Binding of the Retinoblastoma Protein Is Not the Determinant for Stable Repression of Some E2F-Regulated Promoters in Muscle Cells

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Abstract

Permanent silencing of E2F-dependent genes is a hallmark of the irreversible cell cycle exit that characterizes terminally differentiated and senescent cells. The determinant of this silencing during senescence has been proposed to be the binding of the retinoblastoma protein Rb and the consequent methylation of H3K9. During *ex vivo* skeletal muscle differentiation, while most cells terminally differentiate and form myotubes, a subset of myoblasts remains quiescent and can be reinduced by growth factor stimulation to enter the cell cycle. Thus, differentiating cells are composed of two different populations: one in which E2F-dependent genes are permanently repressed and the other not. We observed that, in a manner reminiscent to senescent cells, permanent silencing of the E2F-dependent *cdc6, dhfr*, and *p107* promoters in myotubes was associated with a specific increase in H3K9 trimethylation. To investigate the role of Rb in this process, we developed a reliable method to detect Rb recruitment by chromatin immunoprecipitation. Surprisingly, we observed that Rb was recruited to these promoters more efficiently in quiescent cells than in myotubes. Thus, our data indicate that during muscle differentiation, permanent silencing and H3K9 trimethylation of some E2F-dependent genes are not directly specified by Rb binding, in contrast to what is proposed for senescence. (Mol Cancer Res 2008;6(3):418–25)

Introduction

Skeletal muscle differentiation is a tightly regulated process during which proliferative myoblasts stop to divide and fuse into multinucleated myotubes. Proliferation and differentiation are two mutually exclusive processes. As such, myotubes are terminally differentiated cells that have permanently escaped from the cell cycle. Only experimental conditions can trigger myotubes to reenter the cell cycle and complete G_1-to-S phase transition. As such, ectopically expressed viral proteins such as the adenoviral E1A gene product can trigger DNA synthesis in terminally differentiated myotubes. The recovery to complete DNA synthesis is, in part, due to the ability of E1A to bind to and inhibit pocket proteins such as the retinoblastoma protein (Rb) and, consequently, derepress E2F-responsive genes essential for G_1-S transition (1) because Rb is a key regulator of the E2F family of transcription factors. During G_0-G_1 phases of the cell cycle, Rb (and the other pocket proteins p107 and p130) binds to E2Fs, inhibiting their activity. During progression through the G_1 phase, Rb becomes hyperphosphorylated by cyclin-dependent kinase (cdk)-4-cyclin D1 and cdk2-cyclin E complexes and is released from E2F. Free E2Fs then activate target genes involved in the G_1-to-S phase transition, such as *cyclin A* and *cdk1*, and in S-phase progression, such as *dhfr* and *cdc6*. During muscle terminal differentiation, Rb becomes hypophosphorylated and mRNA and protein levels increase in a MyoD- and cyclic AMP responsive element binding protein–dependent manner (2-4). In addition to its inhibitory role on cell proliferation, Rb is a positive regulator of skeletal muscle differentiation. Indeed, $Rb^{-/-}$ myoblasts fail to terminally differentiate although they can express the early differentiation marker myogenin (5, 6). The other pocket proteins, p107 and p130, cannot substitute for Rb in this process, although p107 was shown to be up-regulated in $Rb^{-/-}$ myoblasts (5).

It is noteworthy that, despite the abundance of hypophosphorylated Rb, p130-E2F complexes were shown to be the predominant E2F complexes in myotubes (3, 7). However, Rb is required both for efficient cell cycle exit and for the permanent repression of E2F-dependent genes. Indeed, two independent studies recently reported that knocking out Rb following myotube formation allowed the reexpression of E2F-dependent genes (although S phase was not restored), showing the importance of Rb in silencing E2F-dependent genes in myotubes (8, 9).

Pocket proteins achieve their inhibitory roles on E2F-dependent genes by two ways. First, they bind to and consequently inhibit the activator domain of E2F-1 to E2F-5. Second, they recruit transcriptional corepressors such as histone-modifying enzymes that locally modify chromatin in the vicinity of the E2F binding sites. As such, histone deacetylation correlates with repression of E2F target genes
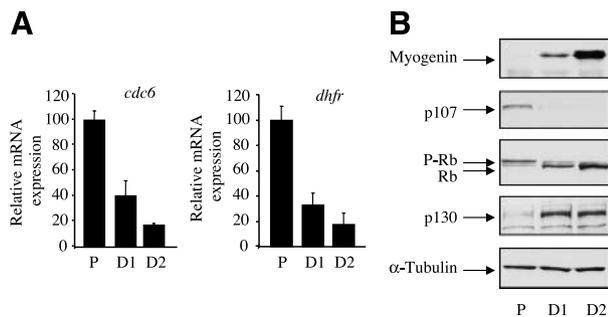
A

B

FIGURE 1. Expression of pocket proteins during C2C12 differentiation. **A.** C2C12 kept in proliferation conditions (see Materials and Methods) or shifted to differentiation medium on confluence for 1 d (*D1*) or 2 d (*D2*) were harvested, and the expression of E2F target mRNAs (*cdc6* and *dhfr*) or control mRNA (*gapdh*) was then measured by reverse transcription followed by quantitative PCR. The amount of *cdc6* and *dhfr* mRNA was divided by the amount of *gapdh* mRNA and calculated relative to 100 in proliferating cells. **B.** Same as in **A**, except that total cell extracts were prepared and analyzed by Western blot with the indicated antibodies.

(10). HDAC1 binds to the pocket domains of Rb, p107, and p130 (11). The histone methyltransferase Suv39H1 binds to both Rb and p130 and, by methylating lysine 9 of histone H3 (H3K9), may be involved in the repression of E2F-dependent genes in quiescent cells (12-14). Accordingly, it was recently shown that E2F-dependent gene extinction in myotubes correlates with an increase of H3K9 methylation by Suv39H1/2 (15). However, how Suv39H is targeted to promoters was not investigated.

During the course of differentiation, a subset of myoblasts escapes from the differentiation program although they exit from the cell cycle and enter into a quiescent state. It has been suggested that these quiescent cells, the so-called "reserve cells," may be similar to satellite cells in muscle. As such, in response to growth factor stimulation, they can reenter the cell cycle and, on induction of differentiation, form myotubes and quiescent cells again (16).

It was shown that p130 preferentially accumulated in undifferentiated quiescent cells that form during differentiation, together with myotubes (17). Thus, serum-starved C2C12 cells are composed of two very different populations of cells with respect to the extent of E2F-dependent gene silencing: myotubes in which E2F-responsive genes are permanently silenced and quiescent cells in which E2F-responsive genes can be reactivated. It is tempting to speculate that the identity of the pocket protein bound to E2F-regulated promoters specifies whether the transcriptional repression is reversible or not. Indeed, p130 binding to E2F-regulated promoters in non-muscular quiescent cells is well documented (18, 19). Moreover, a role for Rb in targeting histone H3 methylation and permanent transcriptional repression to E2F-regulated promoters has already been shown to occur during ras-induced senescence (19).

To test this possibility, we investigated pocket protein binding to E2F-regulated promoters during muscle differentiation. We found that Rb bound equally well to E2F-regulated promoters in quiescent cells compared with myotubes, indicating that Rb binding is not the sole determinant of permanent repression of E2F-regulated promoters during muscle differentiation.

Results

Differentiating C2C12 Cells Expressed Higher Levels of Rb and p130

Whereas the roles of pocket proteins in muscle terminal differentiation and for the concomitant permanent cell cycle exit have been extensively characterized, the binding of these proteins to their most characterized DNA target sequences (i.e., E2F-responsive genes) has never been studied. Yet, in other cell models, the permanent growth arrest induced by senescence correlates with specific recruitment of Rb to E2F-regulated promoters (19).

To study pocket protein binding to E2F-regulated promoters in differentiating myoblasts, we used C2C12 cells, which can be induced to differentiate *in vitro*. Indeed, when shifted at confluence into a medium with low levels of growth factors, C2C12 cells stop proliferating and enter a terminal differentiation program. We found that, as expected, differentiating cells expressed muscle-specific genes, such as *myogenin* and *mck* (data not shown). In addition, the expression of some E2F-regulated genes [dihydrofolate reductase–encoding gene (*dhfr*) and *cdc6*] decreased strongly (Fig. 1A), in parallel with the concomitant cell cycle arrest. To gain a first insight into the identity of the pocket proteins responsible for this repression, we analyzed the expression of all three pocket proteins by Western blot. We found that in differentiating cells, both Rb and p130 levels were increased compared with proliferating myoblasts (Fig. 1B). Moreover, the hypophosphorylated form of Rb, which is presumably the active form of Rb, was largely predominant in differentiating cells. In contrast, p107 levels decreased strongly, and the protein was nearly undetectable on serum withdrawal (Fig. 1B), as expected, because the p107-encoding gene is a target of E2F (20). Thus, altogether, these results are consistent with previously described results (17) and suggest that E2F regulation in differentiating cells is brought about by p130 and/or Rb.

Rb Binding to Some E2F-Regulated Promoters Can Be Analyzed by Chromatin Immunoprecipitation

Whereas binding of p130 and p107 to E2F-regulated promoters has been widely monitored by chromatin immunoprecipitation, Rb binding has always been more elusive. Indeed, many studies failed to detect Rb binding to E2F-regulated promoters by chromatin immunoprecipitation (18, 21-23). To test whether we could detect Rb binding by chromatin immunoprecipitation, we used chromatin prepared from cells induced to differentiate for 2 days because they expressed high levels of Rb and p130 proteins (Fig. 1B). We found that the anti-Rb and anti-p130 antibodies were both able to immunoprecipitate their target protein in chromatin immunoprecipitation conditions (data not shown). To precisely quantify chromatin immunoprecipitation efficiency, we used real-time quantitative PCR to analyze the presence of E2F-regulated promoters (the *dhfr* or *cdc6* promoters) and a control sequence [from the *gapdh* promoter (*gapdh*)]. We first carried out a classic chromatin immunoprecipitation experiment with formaldehyde as a cross-linking product. Using this protocol, we immunoprecipitated more *cdc6* promoter (an E2F-regulated promoter) with the anti-p130 antibody than in the control immunoprecipitates (without antibody; Fig. 2A). Moreover, this

enrichment was specific because it was not observed on a DNA sequence derived from the *gapdh* promoter, which is not bound by E2F. Thus, this strategy allowed us to precisely monitor p130 binding to the *cdc6* promoter. In contrast, the *cdc6* promoter was not enriched in the anti-Rb immunoprecipitates (Fig. 2A), indicating that either Rb does not bind to this promoter or that these experimental settings did not allow us to detect Rb binding.

To distinguish between these two possibilities, we decided to change our cross-linking conditions. To increase the efficiency of Rb cross-linking to DNA, we used products inducing protein/protein cross-links in addition to formaldehyde. We also tested various cross-linking times as well as various formaldehyde concentrations. Most conditions did not allow us to detect Rb binding to the *cdc6* promoter (data not shown). However, in the presence of dimethyl adipimidate (DMA) as a protein/protein cross-linking product followed by a 1% formaldehyde treatment, we immunoprecipitated more E2F-regulated *cdc6* promoter in the anti-Rb immunoprecipitates than in the control immunoprecipitates (Fig. 2B). Moreover, another E2F-regulated promoter (*dhfr*) was similarly enriched in Rb immunoprecipitates, whereas the *gapdh* promoter was only marginally enriched [Fig. 2B; note that in most experiments (see Fig. 5), it was not at all enriched compared with control immunoprecipitates]. Moreover, a sequence derived from the 3′ end of the cdc6-encoding gene and located ∼12 kb away from the E2F binding site was also not enriched in Rb immunoprecipitates (Fig. 2B). Although the enrichment of the *cdc6* and *dhfr* promoter is weak, we believe that it truly reflects Rb binding because (*a*) the antibodies we used are highly specific monoclonal antibodies; (*b*) several sequences not targeted by E2F were not enriched, including not only the *gapdh* promoter (Fig. 2B) but also sequences derived from the 3′ ends of *cdc6* (Fig. 2B) and *dhfr* (data not shown) genes; and (*c*) the use of real-time PCR, which allowed us to quantify background amounts of target sequences (which would seem as empty lanes using conventional PCR), indicates that the control sequences were present in the Rb immunoprecipitates at levels identical to all the DNA sequences we analyzed in the control immunoprecipitates, therefore reflecting background binding to the protein A/protein G beads.

Thus, we conclude from this experiment that these cross-linking conditions allowed us to monitor specific Rb binding to some E2F-regulated promoters. To our knowledge, our data are the first description of chromatin immunoprecipitation experiments monitoring Rb binding to some E2F-regulated promoters by quantitative PCR.

Analysis of Rb Binding to the cdc6 *Promoter during Terminal Differentiation*

We then analyzed Rb binding to the *cdc6* promoter (which reproducibly was the most enriched of the E2F-regulated promoters we analyzed in Rb chromatin immunoprecipitation) during the differentiation process. We prepared DMA and formaldehyde-fixed chromatin from proliferating cells or from cells that have been shifted to differentiation medium for 1, 2, or 3 days. We carried out chromatin immunoprecipitation with anti-Rb antibodies, and we analyzed the presence of *cdc6* promoter or *gapdh* promoter in the Rb immunoprecipitates. We

found that Rb binding to the *cdc6* promoter gradually increased in differentiating cells (Fig. 3), with a maximal binding after 3 days of differentiation induction, which was the latest time analyzed. Analysis of an unrelated sequence (from the *gapdh* promoter) did not show any increase in the same time course. Thus, this experiment indicates that Rb binding to the E2F-regulated *cdc6* promoter was increased when muscle cells stopped proliferating to differentiate.

Quiescent and Terminally Differentiated C2C12 Cells Harbored Distinct Chromatin Marks on Some E2F-Regulated Promoters

In contrast to quiescent cells, terminally differentiated muscle cells have irreversibly exited the cell cycle. Accordingly, E2F-responsive genes are permanently repressed, whereas they can still be activated in quiescent cells. Importantly, these distinct transcriptional states of E2F-regulated promoters (permanent versus non permanent repression) have been shown to be associated with distinct chromatin features, in particular heterochromatic K9 methylation (most likely trimethylation;
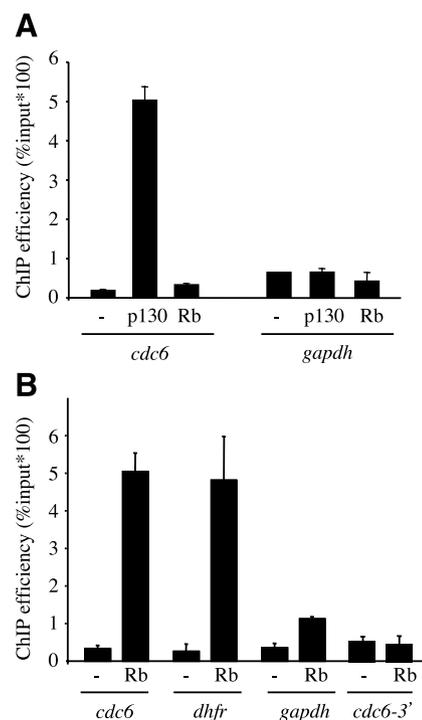

