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P. Rochard, A. Rodier, François Casas, Isabelle Cassar-Malek, S. Marchal-Victorion, et al.. Mitochondrial activity is involved in the regulation of myoblast differentiation through myogenin expression and activity of myogenic factors. *Journal of Biological Chemistry*, 2000, 275 (4), pp.2733-2744. hal-02694643

**HAL Id: hal-02694643**

**<https://hal.inrae.fr/hal-02694643>**

Submitted on 1 Jun 2020

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# Mitochondrial Activity Is Involved in the Regulation of Myoblast Differentiation through Myogenin Expression and Activity of Myogenic Factors\*

(Received for publication, February 17, 1999, and in revised form, October 15, 1999)

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To characterize the regulatory pathways involved in the inhibition of cell differentiation induced by the impairment of mitochondrial activity, we investigated the relationships occurring between organelle activity and myogenesis using an avian myoblast cell line (QM7). The inhibition of mitochondrial translation by chloramphenicol led to a potent block of myoblast differentiation. Carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone and oligomycin, which affect the organelle at different levels, exerted a similar influence. In addition, we provided evidence that this phenomenon was not the result of an alteration in cell viability. Conversely, overexpression of the mitochondrial T3 receptor (p43) stimulated organelle activity and strongly potentiated myoblast differentiation. The involvement of mitochondrial activity in an actual regulation of myogenesis is further supported by results demonstrating that the muscle regulatory gene *myogenin*, in contrast to *CMD1* (chicken *MyoD*) and *myf5*, is a specific transcriptional target of mitochondrial activity. Whereas *myogenin* mRNA and protein levels were down-regulated by chloramphenicol treatment, they were up-regulated by p43 overexpression, in a positive relationship with the expression level of the transgene. We also found that *myogenin* or *CMD1* overexpression in chloramphenicol-treated myoblasts did not restore differentiation, thus indicating that an alteration in mitochondrial activity interferes with the ability of myogenic factors to induce terminal differentiation.

Recent studies emphasize that mitochondria, in addition to their well known involvement in the regulation of energy metabolism, are implicated in the regulation of cell growth and differentiation. In particular, mitochondrial events are involved in the preliminary steps of apoptosis (1), and inhibition of mitochondrial activity, either by deleting mtDNA (*rho*<sup>o</sup> cells)

or by blocking translation in the organelle, has been shown to stop or decrease the proliferation of different cell lines (2–4). Furthermore, the general activity of the organelle, not restricted to energy production, is implicated in such regulation (5, 6). In addition, mitochondrial protein synthesis inhibition is associated with the impairment of differentiation of different cell lines, such as mouse erythroleukemia (7) and mastocytoma cells (8), neurons (9), and human (10), avian (11) or murine myoblasts (12). In agreement with these data, several pathologies are associated with mitochondrial disorders, even if the links between mitochondrial genome rearrangements or activity and pathological symptoms are not always clearly established. Despite these reports, little is known about the molecular mechanisms involved in these regulations. First, the exclusive use of inhibitors of mitochondrial function in previous reports was not fully adapted to demonstrating the occurrence of an actual regulatory pathway involving mitochondrial activity in the regulation of cell differentiation. Second, the nature of the molecular signals underlying the reciprocal cross-talk between mitochondria and the nucleus remains poorly known, even if cytosolic calcium levels have been shown to take part in this mitochondria-to-nucleus retrograde signaling (13, 14).

Skeletal muscle constitutes a valuable model for studying mechanisms involved in the control of cell growth and differentiation. Myoblasts cultured *in vitro* undergo a myogenic development program, including active proliferation, withdrawal from the cell cycle, synthesis of muscle-specific proteins, and fusion into multinucleated myotubes. Experiments with cultured myoblast lines have led to the identification of multiple genetic and environmental factors that influence the establishment and proper differentiation of the myogenic lineage. In particular, the myogenic regulatory factors (MRFs)<sup>1</sup> (*Myf5*, *MyoD*, *Myogenin*, and *MRF4*) form a family of basic helix-loop-helix transcription factors playing key regulatory roles in this process (for review, see Refs. 15 and 16).

In an earlier work, we demonstrated that important changes in mitochondrial activity occurred during avian myoblast differentiation (17). In particular, high mitochondrial activity appears to be associated with the preliminary steps of myogenic differentiation, and its rise just before the onset of terminal differentiation could characterize the irreversible engagement of myoblasts in terminal differentiation.

These data led us to characterize the influence of experimental changes in mitochondrial activity upon *in vitro* myogenic differentiation. Chloramphenicol, which inhibits mitochondrial translation, and therefore the proper assembly of 4 out of 5

\* This work was supported by Institut National de la Recherche Agronomique and by grants from the Association Française contre les Myopathies and the Association de Recherche contre le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by the Ministère de la Recherche et de la Technologie and the Fondation pour la Recherche Médicale.

§ Supported by the Ligue Nationale contre le Cancer.

¶ Supported by the Institut National de la Recherche Agronomique and Direction Générale de l'Enseignement et de la Recherche.

|| Supported by the Association Française contre les Myopathies.

