimmunofluorescence 2 days after the induction of differentiation with an antibody raised against connectin and a fluorescein-conjugated antibody raised against mouse immunoglobulins (×100). When indicated, 0.6 nM T3 was added in the culture medium. A, control cells. B, control cells + T3. C, T3R-expressing cells. D, T3R-expressing cells + T3. E, RXR γ expressing cells. F, RXR γ expressing cells + T3. G, T3R + RXR γ expressing cells. H, T3R + RXR γ expressing cells + T3. These microphotographs are representative of three independent experiments.

**Fig. 11.** Hypothetic scheme involving AP-1 and RXR in the regulation of myoblast differentiation by T3. This hypothesis only considers results obtained using a DR4 TRE, closely related to natural TRES. It is based on the original proposition of Pfahl (16). A, in proliferating myoblasts, a high AP-1 activity is recorded, thus repressing differentiation. In these conditions, according to the hypothesis of this study, c-Jun could function as a bridging molecule between T3R bound to a DR4 TRE and the transcriptional machinery. However, our data demonstrate that c-Jun induces a c-Erb A transcriptional activity in myoblasts but not in HeLa cells. These data suggest that another molecule, which may differ from cell type to cell type, is necessary to stabilize J un binding to the receptor, in agreement with the proposition of Pfahl (16). We propose that a muscle-specific factor (MSF) expressed in proliferative myoblasts plays this role. Such a mechanism could induce the activation of a set of genes involved in myogenic differentiation by T3. B, RXR expression induces formation of a T3R-AP-1 inactive complex either through DR4 or TPA responsive elements. Molecules involved in the bridging between the T3R homodimer and the transcriptional machinery could be directly released by RXR (inducing inactivity of the transcriptional complex) or indirectly (as a consequence of a disruption of the interaction of the transcription complex with DNA induced by RXR). Consequently, AP-1 activity is strongly inhibited, thus derepressing terminal differentiation. In these conditions, T3 responsive proteins synthesized in A could induce terminal differentiation. In differentiated cells, AP-1 activity remains depressed; T3-regulated gene expression is activated by RXR/T3R heterodimer. In this scheme, the T3 transcriptional pathway is always functional in relation to c-Jun (proliferation) or RXR (differentiation) expression. In conjunction with T3R (but probably with other nuclear receptors such as RARs), RXR represses AP-1 activity and overcomes the differentiation block.
X-ray receptor (RXR) expression, which also inhibits AP-1 activity, are involved in the preservation of T3R activity: first, via a DR4 TRE, RXR expression is necessary to induce the inhibition of AP-1 activity by liganded T3R. As a consequence, RXR is not only involved in the stimulation of T3 target genes but also represses another set of genes inhibiting cell differentiation.

The physiological relevance of these data is well illustrated by the observation that RXR strongly potentiates the stimulation of differentiation induced by T3 in control or in c-Erb A overexpressing myoblasts. Because myoblast withdrawal from the cell cycle is the first event of terminal differentiation, an anticipated differentiation would probably result in a reduced number of muscle fibers and consequently an important impairment of muscle development. As previously observed, RXR is not expressed before induction of terminal differentiation in murine myoblasts (33). Our data also indicate that in QM7 cells, RXR is not significantly expressed in proliferating cells. Therefore, RXR absence during the earliest steps of muscle development could provide a protection against such a premature differentiation.

T3 regulates the expression of a large set of genes, involved in developmental processes and in cell metabolism regulation. A lack of RXR expression in proliferating myoblasts inducing a T3 cell proliferation inefficiency would probably severely impair cell metabolism and viability if we consider that direct repeats are the most frequently described TREs. Interestingly, AP-1 activity could restore the T3R transcriptional activity. Therefore, our data indicate that alternative mechanisms are involved in the preservation of T3R activity: first, via AP-1 activity, which also inhibits differentiation, and second via RXR expression, which also inhibits AP-1 activity through a T3R-related mechanism and probably derepresses differentiation (Fig. 11).

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