FIGURE 2. Detection of Rb binding by chromatin immunoprecipitation. **A.** C2C12 cells cultured in differentiation conditions for 2 d were harvested using standard chromatin immunoprecipitation (*ChIP*) conditions (cross-linking with 1% formaldehyde for 15 min). Sonicated chromatin was then subjected to a chromatin immunoprecipitation analysis using, as indicated, anti-p130 or anti-Rb antibodies, as well as a no antibody control (−). The amounts of *cdc6* promoter or *gapdh* promoter in the inputs and in the immunoprecipitates were then measured by quantitative PCR. Result of a typical experiment is shown. **B.** C2C12 cells cultured in differentiation conditions for 2 d were treated with DMA (a protein/protein cross-linking agent) before formaldehyde treatment (see Materials and Methods). Sonicated chromatin was then analyzed by chromatin immunoprecipitation with anti-Rb antibodies or no antibody as a control (−). The amounts of *cdc6*, *dhfr*, and *gapdh* promoters, as well as cdc6 3′ sequence, in the inputs and in the immunoprecipitates were then measured by quantitative PCR.
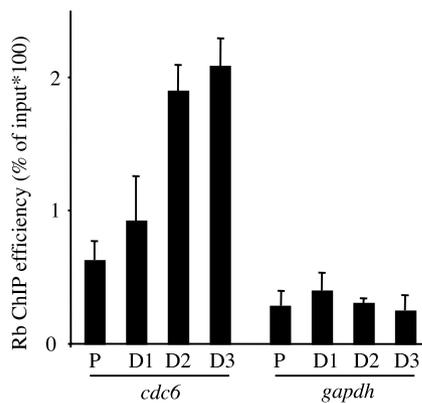
FIGURE 3. Evolution of Rb binding to E2F-regulated promoters during C2C12 differentiation. C2C12 cells were cultured in proliferation conditions (*P*) or in differentiation conditions for 1 d (*D1*), 2 d (*D2*), or 3 d (*D3*), and then subjected to a DMA/formaldehyde cross-linking. Sonicated chromatin was then immunoprecipitated with anti-Rb antibodies, and the amounts of *cdc6* promoter or control *gapdh* promoter in the inputs and immunopre-cipitates were measured by quantitative PCR.

ref. 15). However, in this study, terminally differentiated cells and quiescent cells did not originate from the same cell lines.

Interestingly, it is possible to recapitulate these two different cell fates using C2C12. Indeed, when shifted to differentiation medium, confluent C2C12 cells stop proliferating and most cells undergo a terminal differentiation program characterized by the expression of muscle-specific genes, a permanent repression of E2F-responsive promoters, and cell fusion leading to myotube formation. However, a low proportion of cells stop proliferating but do not express muscle-specific genes. Rather, they enter quiescence and can be reinduced to enter cell cycle following serum addition. These two populations can be efficiently separated by controlled trypsin treatment. Indeed, on mild trypsin digestion, myotubes can be harvested whereas quiescent cells remain adherent, therefore allowing us to collect terminally differentiated cells (myotubes) and quiescent myoblasts from the same culture dishes.

We took advantage of this system in which any indirect effect of the culture conditions can be ruled out to investigate the determinants of permanent repression of E2F-dependent genes during terminal differentiation.

Reverse transcription followed by real-time PCR analysis indicates that trypsin-sensitive cells were terminally differentiated because they expressed muscle-specific genes, such as *myogenin* and *mck*, and E2F-regulated genes (such as *cdc6* and *dhfr*) were repressed (Fig. 4A and B). Cells that were resistant to trypsin digestion harbored features characteristic of quiescent cells: E2F-regulated promoters were repressed, as expected, because these cells have exited the cell cycle, but muscle-specific genes were not activated; these cells thus harbored features of quiescent myoblasts (Fig. 4A and B).

We therefore prepared chromatin from these two cell populations, as well as from control proliferating C2C12 cells, and analyzed the presence of heterochromatic marks (H3K9 trimethylation) by chromatin immunoprecipitation (Fig. 4C). We found that trimethylation of H3K9 increased in myotubes compared with proliferating cells on various E2F-responsive promoters (*cdc6, dhfr*, and *p107* promoters). Although this

increase is weak (~ 2-fold), it is similar to previously published results (15). Moreover, this increase was specific because it was not observed on the control *gapdh* promoter. Most interestingly, the presence of this heterochromatic mark was not increased in quiescent cells on any of the E2F-regulated promoters we analyzed compared with proliferating cells. Thus, this result indicates that in C2C12 cells, the presence of trimethylation of H3K9 on some E2F-responsive promoters correlates with their permanent repression.

Binding of Rb to Some E2F-Regulated Promoters Did Not Correlate with Permanent Cell Cycle Exit

Rb has been shown to bind to E2F-regulated promoters only when these promoters are permanently repressed (19). It has been proposed to mediate this stable repression by targeting chromatin marks specific for heterochromatin on E2F-regulated promoters, thereby permanently preventing them from being transcribed. To test whether Rb binding would correlate with the presence of heterochromatic marks on E2F-regulated promoters, we prepared DMA and formaldehyde–cross-linked chromatin from quiescent or terminally differentiated cells (separated by mild trypsin digestion) as well as from proliferating myoblasts as a control. We then carried out chromatin immunoprecipitation analysis with anti-Rb anti-bodies (Fig. 5A). We found that Rb binding to the E2F-regulated *cdc6* promoter increased in myotubes compared with proliferating C2C12 cells (*top graph*). Strikingly, however, binding of Rb to the *cdc6* promoter was higher, if anything, in quiescent cells than in myotubes (Fig. 5A, *bottom graph*). Similar results were obtained for the *dhfr* and *p107* promoters (Fig. 5B). Therefore, these data indicate that the status of an E2F-regulated promoter with respect to permanent or non-permanent silencing does not correlate with the binding of Rb protein. Consequently, binding of Rb to E2F-regulated promoters is not the sole determinant of their permanent repression in myotubes.

Discussion

In this article, we analyze the involvement of the retinoblastoma protein in the regulation of some E2F-regulated promoters during muscle terminal differentiation. We first set up a reliable method to detect Rb binding to E2F-regulated promoters by chromatin immunoprecipitation. Indeed, although it was generally proposed that E2F-regulated genes were an important target of the retinoblastoma protein, previous studies detected only very low or even no binding of Rb to E2F-regulated promoters, whereas binding of the two other pocket proteins was easy to detect (18, 21-23). Because of these limitations, binding of Rb to E2F-regulated promoters was, to our knowledge, never assessed by quantitative PCR, although this technique is an important improvement because it allows quantification of background levels of target sequences (which is crucial when the chromatin immunoprecipitation signals are weak). Here, we show that the addition of a protein/protein cross-linking step before the classic formaldehyde cross-link allowed us to reliably and quantitatively measure binding of Rb to at least three E2F-targeted promoters. This method should prove very useful for fundamental as well as cancer-related

studies, considering the importance of the Rb pathway in cancer.

We use our method to investigate Rb binding to some E2F-regulated promoters during the course of muscle cell terminal differentiation. Indeed, terminally differentiated muscle cells stop proliferating and cannot be induced to enter the cell cycle again. Interestingly, Rb expression is required for this stable cell cycle exit, and Rb cannot be compensated by other members of the pocket proteins family (5). Moreover, in terminally differentiated cells, E2F-responsive genes are stably repressed and have been proposed to harbor specific heterochromatic marks (15).

In vitro cultured C2C12 cells represent a nice model to study muscle terminal differentiation because they can be induced to differentiate *in vitro* on shifting to low serum. Moreover, it is possible to compare directly differentiated cells that have irreversibly exited the cell cycle and undifferentiated quiescent cells that have stopped proliferating but can still be induced to

enter the cell cycle. E2F-responsive genes are regulated accordingly, that is, they are stably repressed in terminally differentiated cells, but can be reactivated in quiescent cells. Importantly, because these cells are harvested from the same dish, one can rule out any indirect effect originating from the culture conditions. We found that Rb binding is not the determinant for this differential regulation, at least for the three E2F-target genes we analyzed (*cdc6, dhfr,* and *p107*), because Rb binding in quiescent cells is comparable with that in terminally differentiated cells. Of course, it would be better to analyze more E2F-targeted genes than the three we analyzed. However, E2F-targeted promoters are very G/C rich and are therefore very difficult to analyze by real-time PCR. Along this line, we never succeeded to analyze the *cyclin E* promoter, which would have been very interesting because it has previously been shown to be targeted by Rb in quiescent cells (24). Nevertheless, because we found very similar results for the three E2F-regulated genes we analyzed, with respect to their
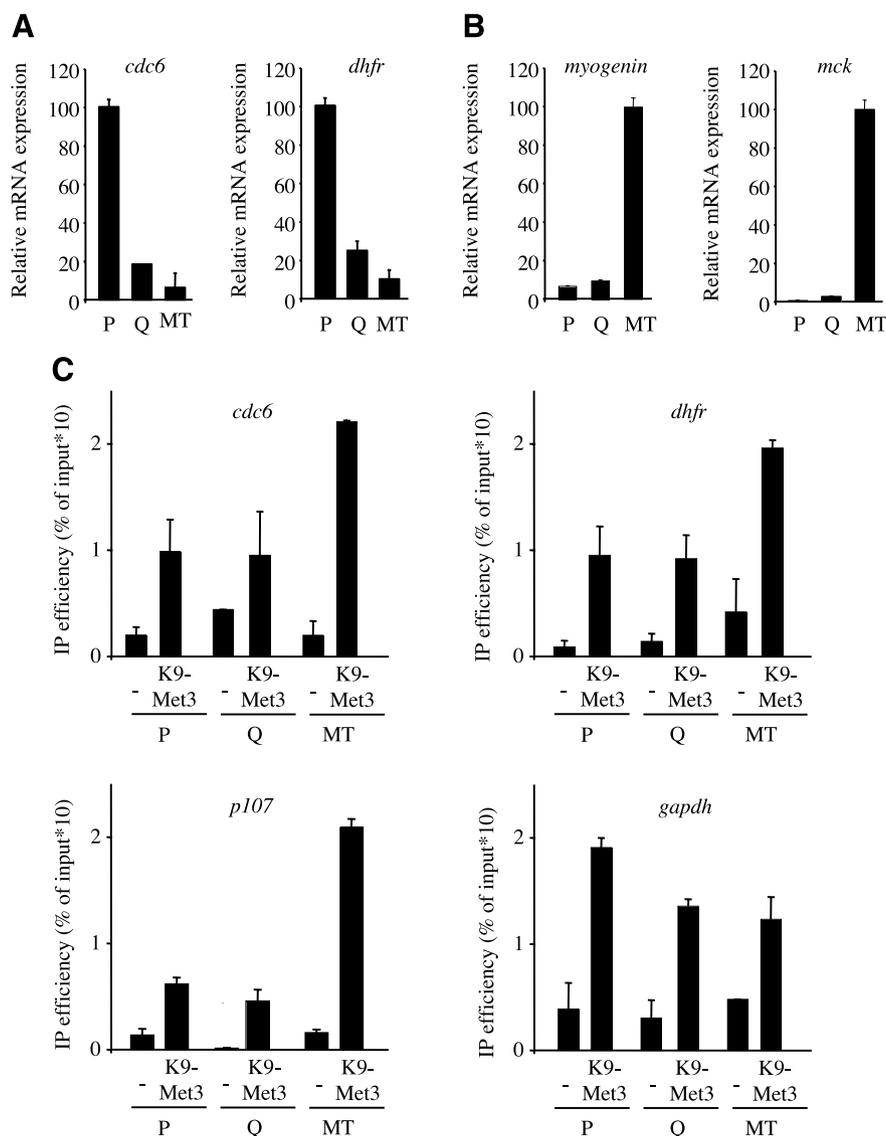

FIGURE 4. Validation of quiescent and differentiated C2C12 cells. **A.** C2C12 cells were either cultured in proliferation conditions (*P*) or in differentiation conditions for 3 to 4 d. This latter culture was then treated with trypsin to collect either fully differentiated cells (myotubes, *MT*) or quiescent nondifferentiated cells (*Q*), as described in Materials and Methods. Total mRNA was then extracted and reverse transcribed, and the expression of *cdc6, dhfr,* and *gapdh* mRNA was measured by quantitative PCR. The amounts of *cdc6* and *dhfr* mRNA were divided by the amount of *gapdh* mRNA and calculated relative to 100 in proliferating cells. **B.** Same as in **A,** except that *myogenin* and *mck* (muscle creatine kinase) mRNA amounts were divided by the amount of *gapdh* mRNA and calculated relative to 100 in myotubes. **C.** Chromatin from formaldehyde−cross-linked proliferating, quiescent, or terminally differentiated C2C12 cells was prepared and subjected to a chromatin immunoprecipitation analysis with antibodies recognizing trimethylated H3K9 (*K9-Met3*) or no antibody as a control (−). The amounts of *cdc6* promoter, *dhfr* promoter, *p107* promoter (three E2F-regulated genes), or control *gapdh* promoter in the inputs and in the immunoprecipitates were then measured by quantitative PCR.

FIGURE 5. Rb binds to E2F-regulated promoters in quiescent and differentiated C2C12 cells. **A.** Proliferating (*P*), quiescent (*Q*), and differentiated (*MT*) C2C12 cells were cross-linked with DMA and subsequently with formaldehyde. Sonicated chromatin was then subjected to a chromatin immunoprecipitation assay with anti-Rb antibodies or no antibody as a control (−). The amounts of *cdc6* promoter or control *gapdh* promoter in the inputs and immunoprecipitates were measured by quantitative PCR. Immunoprecipitation efficiency was calculated relative to 100 for the amount of *cdc6* promoter immunoprecipitated by the anti-Rb antibodies from myotubes chromatin. Note that to reduce the number of samples, quiescent or proliferating cells were analyzed in separate experiments (and are thus shown in separate graphs). Columns, mean from two independent experiments; bars, SD. **B.** Same as in **A,** except that only quiescent and differentiated C2C12 cells were used and that *p107* and *dhfr* promoters were analyzed. Immunoprecipitation efficiency was calculated relative to 100 for the amount of promoter immunoprecipitated by the anti-Rb antibodies from myotubes chromatin.
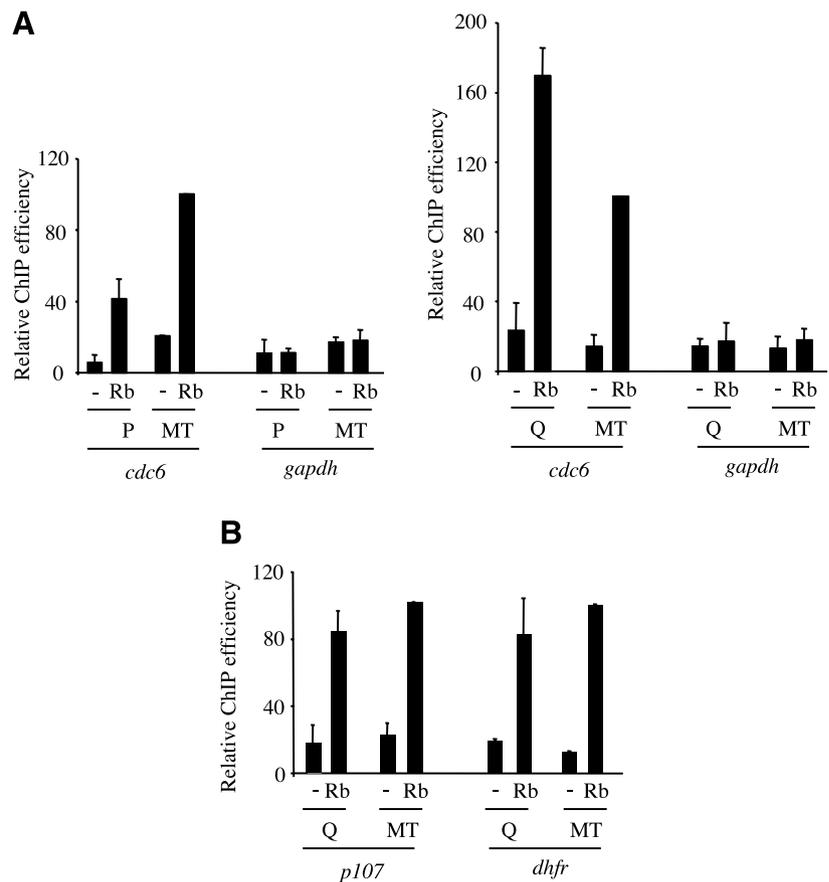
transcriptional regulation, to the presence of heterochromatic marks on their promoters, and to Rb binding to their promoters, we believe that our findings are likely to be true for most, if not all, E2F-regulated promoters. It remains possible, however, that a subset of E2F-regulated promoters are bound by Rb in myotubes but not in myoblasts. Large-scale studies such as chromatin immunoprecipitation on chips could help to resolve this point.

Our results thus stand in contrast to those of Narita et al. (19), who found that Rb binding to E2F-regulated promoters strictly correlated with irreversible growth arrest in ras-induced senescent IMR90 cells and was not observed in the corresponding quiescent cells. This discrepancy could be due to an intrinsic difference between cell types. Note, however, that in this study, quiescent cells and senescent cells were obtained through very different technical procedures, and one cannot rule out the possibility that Rb binding during senescence is only an indirect consequence of the experimental settings leading to senescence (such as c-ras expression). Our results show unambiguously that in differentiating muscle cells, there is no correlation between Rb binding to the E2F-regulated promoters we analyzed and their stable repression.

Our results stand in apparent discrepancy with the observation that Rb is required for the stable repression of E2F-responsive genes during muscle differentiation. However,

it is possible that although Rb binds to E2F-regulated promoters both in quiescent and in differentiating cells, it can only induce stable repression in differentiating cells; the determinant of stable repression would not be Rb binding but rather the identity of cofactors recruited to Rb to E2F-regulated promoters. What could be these cofactors? It has previously been proposed that stable repression of E2F-regulated promoters during muscle differentiation is due to the establishment of a "heterochromatic-like" structure on these promoters (25). Indeed, by comparing quiescent fibroblasts with differentiated C2C12 cells, Ait-Si-Ali et al. (15) observed that silencing of E2F-responsive genes correlated with the presence of a heterochromatic mark (H3K9 methylation). Moreover, it required the expression of Suv39H1, an enzyme responsible for H3K9 trimethylation in heterochromatin. Interestingly, we confirmed these results using quiescent C2C12 cells; we found that quiescent C2C12 cells and differentiated C2C12 cells harbored distinct levels of trimethylated H3K9 on E2F-regulated promoters. It is thus tempting to speculate that the determinant of stable repression is the Rb-mediated recruitment of Suv39H1 to E2F-regulated promoters. It would thus be very informative to investigate the regulation of Rb/Suv39H1 interaction and how it relates to muscle differentiation. It has to be noted, however, that the other pocket proteins can also interact with Suv39H1 (13), and it would thus be unclear why they would not be able to compensate for the absence of Rb.

Noteworthy, methylated H3K9 represents a docking site for HP1 proteins (HP1α, HP1β, and HP1γ) that play essential roles in establishing and maintaining heterochromatin-mediated gene silencing. Whether HP1 binds to E2F target genes only in muscle differentiated cells is not known. However, HP1 can directly bind not only to Rb (14) but also to p130 (26). As such, similarly to Suv39H1, it would be unclear why p130 could not compensate for the absence of Rb in stably repressing E2F-dependent genes in myotubes. Nevertheless, it was recently shown that stable repression of E2F-dependent genes in terminally differentiated neurons relies on the identity of HP1 isoforms recruited on their promoters. Indeed, Panteleeva et al. (26) clearly evidenced a molecular switch from HP1γ to HP1α isoform during terminal differentiation of cerebellar granule neurons, both *ex vivo* and *in vivo*. They further showed that the binding of HP1α at E2Fs sites only in terminally differentiated neurons correlated with a higher compaction of chromatin surrounding these sites. Because the authors compared early differentiating neurons with terminally differentiated neurons, it remains to be determined if differential binding of HP1 isoforms may dictate reversible versus permanent silencing of E2F-dependent genes that characterize quiescent and terminally differentiated cells, respectively. Although our results show that Rb binding is not the determinant of whether E2F target gene promoters are permanently silenced or reversibly repressed, it is firmly established that Rb is required for this permanent silencing in myotubes (8, 9). This apparent paradox may be explained, in part, by a recent report showing that the cdk inhibitor p21, which is a positive upstream regulator of Rb, is essential to sustain the nonproliferative state of myotubes. Indeed, it was shown that derepression of cyclin D-cdk4/cdk6 complexes (through inhibition of p21 expression) was sufficient to induce DNA synthesis in C2C12 differentiated cells (27). Even if the link with Rb has not been investigated, cdk4-cyclin D3 complex present in myotubes may phosphorylate Rb and dissociate it from E2F target genes, resulting in the activation of E2F-dependent genes. These results suggest that a determinant of the permanent block may function upstream of Rb binding to maintain p21 expression.

Our results thus underline the importance of characterizing the proteins interacting with Rb and the determinants of their interaction. Strikingly, Rb is extensively modified by posttranslational modifications, including phosphorylation, acetylation, sumoylation, and ubiquitinylation (28-31). These dynamic modifications are ideal candidates for regulating the identity of Rb cofactors and subsequently the chromatic marks induced on E2F-regulated promoters by the presence of Rb in various conditions.

Materials and Methods

Cell Culture

C2C12 cells were cultured in DMEM/Ham's F-12 medium supplemented with antibiotics and 15% FCS (Invitrogen). Cells were grown at 37°C under a humidified atmosphere with 5% CO_2.

Differentiation was induced by shifting cells to a low-serum medium (DMEM supplemented with 2% horse serum). After 4 d in differentiation medium, myotubes and quiescent cells were separated by mild trypsinization as previously described (17).

Antibodies

The antibodies used were mouse monoclonal anti-myogenin (F5D, hybridomas), rabbit anti-p107 (C-18, Santa Cruz), rabbit anti-p130 (C-20, Santa Cruz), mouse monoclonal anti-Rb (XZ55 and G3-245, PharMingen), and mouse monoclonal anti-tubulin (DM1A, Sigma) antibodies. Western blots were done following standard procedures.

RNA Extraction and Reverse Transcription

Total RNA was extracted using the RNeasy mini kit as described by the supplier (Qiagen). One microgram of each purified RNA preparation was reversed transcribed for 50 min at 42°C in a 20-μL reaction containing 0.5 μg of random primers (Promega), 1× avian myeloblastosis virus reverse transcriptase buffer, 10 units of avian myeloblastosis virus reverse transcriptase (Promega), 40 units of rRNasin (Promega), 10 mmol/L DTT, and 0.5 mmol/L deoxynucleotides (Promega). The reaction was stopped by incubating the samples for 15 min at 70°C, and cDNAs were analyzed by real-time quantitative PCR.

Chromatin Immunoprecipitation

For Rb immunoprecipitation, cross-linking of cells was carried out essentially as described (32). Briefly, cells were collected by trypsinization, washed twice in PBS, and resuspended in 10 mmol/L DMA (Pierce), 0.25% DMSO for 45 min under agitation at room temperature. Cells were then washed once with PBS and incubated with 1% formaldehyde for 15 min. For p130 chromatin immunoprecipitation, formaldehyde was added to the culture medium to a final concentration of 1% and cross-linking was allowed to proceed for 15 min at room temperature. In both cases, cross-linking was stopped by adding 0.125 mol/L glycine for 5 min. The preparation of chromatin and the immunoprecipitation steps were done as previously described (33). Nuclei extracts were sonicated to obtain DNA fragments of about 500 to 1,000 pb. Immunoprecipitation was carried out with 7.5 μg of each anti-Rb antibody (XZ55 and G3-245, PharMingen) or 4 μg of anti-p130 antibody (C-20, Santa Cruz) or without antibody. Following cross-link reversion, the immunoprecipitated DNA and input DNA were purified on a GFX column (GE Healthcare Biosciences) and analyzed by real-time quantitative PCR.

Quantitative PCR

Real-time quantitative PCR was carried out according to the manufacturer's instructions using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) on an ICyclerQ (Bio-Rad) real-time PCR device. Samples were analyzed in triplicate, and the mean and SD were calculated.

For cDNA quantification, the following primers were used: *gapdh* forward, 5′-AGGTTGTCTCCTGCGACTTC-3′; *gapdh* reverse, 5′-ATGTAGGCCATGAGGTCCAC-3′; *cdc6* forward, 5′ CAGTGCTGCGATCCAGTTCT 3′; *cdc6* reverse, 5′-CCTGACATCCGACTCCACAA-3′; *dhfr* forward, 5′-GGTAGGAAAACCTGGTTCTC-3′; *dhfr* reverse, 5′-CAGAACTGCCTCCGACTATC-3′; *myogenin* forward,

5′-CTACAGGCCTTGCTCAGCTC-3′; *myogenin* reverse 5′-AGATTGTGGGCCTCTGTAGG-3′; *mck* forward, 5′-GATTCTCACTCGCCTTCGTC-3′; *mck* reverse, 5′-GCCCTTTTCCAGCTTCTTCT-3′.

For chromatin immunoprecipitation experiments, the following primers that amplify gene promoter (P) were used: *dhfr*-P forward, 5′-GCCTAAGCTGCGCAAGTGGT-3′; *dhfr*-P reverse, 5′-GTCTCCGTTCTTGCCAATCC-3′; *cdc6*-P forward, 5′-TGATGAGTGACAACTAATCAG-3′; *cdc6*-P reverse, 5′-GAGCTTTGCACTCTTCAGG-3′; *gapdh*-P forward, 5′-CTCTGAGCCTCCTCCAATTC-3′; *gapdh*-P reverse, 5′-GCTACGCCATAGGTCAGGAT-3′; *p107*-P forward, 5′-TCTCTGACGTGGCGCTTCTA-3′; *p107*-P reverse, 5′-GAAGCCTTCTCCATGACACT-3′; *cdc6*-3′ forward, CCGATGTTCGTCATCACAGA; *cdc6*-3′ reverse, CCTGGAAGTGCCATGAGTAA.

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