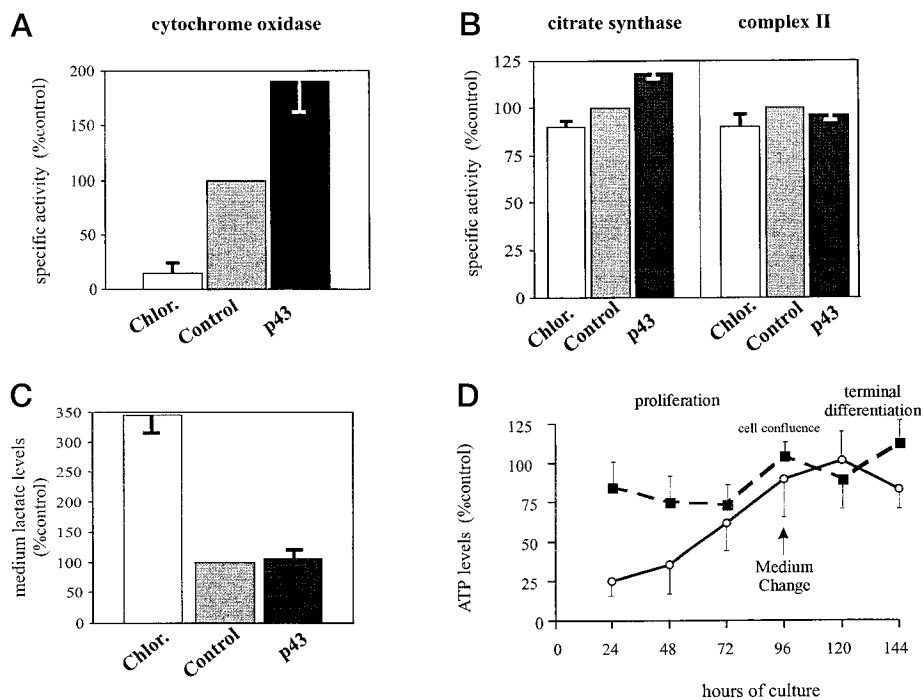
\*\* Supported by the Fondation pour la Recherche Médicale.

‡‡ Supported by the Ligue Régionale contre le Cancer.

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<sup>1</sup> The abbreviations used are: MRF, myogenic regulatory factor; AchR $\alpha$ , acetylcholine receptor  $\alpha$  subunit; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone.

**FIG. 1. Influence of chloramphenicol or p43 overexpression on mitochondrial activity.** QM7 myoblasts treated with chloramphenicol (100  $\mu\text{g/ml}$ ) (□) or overexpressing the mitochondrial T3 receptor (■) were grown for 72 h before being harvested. Cytochrome oxidase-specific activities (A) and citrate synthase- and complex II-specific activities (B) were determined as described under "Experimental Procedures." Results are expressed relative to the control values obtained in normal QM7 and in myoblasts transfected with the pIRV empty vector, respectively. Data are the mean  $\pm$  S.E. of six separate experiments. For lactate production measurements (C), the medium was changed at 72 h of culture, and an aliquot was taken 24 h later. As for ATP level determination (D), results were normalized relative to the protein content of the dish and expressed relative to the corresponding control (○, chloramphenicol-treated myoblasts; ■, p43-overexpressing myoblasts). Data are the mean  $\pm$  S.E. of four separate experiments.



respiratory chain complexes, was used to inhibit the activity of the organelle. Conversely, mitochondrial activity was stimulated by overexpression of the mitochondrial T3 receptor (p43) (18). This overexpression stimulates mitochondrial genome transcription (19) and consequently the activity of the enzymes partly encoded by the mitochondrial genome (respiratory chain).

In this study, we took advantage of these two effectors leading to reciprocal changes in mitochondrial activity to define more accurately the relationships occurring between the organelle and myoblast differentiation, in the search for molecular mechanisms involved in the cross-talk between mitochondria and the nucleus.

#### EXPERIMENTAL PROCEDURES

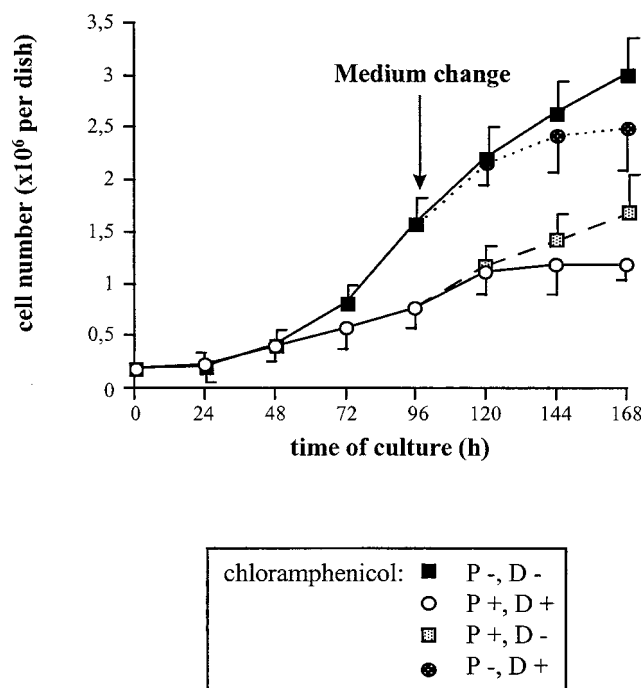
**Cell Cultures**—Quail myoblasts of the QM7 cell line (20) were seeded at a plating density of 7000 cells/cm<sup>2</sup> in 60- or 100-mm coated dishes. They were grown in Earle 199 medium, supplemented with L-glutamin (4 mM), tryptose phosphate broth (0.2%), gentamycin (100 IU/ml), glucose (final concentration, 4500  $\mu\text{g/liter}$ ), and fetal calf serum (10%). Terminal differentiation was induced after 96 h of culture at cell confluence by lowering the medium serum concentration (0.5%).

Chloramphenicol (100  $\mu\text{g/ml}$ ) was added when indicated to inhibit mitochondrial protein synthesis. FCCP (2.5 mM) or oligomycin (1  $\mu\text{g/ml}$ ) was added when indicated to inhibit mitochondrial membrane potential or complex V of respiratory chain activity.

Cell viability was assessed during proliferation and differentiation by observation of trypan blue exclusion after incubation of the cells with 0.04% of dye (Sigma) for 45 min.

**Stable and Transient Transfection**—QM7 myoblasts constitutively expressing p43 were obtained by stable transfection of pIRV  $\Delta$ 1 expression vector, constructed by inserting the p43 coding sequence (21) into the *EcoRI* site of the pIRV vector. Control myoblasts were obtained by simultaneous stable transfection of pIRV "empty" vector. Transient overexpression of CMD1 and myogenin was obtained by transfection of pRSV-CMD1 and pRSV-MgN expression vectors. Control myoblasts were transfected with pRSV-(A)<sub>n</sub> empty vector. 10  $\mu\text{g}$  (stable transfection) of each plasmid also carrying G418 resistance or 2  $\mu\text{g}$  (transient transfection) was transfected using the calcium phosphate procedure 24 h after plating. The medium was changed 24 h later after three phosphate-buffered saline washes, and amplification was performed after about 10 days of G418 selection for stable transfection.

**Measurement of Metabolic Parameters**—Cells plated in coated dishes (7000 cells/cm<sup>2</sup>) were harvested in 1.5 ml of TNE (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, pH 8.0), and then centrifuged for 5 min at

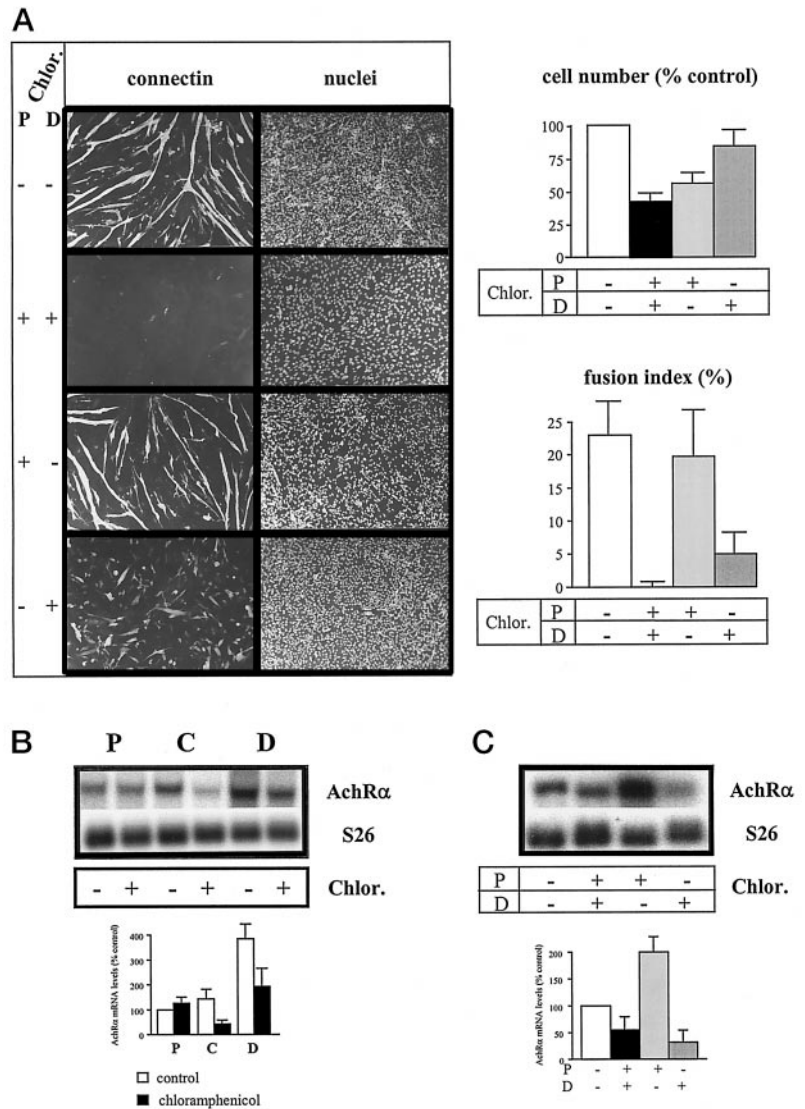


**FIG. 2. Influence of chloramphenicol treatment upon myoblast proliferation.** Nuclei were counted at the indicated time. QM7 myoblasts were cultured in a medium containing chloramphenicol during proliferation (P) and/or terminal differentiation (D) as indicated. Data are the mean  $\pm$  S.E. of three separate experiments.

12,000  $\times g$ . For enzymatic activity determination, the pellet was resuspended in 50–150  $\mu\text{l}$  of lysis buffer (10 mM Tris, pH 7.8) and lysed by three cycles of freezing/thawing. Total proteins were measured on an aliquot using the Bio-Rad protein assay kit. Citrate synthase activity was measured according to Bergmeyer *et al.* (22). Cytochrome oxidase was measured as described by Warthon and Tzagaloff (23). Enzymatic activities were measured as specific activities, and expressed relative to control levels, recorded in "normal" and pIRV-transfected myoblasts for chloramphenicol-treated and p43-overexpressing myoblasts, respectively. ATP levels were measured using the ATP HS II bioluminescence



**FIG. 3. Influence of inhibition of mitochondrial activity by chloramphenicol on QM7 myoblast differentiation.** *A*, immunofluorescence staining 72 h after the medium change inducing terminal differentiation with connectin antibody of control and chloramphenicol-treated myoblasts. QM7 myoblasts were cultured in a medium containing chloramphenicol (*Chlor.*) during proliferation (*P*) and/or terminal differentiation (*D*) as indicated. Fusion index values are the mean  $\pm$  S.E. of three separate experiments. Nuclei were stained with Hoechst 33258. Microphotographs of a typical experiment are shown ( $\times 100$ ). *B*, AchR $\alpha$  mRNA levels. 15  $\mu$ g of total RNAs isolated from control or chloramphenicol-treated (*Chlor.*) myoblasts at the indicated time (proliferation (*P*), cell confluence (*C*), and terminal differentiation (*D*)) was analyzed by Northern blot for AchR $\alpha$  mRNA. *C*, chloramphenicol removal led to restoration of AchR $\alpha$  mRNA levels. Conversely, when the drug was added at the onset of terminal differentiation, these levels were strongly down-regulated. QM7 myoblasts were cultured in a medium containing chloramphenicol (*Chlor.*) during proliferation (*P*) and/or terminal differentiation (*D*) as indicated. Quantitation was performed using a STORM phosphorimager (Molecular Dynamics) and normalized in relation to S26 levels. Results are expressed as percentages of the control value recorded in proliferating myoblasts. Data are the mean  $\pm$  S.E. of five separate experiments. Typical Northern blots are shown.



assay kit (Roche Molecular Biochemicals). Lactate levels were measured on an aliquot of culture medium using the L-Lactic acid determination kit (Roche Molecular Biochemicals). These two parameters were normalized in relation to the amount of total cellular proteins; they were expressed relative to their respective control at the same stage of the culture for chloramphenicol-treated or p43-overexpressing myoblasts.

**Cell Counting**—At appropriate times, myoblasts were fixed using 100% methanol and stained with Giemsa.

**Cytoimmunofluorescence Studies**—Myoblast differentiation was assessed by morphological changes and accumulation of connectin, a muscle-specific marker. After methanol fixation and appropriate washings, cells were stained with an antibody raised against connectin and a fluorescein-conjugated antibody raised against mouse immunoglobulins. Nuclei were stained with Hoechst 33258 (1  $\mu$ g/ml).

The fusion index (percentage of nuclei incorporated into myotubes relative to the total number) was used to quantify terminal differentiation.

**RNA Level Analysis**—RNA levels were monitored by Northern blots. Total RNAs were isolated after 48 (proliferation), 96 (cell confluence) and 168 (terminal differentiation) h of culture, as described by Chomczynski and Sacchi (24), and 15  $\mu$ g was loaded in each lane. Membranes were hybridized with cDNA probes labeled using the Megaprime DNA labeling system (Amersham Pharmacia Biotech). Quantitation was performed using a STORM phosphorimager (Molecular Dynamics) and normalized in relation to S26 levels.

**Determination of Myogenin mRNA Half-life**—QM7 myoblasts were pretreated with chloramphenicol at the medium change inducing terminal differentiation. 36 h later, actinomycin D (5  $\mu$ g/ml) was added to the medium. Total RNAs were isolated at the indicated times.

**Protein Level Analysis**—Protein levels were monitored by Western blot performed on nuclear extracts. Nuclei were collected as described by Schreiber *et al.* (25); 50  $\mu$ g of nuclear proteins were run on 10% SDS-PAGE mini-gels and transferred onto nitrocellulose membrane. Membranes were probed with anti-MyoD (C20, Santa Cruz Biotechnology, diluted at 1:200) or anti-myogenin (M-225, Santa Cruz Biotechnology, diluted at 1:100) antibodies. These antibodies were further detected by ECF (Amersham Pharmacia Biotech) and quantitation was performed using a STORM phosphorimager (Molecular Dynamics).

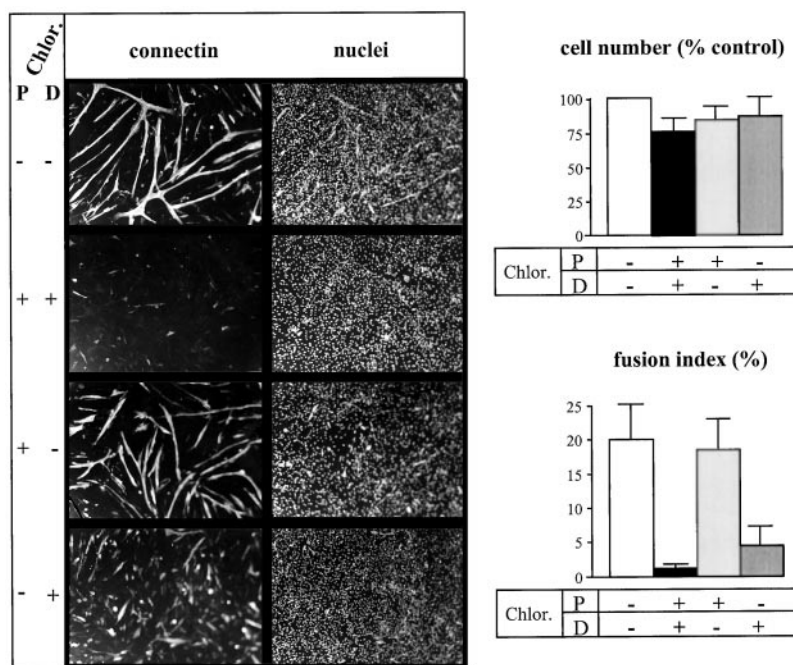
**Statistical Analysis**—Statistical analysis was performed using the paired T test (26).

## RESULTS

**Influence of Chloramphenicol or p43 Overexpression on Mitochondrial Activity**—To validate our experimental approach, we first characterized the influence of chloramphenicol treatment or p43 overexpression on mitochondrial activity. As expected, myoblasts cultured in the presence of chloramphenicol showed an impressive decrease in cytochrome oxidase activity, an enzyme partly encoded by the mitochondrial genome ( $p < 0.001$ ; Fig. 1A). On the other hand, mitochondrial T3 receptor (p43) overexpression led to a significant stimulation of cytochrome oxidase activity ( $p < 0.001$ ; Fig. 1A). Furthermore, neither chloramphenicol nor p43 overexpression affected the activity of citrate synthase or of the complex II of the respiratory chain, encoded only by nuclear genes (Fig. 1B).

We then addressed the question of whether QM7 myoblasts

**FIG. 4. Chloramphenicol-induced block in the differentiation program is not the result of a decrease in cell numbers in treated dishes.** To reach a similar number of cells at confluence, QM7 myoblasts exposed to chloramphenicol (*Chlor.*) during proliferation (*P*) (*P*<sup>+</sup>, *D*<sup>+</sup> and *P*<sup>-</sup>, *D*<sup>-</sup>) were seeded at twice the plating density of the control or *P*<sup>-</sup>, *D*<sup>+</sup> myoblasts. Immunofluorescence staining with connectin antibody, 72 h after the medium change inducing terminal differentiation (*D*). Fusion index values are the mean  $\pm$  S.E. of three separate experiments. Nuclei were stained with Hoechst 33258. Microphotographs of a typical experiment are shown ( $\times 100$ ).



could respond to the inhibition of aerobic metabolism induced by chloramphenicol by activating anaerobic pathways. Lactate accumulation in the culture medium between the third and fourth day of culture, normalized to the amount of total cellular proteins, was found to be strongly increased in chloramphenicol-treated cells relative to control myoblasts. However, it remained unaffected in p43-overexpressing cells (Fig. 1C).

We next studied whether these changes in metabolic activity could induce changes in intracellular ATP levels. Chloramphenicol-treated myoblasts showed a strong reduction in ATP levels at the onset of the culture, but the difference between control and treated myoblasts progressively decreased and ceased to be significant after 72 h of culture and during terminal differentiation (Fig. 1D). No major changes in intracellular ATP levels were induced by p43 overexpression (Fig. 1D).

We also studied the influence of chloramphenicol exposure on QM7 myoblast viability in long term culture. Cell growth was maintained, although at a slower rate, for at least 3 months in the presence of the drug.

Lastly, macro arrays experiments showed that chloramphenicol treatment did not affect general nuclear gene expression (data not shown).

This set of data therefore indicates that chloramphenicol inhibited mitochondrial activity without affecting cell viability. Inhibition of mitochondrial protein synthesis did not induce a permanent alteration in intracellular ATP levels, as they were restored just before and during terminal differentiation. Conversely, as expected (19), the activity of the organelle was stimulated by overexpression of the mitochondrial T3 receptor, without influencing ATP levels.

**Influence of Changes in Mitochondrial Activity on QM7 Myoblast Proliferation and Differentiation**—Previous studies have suggested that mitochondrial activity could be involved in the regulation of muscle differentiation (10–12, 27, 28). In addition, we have shown that important changes in mitochondrial activity occur just before the onset of differentiation, and are not observed in differentiation-deficient myoblasts (17). Furthermore, expression of the *v-erb A* oncogene induces significant changes in this activity (17) before stimulating myoblast differentiation (29). To clarify further the relationships observed between mitochondrial activity and muscle dif-

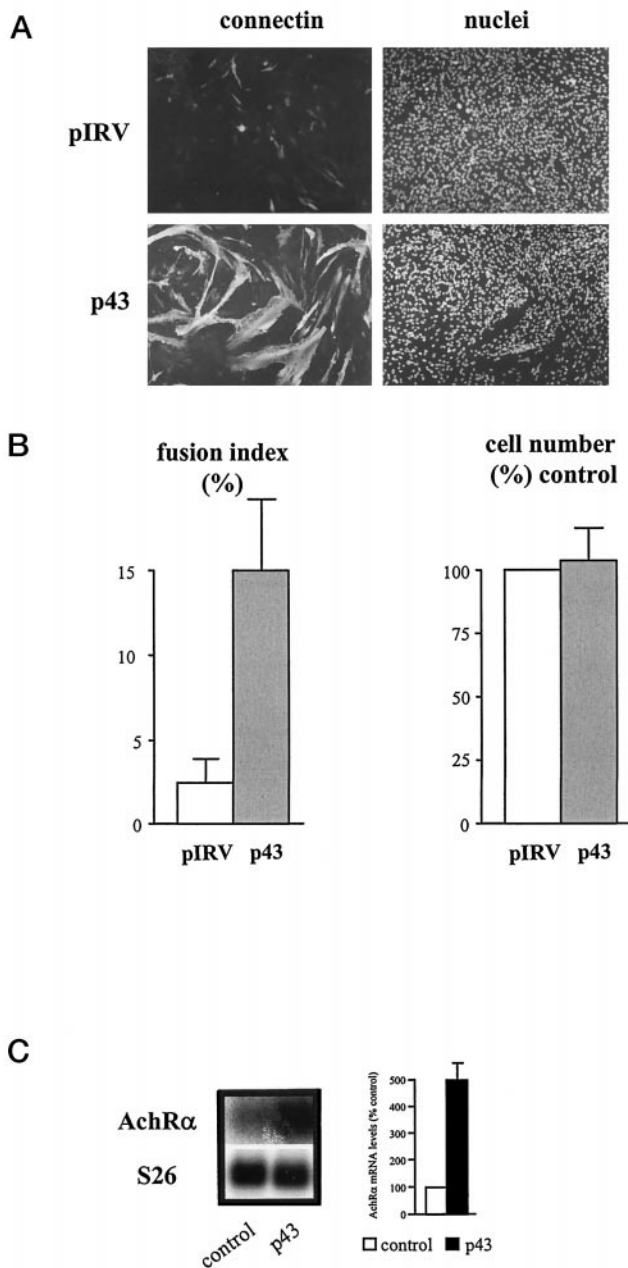
ferentiation, we investigated the influence of chloramphenicol or p43 overexpression on QM7 myoblast proliferation and differentiation.

The drug exerted a marked negative influence on myoblast proliferation. Cell numbers, assessed by counting nuclei in control or chloramphenicol-treated myoblasts, increased exponentially in all cultures until the medium change; proliferation was still maintained, at a lower rate, in control cells after serum removal (Fig. 2). 72 h after plating, chloramphenicol significantly decreased cell proliferation and hence myoblast numbers (47 and 39% of the control value at cell confluence and after 72 h in the differentiating medium, respectively;  $p < 0.001$ ; Fig. 2). This influence was not the result of a decrease in cell viability, as assessed by culture observation, FACS, or trypan blue exclusion experiments, in either proliferative or differentiating medium (data not shown). These observations are in agreement with previous results of King and Attardi (30), who reported that mtDNA-less (*rho*<sup>0</sup>) cells were still able to proliferate, at a slower rate than the wild type parental line, when the medium was complemented with uridine (a component of tryptose phosphate broth used for QM7 culture) and pyruvate (present in Earle 199 medium). Addition of chloramphenicol at cell confluence slightly decreased the proliferation rate observed in the differentiating medium. Conversely, removal of the drug at this stage increased proliferation of myoblasts previously treated with chloramphenicol (Fig. 2).

Myogenic differentiation was assessed in cytoimmunofluorescence experiments, enabling the study of morphological (myotube formation) and biochemical (synthesis of connectin, an early marker of differentiation) criteria. Quantitative data were obtained by measuring the fusion index. In control cultures, myoblasts began to fuse into multinucleated myotubes 24 h after the induction of terminal differentiation, and they accumulated muscle-specific proteins. Maximal differentiation was observed 72 h after the medium change.

In agreement with previous studies on avian, human, or mouse myoblasts (10–12), our data demonstrated that impairment of mitochondrial protein synthesis throughout the culture inhibited QM7 myoblast differentiation: myotube formation was almost fully abrogated (fusion index, 0.5% versus 23% in control myoblasts;  $p < 0.001$ ), and connectin accumulation was





**FIG. 5. Influence of overexpression of the mitochondrial T3 receptor (p43) on QM7 myoblast differentiation.** *A*, immunofluorescence staining with connectin antibody of 6-day cultures in 10% serum of pIRV or pIRVΔ1 stably transfected myoblasts. Fusion index values are the mean  $\pm$  S.E. of three separate experiments. Nuclei were stained with Hoechst 33258. Microphotographs of a typical experiment are shown ( $\times 100$ ). *B*, AchR $\alpha$  mRNA levels. 15  $\mu$ g of total RNAs isolated from control or p43-overexpressing myoblasts at cell confluence was analyzed by Northern blot for AchR $\alpha$  mRNA. Quantitation was performed using a STORM phosphorimager (Molecular Dynamics) and normalized in relation to S26 levels. Results are expressed as percentages of the control value. Data are the mean  $\pm$  S.E. of five separate experiments. Typical Northern blots are shown.

at the brink of detection (Fig. 3A). Moreover, acetylcholine receptor  $\alpha$  subunit (AchR $\alpha$ ) mRNA levels were shown to be down-regulated, although less drastically, under exposure to chloramphenicol (29 and 50% of control values at cell confluence and during terminal differentiation, respectively;  $p < 0.01$ ; Fig. 3B). Such a difference in the influence of mitochondrial protein synthesis inhibition on two different aspects of terminal differentiation has already been recorded by Korohoda *et al.* (11), who found that the increase in creatine kinase

activity was moderately inhibited when fusion was almost completely blocked under exposure to chloramphenicol. Moreover, such partial disjunction of morphological and biochemical differentiation has been reported elsewhere (31).

As previously demonstrated (11), we also observed that inhibition of differentiation by chloramphenicol appeared fully reversible: myotube formation, connectin accumulation, and AchR $\alpha$  levels were restored upon removal of the drug at the induction of terminal differentiation (fusion index, 20 *versus* 23% in control myoblasts; not significant; Fig. 3, A and C). This reversibility was also observed with myoblasts passaged for 3 months in the presence of the drug (data not shown), demonstrating that exposure to chloramphenicol did not alter the myogenic commitment of QM7 cells. Moreover, addition of chloramphenicol to the culture medium only at the onset of terminal differentiation led to an inhibition of myogenesis similar to that recorded with a treatment performed throughout culture (fusion index, 5%;  $p < 0.01$ ; Fig. 3, A and C). These last results suggest that the chloramphenicol-induced block in the differentiation program is probably not the result of a reduction in cell numbers in treated dishes.

To confirm this hypothesis, we increased plating density of myoblasts exposed to the drug during the proliferation phase. In these conditions, cell numbers did not differ in control or treated cultures at cell confluence, and as previously observed (Fig. 3), chloramphenicol reversibly abrogated QM7 myoblast differentiation (Fig. 4).

All of these data establish that the influence of inhibition of mitochondrial activity upon myogenesis is not the result of a "nonspecific" alteration in cell viability or proliferation but clearly involves a block in the differentiation program.

Conversely, myogenic differentiation of p43-overexpressing myoblasts was stimulated, even in proliferative conditions (10% serum), where these cells, in contrast to (pIRV empty vector stably transfected) control myoblasts, had already undergone fusion and connectin synthesis (fusion index, 15 *versus* 2.6% for control myoblasts;  $p < 0.001$ ; Fig. 5A). In the same way, AchR $\alpha$  mRNA levels were up-regulated at cell confluence in QM7 myoblasts overexpressing p43 (5-fold increase;  $p < 0.01$ ; Fig. 5B).

These data clearly demonstrate that mitochondrial activity is involved in the regulation of myoblast differentiation. As terminal differentiation is the result of important changes in the expression of a set of nuclear genes, our results also suggest that mitochondrial activity is able to influence, directly or indirectly, regulation of myogenesis regulatory genes.

*Influence of Changes in Mitochondrial Activity on Myogenic Regulatory Factor Expression*—In an attempt to determine how mitochondrial activity could affect myogenic differentiation, we performed Northern and Western blot experiments with cDNA probes and antibodies directed against MRFs.

We found that at every stage of culture studied, neither CMD1 (the avian homologue of MyoD) nor myf5 mRNA levels were influenced by chloramphenicol treatment (Fig. 6A). Similarly, they did not differ in control myoblasts and in p43-overexpressing cells, either at cell confluence (Fig. 6B) or at any stage of the culture (data not shown). However, Western blot experiments indicated that CMD1 is posttranscriptionally regulated in QM7 myoblasts. Whereas its mRNA level did not significantly change throughout culture, the CMD1 protein levels rose during terminal differentiation (Fig. 6, A and C). Moreover, chloramphenicol treatment seems to interfere with this regulation, as it decreased the CMD1 protein levels at cell confluence (19% of control value;  $p < 0.001$ ). However, this regulation is not a major explanation of the differentiation block induced by chloramphenicol, as further restoration of the

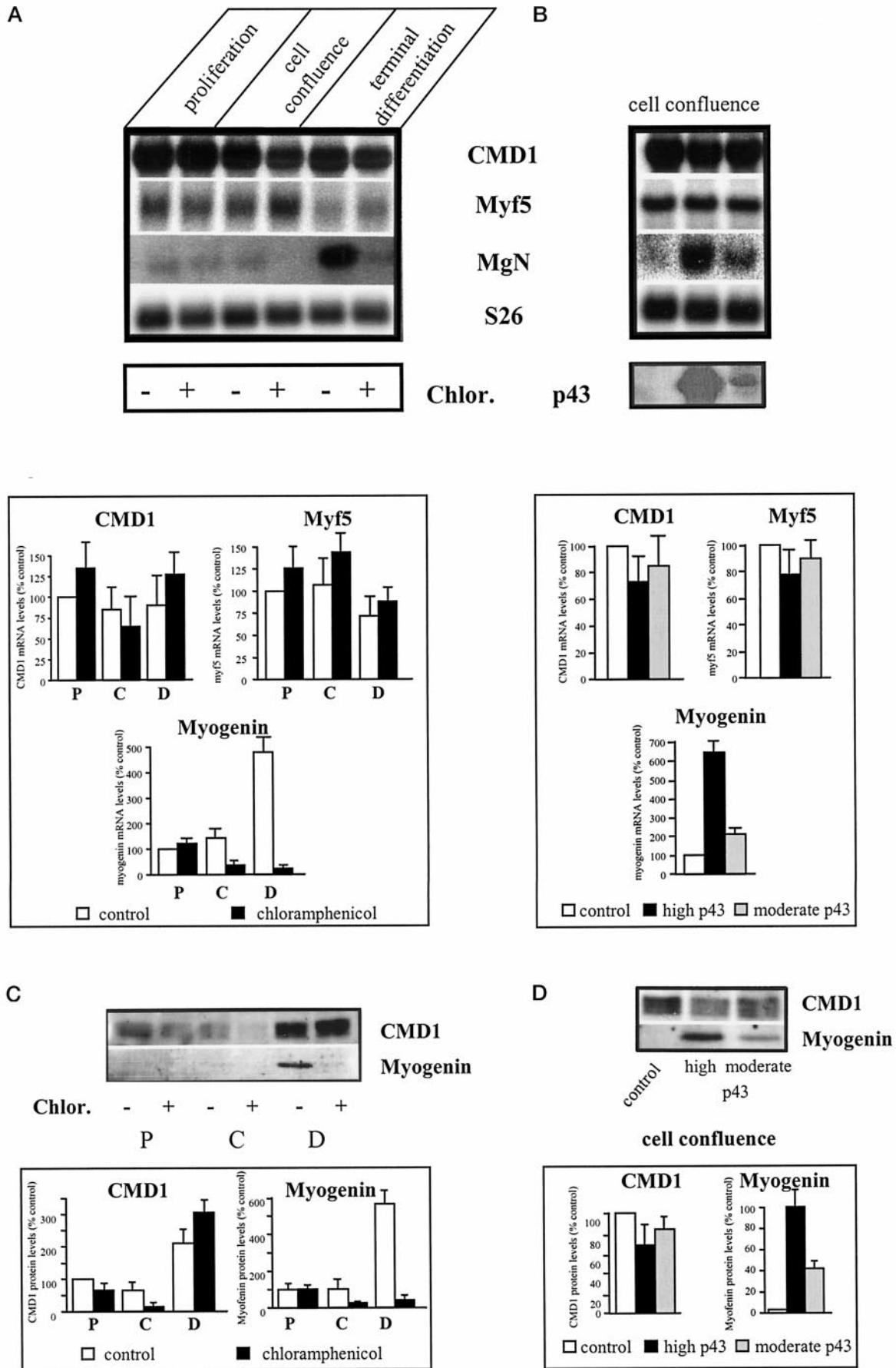


FIG. 6. Influence of experimental changes in mitochondrial activity on myogenic regulatory factor levels during QM7 myoblast differentiation. 15  $\mu$ g of total RNAs or 50  $\mu$ g of nuclear proteins isolated from control or chloramphenicol-treated (*Chlor.*) myoblasts at the

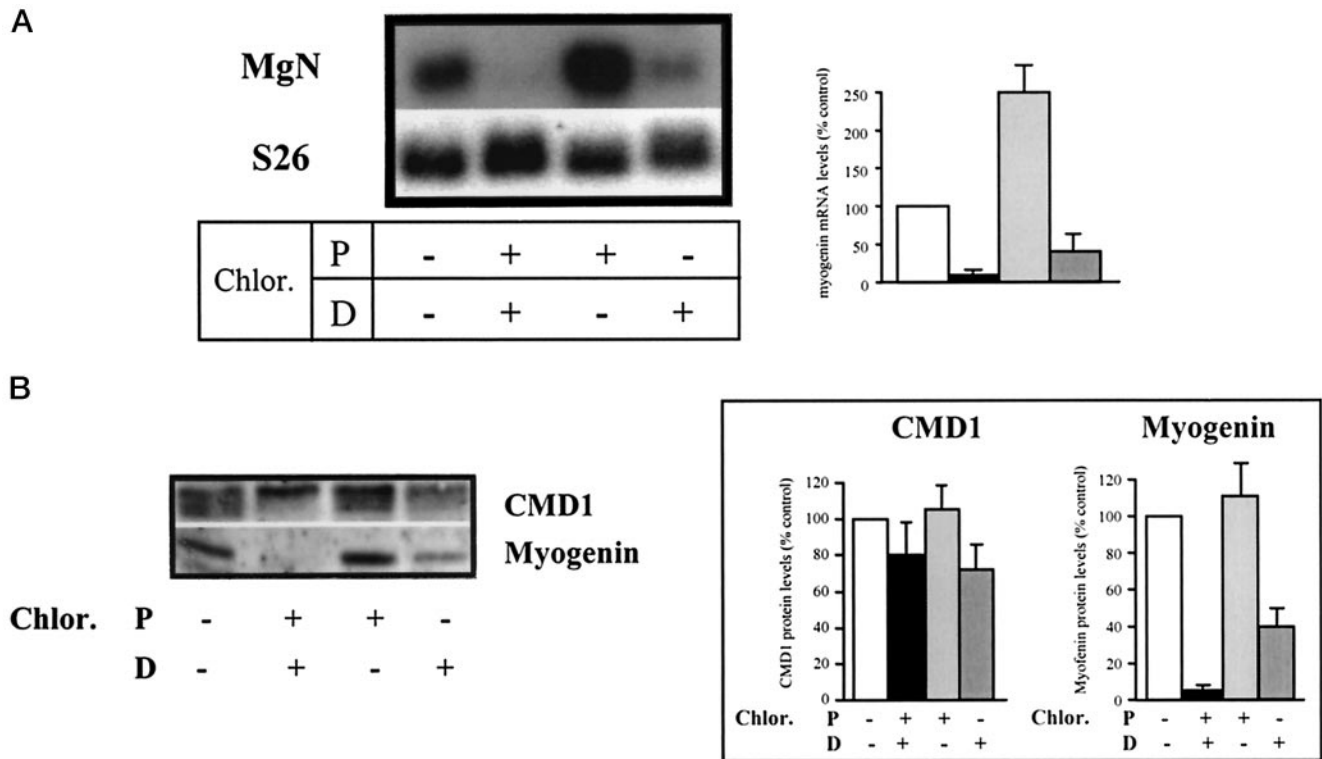


FIG. 7. **Reversibility of chloramphenicol influence on myogenin levels.** Northern (A) and Western (B) blot experiments performed on RNAs or proteins isolated 72 h after the induction of terminal differentiation. QM7 myoblasts were cultured in a medium containing chloramphenicol (*Chlor.*) during proliferation (P) and/or terminal differentiation (D) as indicated. Quantitation was performed using a STORM phosphorimager (Molecular Dynamics) and normalized in relation to S26 levels for mRNAs. Results are expressed as percentages of the control value. Data are the mean  $\pm$  S.E. of five (A) or three (B) separate experiments. Typical blots are shown.

CMD1 protein level after the medium change did not restore differentiation. In addition, despite this decrease, chloramphenicol removal at cell confluence rapidly induced terminal differentiation. We also observed in cytoimmunofluorescence studies that nuclear localization of CMD1 was not influenced by chloramphenicol treatment (data not shown).

As expected (32), myogenin mRNA and protein levels were at the brink of detection in proliferative myoblasts and were strongly up-regulated during terminal differentiation. However, chloramphenicol exposure drastically reduced myogenin levels at cell confluence and during terminal differentiation (to 26 and 4.8% (mRNA) and 21 and 9.6% (protein) of control values, respectively;  $p < 0.001$ ; Fig. 6, A and C). Furthermore, chloramphenicol removal led to the restoration of myogenin expression (Fig. 7). