ATF site of human RB gene promoter is a responsive element of myogenic differentiation

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Abstract RB mRNA increases during terminal differentiation of C2 myoblasts. We demonstrate that RB promoter activity increases about 4-fold during differentiation. The increase of RB promoter activity was reduced when a point mutation was designed in the ATF site. In a gel shift assay of the ATF site, two specific bands were observed. One of them, with the lower mobility, disappeared during differentiation. This band reacted with an antibody against ATF-1. We cotransfected an RB promoter-luciferase plasmid with the TREB36/ATF-1 plasmid. ATF-1 suppressed the activity of the wild-type RB promoter but not of that with a point mutation at the ATF site. These results suggest that the ATF site of the RB promoter is a responsive element during myogenic differentiation of C2 cells. We hypothesize that RB promoter activity is stimulated partially due to the dissociation of ATF-1, which suppresses the promoter activity through the ATF site in C2 myoblasts.

Key words: RB gene promoter; Myogenic differentiation; ATF

1. Introduction

The retinoblastoma susceptibility gene (RB) is the first identified prototype of a tumor suppressor gene [1]. Inactivation of the RB is associated with the etiology of a number of human tumors [2]. Expression of the RB protein in RB-negative cells suppresses the neoplastic phenotype and arrests the cells in late G1 phase [3,4]. Thus, the RB protein appears to play an important role in suppressing cell growth.

The RB protein is a nuclear phosphoprotein that undergoes cell cycle dependent changes in its phosphorylation status. The activity of the RB protein has been supposed to be controlled by its phosphorylation [2]. However, during terminal differentiation of the mouse skeletal muscle cell line C2, both the mRNA and protein of the RB have been markedly induced [5]. Also in other cell lines, such as mouse skeletal myoblast S2 cells, mouse erythroleukemic MEL cells and mouse embryonal carcinoma P19 cells, the RB mRNA increases during differentiation [6–8]. However, these studies did not describe whether the increase occurred at transcriptional or post-transcriptional level.

Skeletal muscle cells are an excellent system for investigating the molecular mechanism of differentiation, because they exhibit permanent withdrawal from the cell cycle and the remarkable phenotype to form multinucleated myotubes [9]. The terminal differentiation of muscle cells requires RB protein, because multinucleated myotubes from mice deficient in the RB gene cannot maintain the differentiated state and are induced by serum to reenter the cell cycle [10]. In myogenic differentiation, the RB protein must interact directly through a pocket domain with MyoD protein [11]. Moreover, the RB gene is transactivated by MyoD at the transcriptional level [12]. From these perspectives, the RB protein is an important regulator of muscle differentiation.

In the present study, we found that RB promoter activity increased about 4-fold during the differentiation of mouse skeletal muscle C2 cells. We identified a responsive element during muscle differentiation as an ATF site, where we found oncogenic germ-line mutation in one hereditary retinoblastoma family [13]. In a gel shift assay of the ATF site, a band that disappeared during myogenesis reacted with an antibody against ATF-1. TREB36/ATF-1 protein [14] suppressed the RB promoter activity specifically through the ATF site in cotransfection experiments. The increase of RB promoter activity during muscle differentiation of C2 cells is likely to be ascribed to the dissociation of ATF-1 protein suppressing the RB promoter.

2. Materials and methods

2.1. Cell lines and culture conditions

The mouse skeletal muscle cell line C2 [15] was maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's (DME) medium containing 10% fetal calf serum (growth medium). To induce terminal differentiation, 2×10^5 cells were plated in the growth medium on 3.5 cm diameter dishes and maintained for 24 h, then the medium was changed to DME medium containing 5% horse serum (differentiation medium).

2.2. Plasmid preparation and mutagenesis of the RB promoter

The wild-type human RB promoter-luciferase fusion plasmid, pXRP1, and the various mutant RB promoter-luciferase fusion plasmids were generated as described [13,16]. We confirmed by sequencing that the RB promoter constructs had no cloning-induced point mutations.

2.3. Stable transfection

Wild and mutant human RB promoter-luciferase fusion plasmids, and a plasmid conferring neomycin resistance, pSV2neo, were cotransfected into C2 mycoblasts by the Chen-Okayama method as described [17]. About 3 weeks later, the G418-resistant cells were isolated as a mixture of multiple colonies, and were expanded in a manner that was independent of the copy number and the integration locus in order to avoid clonal heterogeneity [18].

2.4. Luciferase assay

Cells were maintained in 3.5 cm diameter culture dishes, washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), then cell lysates were collected and stored at −70°C. Luciferase activity

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was measured under the conditions at the time as described [19], and was standardized by the amount of cellular protein in all samples or chloramphenicol acetyltransferase (CAT) activity driven by pSV2CAT as an internal control for transfection efficiency in some transient transfection experiments (data not shown). The luciferase assays were performed in triplicate and all experiments were repeated at least three times.

2.5. Gel shift assay

We prepared a nuclear extract and a whole cell extract from C2 myoblasts and C2 myotubes as described [20,16]. Radiolabeled double-stranded DNA was generated by annealing complementary oligomers with 5′ overhangs then filling in the recessed 3′ ends with dNTPs including ^{32}P-labeled dCTP, using the Klenow fragment of DNA polymerase I. All assayed sequences have flanked partial *Hin*dIII and *Sal*I recognition sequences not present in the RB gene. The DNA fragments had the following sequences (complete sense strand) (Fig. 2A): ATF-WT (wild-type ATF site from the RB promoter), AGCTCGGAAGTGACGTTTTCCCTCGA; ATF-MT (mutant ATF site from the RB promoter), AGCTCGGAAGTGACTTTTTCCCTCGA; RBF-1 (RBF-1/E4TF1 site from the RB promoter) [13,21], AGCTGCCGCGGGCGGAAGTTCGA; RBF-1+ATF (from the RBF-1/E4TF1 site to the ATF site from the RB promoter), AGCTGCCGCGGGCGGAAGTGACGTTTTCCCTCGA. Protein and DNA binding reactions were carried out as follows. 1 ng of radiolabeled oligomer DNA, 7 μg of nuclear extracts, and 1 μg of poly(dIdC) were incubated in a buffer containing 20 mM HEPES (pH 7.4), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, and 0.1% Nonidet P-40 for 30 min at room temperature. When the anti-ATF-1, anti-ATF-2, or anti-CREB-1 antibody (Santa Cruz Biotechnology) was used, 1 μg of the antibody was added and incubated for an additional 30 min at room temperature. Subsequently, reaction mixtures were loaded onto 4% polyacrylamide gels (acrylamide:bisacrylamide = 20:1) and run in 0.25×Tris-borate-EDTA buffer at room temperature at 200 V for 1.5 h.

2.6. Western blotting analysis

Nuclear cell extracts were analyzed with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyldifluoride membrane (Millipore). Membranes were washed with rinse buffer (PBS containing 0.1% Triton-X and 0.1% Tween-20) at room temperature for 15 min and incubated in blocking buffer (5% nonfat milk in rinse buffer). The membrane was then incubated with purified anti-ATF-1 antibody (0.5 μg/ml) in rinse buffer for 1 h at room temperature and washed three times with rinse buffer. Next, the membrane was incubated with 1:4000 diluted anti-mouse IgG, horseradish peroxidase-linked whole antibody (Amersham) in rinse buffer for 1 h at room temperature. After three washes, membranes were developed with enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham).

2.7. Transient transfection

DNA was transfected into cells by the calcium phosphate coprecipitation method as described [22]. Each transfection mixture contained 2 μg of the RB promoter-luciferase fusion plasmid as a reporter and 4 μg of the TREB36/ATF-1 expression plasmid [23] as an effector. After a 48 h incubation, the cells were harvested, and the cell lysates were used for the luciferase assay as described above. The same results were obtained when luciferase activity was standardized by the amount of cellular protein or CAT activity driven by pSV2CAT as an internal control.

3. Results

To investigate whether or not the expression of the RB gene is regulated at the transcriptional level, we established mouse skeletal myoblast C2 cells, in which human RB promoter-luciferase fusion plasmids were stably transfected. We traced changes in the RB promoter activity during myogenesis of these cells by means of a luciferase assay. As shown in Fig. 1A, the RB promoter activity increased about 2.9-fold 24 h after changing to the differentiation medium, and about 4.8-fold in the terminally differentiated myotubes 96 h after the shift. The increase in the activity correlates with the accumulation of RB mRNA during myogenic differentiation, which

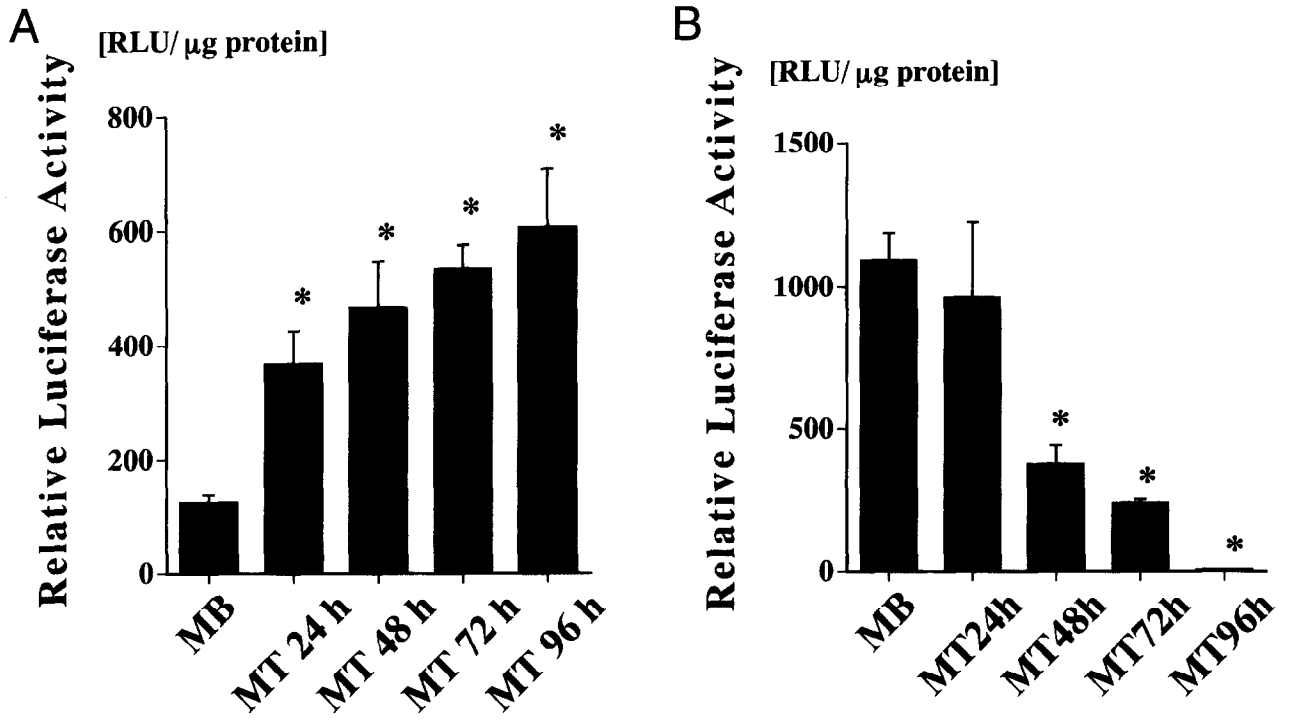


Fig. 1. Promoter activities of RB and cyclin A during muscle cell differentiation. A: The RB promoter activity increases during muscle cell differentiation. B: The cyclin A promoter activity decreases during muscle cell differentiation. Relative luciferase activity is shown as raw light units (RLU) per μg protein in cell lysates. The luciferase assays were performed in triplicate and the data are shown as means ± S.E. Significance was calculated against the value from myoblasts. *$P < 0.05$. MB: myoblasts, MT: myotubes.

has been reported [5]. On the other hand, in the C2 myoblasts into which a cyclin A promoter-luciferase fusion plasmid [14] was stably transfected, the cyclin A promoter activity decreased markedly, as the cells differentiated into myotubes (Fig. 1B). The decrease in cyclin A promoter activity was consistent with reports that cyclin A mRNA decreased during myogenic differentiation [24]. In addition, when we used human osteosarcoma MG63 cells into which human RB promoter-luciferase fusion plasmids were stably transfected, the RB promoter activity did not increase by serum depletion (data not shown). These results indicate that during muscle cell differentiation, human RB promoter activity specifically increases.

To elucidate which promoter element is involved in the upregulation of the RB promoter in muscle cell differentiation, we generated a series of plasmid constructs of deleted or point mutated RB promoters as shown in Fig. 2A [13,16]. We then stably transfected a series of mutant RB promoters-luciferase fusion plasmids and identified the differentiation responsive element of the RB promoter. Luciferase activity was assayed using the lysates prepared from C2 myoblasts and from C2 myotubes. We compared the relative luciferase activity of C2 myotubes with that of C2 myoblasts in each established cell line (Fig. 2B). The promoter activity with each mutation in the stably transfected cell lines does not necessarily reflect the exact effect of each mutation, perhaps because stably transfected copies of each plasmid might differ in each established cell line. Therefore, as shown in Fig. 2B, we determined the fold induction of the RB promoter activities during differentiation, showing ratios of luciferase activities in myotubes versus myoblasts for different RB promoter constructs. During muscle cell differentiation, wild-type RB promoter activity from the pXRP1 plasmid increased about 3.7-fold. In contrast, the activity of pXRP2, including the point mutation at the ATF site, increased only 1.4-fold. The activities of the other deleted or mutated plasmids increased 2.4–4.5-fold. These results suggest that the ATF site is a muscle differentiation-responsive element.

To investigate changes in the nuclear factors that bind to the ATF and RBF-1/E4TF1 sites of the RB promoter during muscle cell differentiation, we prepared nuclear extracts from C2 myoblasts and C2 myotubes. Incubation of the nuclear extracts with ^{32}P-labelled oligonucleotides resulted in the formation of a specific protein-DNA complex as shown in Fig. 3A. When we used the wild-type RBF-1+ATF sequences from the RB promoter (RBF-1+ATF) as a probe, seven specific bands were detected in the extracts from C2 myoblasts (Fig. 3A, lane 1). The intensity of band A decreased in the presence of competing wild-type ATF sequence from the RB promoter, designated ATF-WT (Fig. 3A, lane 2). Band A was therefore thought to be a member of the ATF family. In contrast, the intensity of other bands was reduced in the presence of a competing wild-type RBF-1 sequence (Fig. 3A, lane 3). They consequently seemed to be proteins binding to the RBF-1/E4TF1 site. However, in C2 myotubes, band A was barely detectable (Fig. 3A, lane 7). The disappearance of band A suggested its implication in enhancing RB promoter activity. To identify the band A protein regarded as a member of the ATF family, we added two anti-ATF antibodies to the nuclear extracts from C2 myoblasts and C2 myotubes, and performed a supershift assay using the RBF-1+ATF sequences as a probe. Interestingly, co-incubation with the antibody to ATF-1 supershifted band A (Fig. 3A, lane 5), whereas that to ATF-2 did not (Fig. 3A, lane 6). When we used ATF-WT as a probe in the gel shift assay, there were at least two specific bands from the nuclear extracts of C2 myoblasts (labelled A and B, shown in Fig. 3B, lane 1). The intensity of these two bands was reduced in the presence of competing ATF-WT (Fig. 3B, lane 2), but not in the presence of the competing mutant ATF sequence, ATF-MT (Fig. 3B, lane 3). During muscle cell differentiation, band A declined and was barely evident in C2 myotubes (Fig. 3B, lane 7). When anti-ATF antibodies were added to the nuclear extracts from C2 myoblasts and C2 myotubes, the antibody against ATF-1 supershifted band A (Fig. 3B, lane 4), whereas the antibodies against ATF-2 and CREB-1 did not (Fig. 3B, lanes 5, 6). Additionally, we confirmed in the gel shift assay of the ATF site that the disappearance of the band A correlated with the

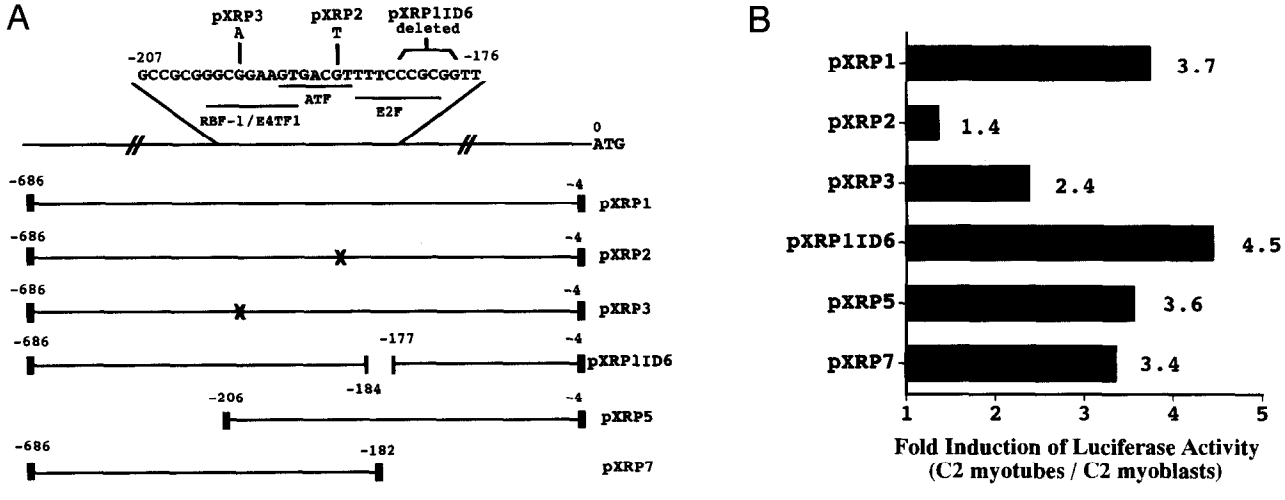
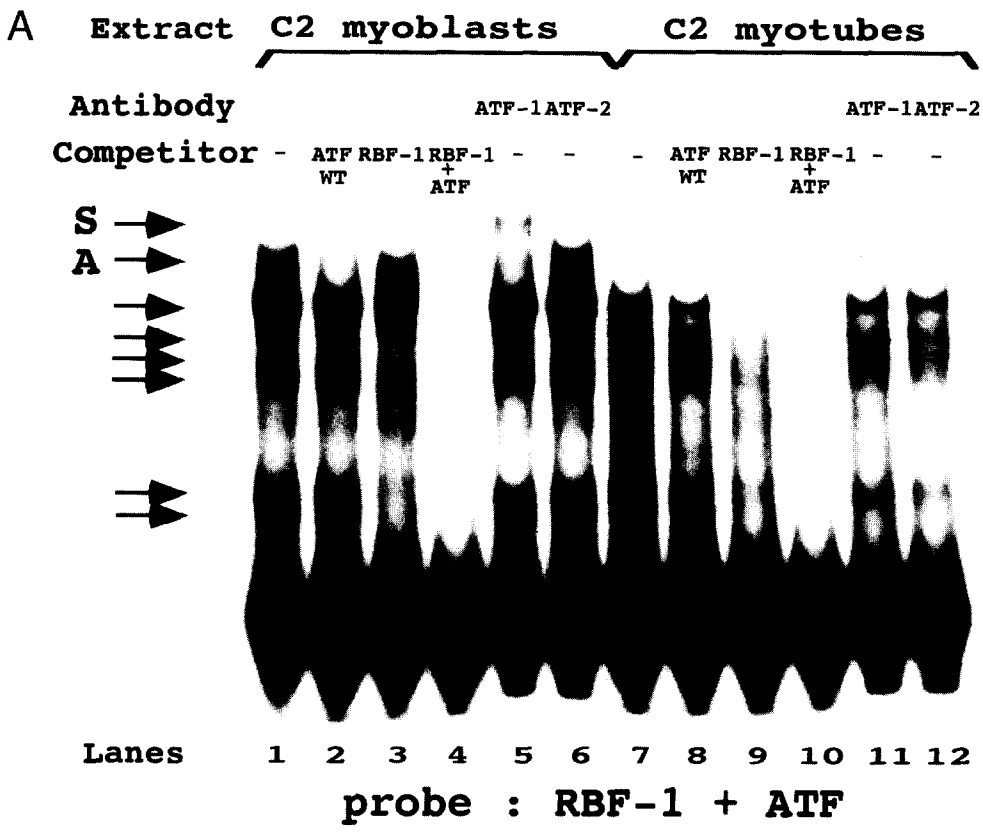
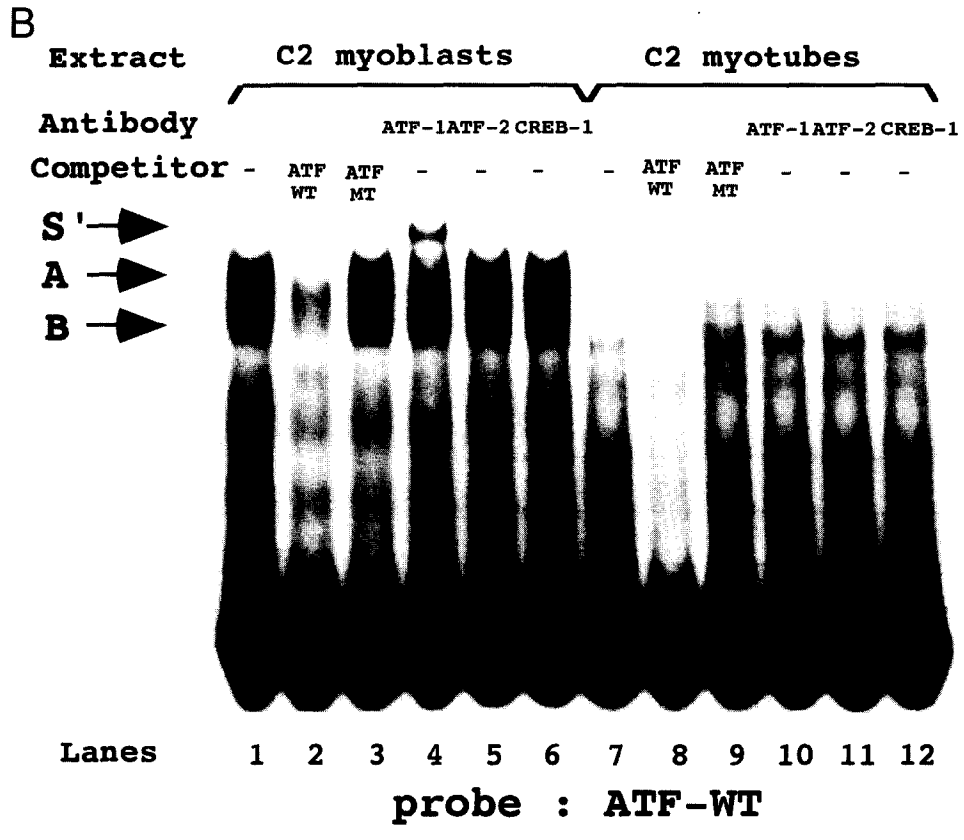


Fig. 2. Mapping of the responsive element during muscle cell differentiation. A: The sequence of the essential RB promoter region and the schematic diagram of the wild-type and several mutants. The sequence is numbered using the first base of the initiating methionine codon as 0. B: The enhancement of the RB promoter activity in the wild-type and the mutant RB promoters during muscle cell differentiation. Shown is the relative luciferase activity (the activity in myotubes/that in myoblasts) indicated as fold induction. Data are shown as means of four independent experiments.


A
Extract
C2 myoblasts
C2 myotubes
Antibody
ATF-1 ATF-2
ATF-1 ATF-2
Competitor
- ATF WT RBF-1 RBF-1 + ATF - - - ATF WT RBF-1 RBF-1 + ATF - -
S
A
Lanes 1 2 3 4 5 6 7 8 9 10 11 12
probe : RBF-1 + ATF


B
Extract
C2 myoblasts
C2 myotubes
Antibody
ATF-1 ATF-2 CREB-1
ATF-1 ATF-2 CREB-1
Competitor
- ATF WT ATF MT - - - - ATF WT ATF MT - - -
S'
A
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Lanes 1 2 3 4 5 6 7 8 9 10 11 12
probe : ATF-WT

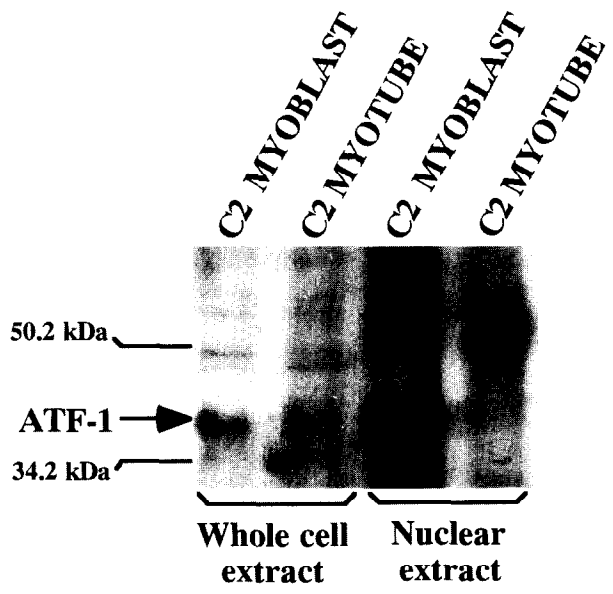

Fig. 4. Down-regulation of TREB36/ATF-1 protein during muscle cell differentiation. Whole cell extracts and nuclear extracts were prepared from C2 myoblasts and C2 myotubes. After 20 μg of whole cell extracts or nuclear extracts per lane were analyzed by SDS-PAGE, they were transferred to nitrocellulose membrane and the Western blotting analysis was performed using the antibody to ATF-1 as described in Section 2. These findings were confirmed by two independent samples of C2 myoblasts and C2 myotubes.

gradual induction of RB promoter activity during terminal differentiation (data not shown). On the other hand, when we used the RBF-1/E4TF-1 sequence as a probe, there was little difference in the band profiles in the gel shift assay performed during muscle cell differentiation (data not shown). These data suggest that the protein dissociated from the ATF site of the RB promoter during differentiation is related to ATF-1.

To examine the expression of ATF-1 protein during myogenesis, we performed Western blotting analysis using nuclear extracts or whole cell extracts of C2 cells. As shown in Fig. 4, ATF-1 protein markedly decreased during muscle cell differentiation in both whole cell and nuclear extracts of C2 cells. The reduction of ATF-1 protein during differentiation was more evident in nuclear extracts than in whole cell extracts, suggesting that the nuclear distribution of ATF-1 protein was reduced during myogenic differentiation. Therefore, we conclude that the dissociation of ATF-1 protein from the ATF site of the RB promoter during myogenesis is caused by the down-regulation of ATF-1 protein during myogenesis.

We then postulated that ATF-1 binds to the ATF site and suppresses RB promoter activity through the site in C2 myoblasts. To clarify the role of ATF-1 in the transcription of the RB gene in muscle cells, we cotransfected the wild-type RB promoter-luciferase fusion plasmid, pXRP1, with the TREB36/ATF-1 expression plasmid, pCG-T36, in C2 myoblasts. As shown in Fig. 5, the expression of TREB36/ATF-1 down-regulated the RB promoter activity to about 37% of the control, whereas TREB36/ATF-1 expression did not suppress the RB promoter activity when the ATF site was mutated (pXRP2). Therefore, the TREB36/ATF-1 protein specifically suppresses the RB promoter through its ATF site in C2 myoblasts.

4. Discussion

The expression of the RB gene has been shown to increase at the mRNA level during differentiation in mouse skeletal muscle C2 cells [5]. In the present study, we found that RB promoter activity increases from the early stage of differentiation and reaches about 4-fold in terminally differentiated C2 cells. We then identified the ATF site as a responsive element of the RB promoter during myogenic differentiation of C2 cells. We and others have described three major transcriptional factor-binding sites, RBF-1/E4TF1 site overlapping the Sp-1 site [13,21], ATF site [13], and E2F site [16,25] in the RB promoter. Human and mouse RB promoters display high homology in the three major sites described above [26]. Furthermore, germ-line mutations in the RBF-1/E4TF1 and ATF sites in the human RB promoter result in hereditary retinoblastoma because of a quantitative decrease in the activity of the RB promoter using African monkey kidney CV-1 cells [13]. Also in this study, the mutation at the ATF site actually reduced the RB promoter activity in C2 mouse myoblast cells. These findings indicate that the ATF site of the RB promoter is an essential transactivating site suggesting the existence of a transactivator on the site. However, in this study, during skeletal muscle cell differentiation, the RB promoter activity increased through the ATF site, whereas ATF-1 was depleted in the gel shift assay. In addition, TREB36/ATF-1 suppressed the RB promoter activity through the ATF site. Thus, we hypothesize that the ATF-1 specifically binding to the ATF site of the RB promoter in C2 myoblasts has a suppressive effect on the RB promoter. During muscle differentiation, the ATF-1 protein decreases and thus RB promoter activity might increase, being free from the suppression by ATF-1. It should be noted that ATF-1 works as a suppressor on the ATF site of the RB promoter in C2

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Fig. 3. A: Gel shift assay of sequences including the RBF-1+ATF site from the promoter of the RB gene. Lanes 1–6, nuclear extracts from C2 myoblasts; lanes 7–12, nuclear extracts from C2 myotubes; lanes 1 and 7, radiolabeled, wild-type of the RBF-1+ATF sequences with nuclear extracts; lanes 2 and 8, the same as lanes 1 and 7, but with 20-fold molar excess of the competitors of the ATF sequence; lanes 3 and 9, the same as lanes 1 and 7, but with 20-fold molar excess of the competitors of the RBF-1 sequence; lanes 4 and 10, the same as lanes 1 and 7, but with 20-fold molar excess of the competitors of the RBF-1+ATF sequences; lanes 5 and 11, the same as lanes 1 and 7, but with 1 μg of anti-ATF-1 antibody; lanes 6 and 12, the same as lanes 1 and 7, but with 1 μg of anti-ATF-2 antibody. The arrow labeled A indicates a complex binding to the ATF site. Other complexes bound to the RBF-1 site. The arrow labeled S indicates a supershifted band. B: Gel shift assay of sequences including the ATF site from the RB promoter. Lanes 1–6, nuclear extracts from C2 myoblasts; lanes 7–12, nuclear extracts from C2 myotubes; lanes 1 and 7, radiolabeled, wild-type of the ATF sequence with nuclear extracts; lanes 2 and 8, the same as lanes 1 and 7, but with 20-fold molar excess of the competitors of the ATF sequence; lanes 3 and 9, the same as lanes 1 and 7, but with 20-fold molar excess of the mutant ATF sequence; lanes 4–6 and 10–12 demonstrate the effect of anti-ATF-1 (TREB36), ATF-2 or CREB-1 antibody on the ATF complexes. Lanes 4 and 10, the same as lanes 1 and 7, but with 1 μg of anti-ATF-1 antibody; lanes 5 and 11, the same as lanes 1 and 7, but with 1 μg of anti-ATF-2 antibody; lanes 6 and 12, the same as lanes 1 and 7, but with 1 μg of anti-CREB-1 antibody. The arrows labeled A and B indicate complexes binding to the ATF site. The arrow labelled S′ indicates a supershifted band.

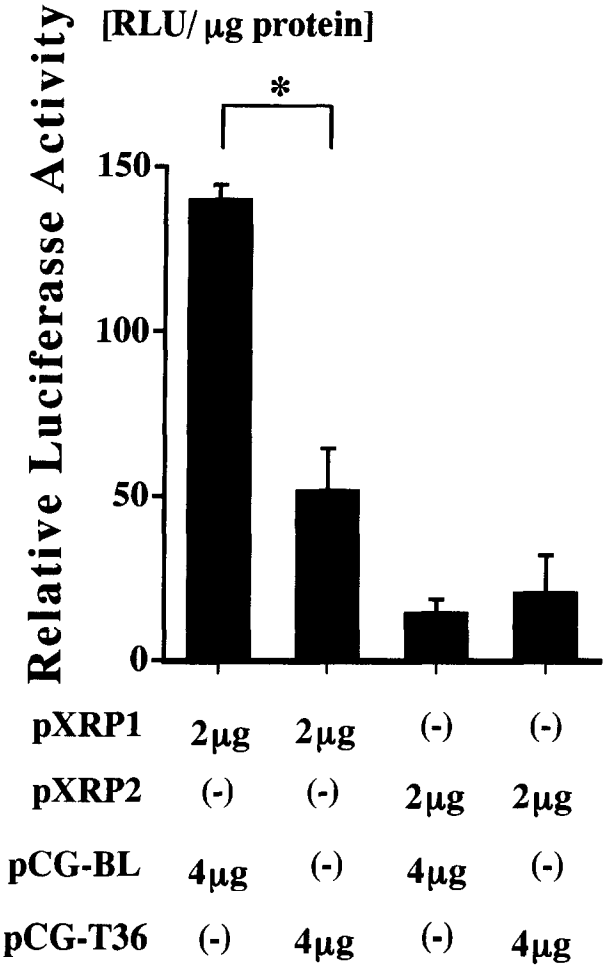

Fig. 5. The TREB36/ATF-1 protein suppresses the RB promoter activity in the C2 myoblasts. The TREB36/ATF-1 expression plasmid, pCG-T36, or its empty vector, pCG-BL, was cotransfected with the wild-type (pXRP1) or mutant ATF (pXRP2) of the RB promoter-luciferase fusion plasmid in the C2 myoblasts. Relative luciferase activity is shown by raw light units (RLU) per μg protein in cell lysates. The luciferase assay was performed in triplicate and the data are shown as means ± S.E. $^*P < 0.05$.

myoblasts, whereas the ATF site is a positive transcriptional site [13].

Consistent with this finding, in CCL64 mink lung epithelial cells, ATF-1 slightly down-regulates the RB promoter activity [27]. On the other hand, although ATF-2 had been reported to activate the RB promoter [27], it could not enhance RB promoter activity in C2 cells (data not shown) and did not bind to the ATF site of the RB promoter (Fig. 3B). In addition, CREB-1 protein did not bind to the ATF site as shown in Fig. 3B. These results suggest that the activators on the ATF site might be some other ATF family proteins including novel ones. One possibility is that the band B protein shown in Fig. 3B may be an ATF protein activating the ATF site of the RB promoter. On the other hand, Shiio et al. suggest that Bcl-3 enhances the RB gene promoter through the E4TF1 site during muscle differentiation [28]. However, expression of Bcl-3 mRNA is highest at 48 h, and then it gradually decreases. Therefore, the induction of Bcl-3 might be involved in early induction of RB mRNA, and we propose that the dissociation of ATF-1 partially contributes to the expression of the RB gene during terminal differentiation of C2 cells. As the next step, we plan to examine if the increase of the RB promoter activity during differentiation in other cell lines is caused by the same mechanism.

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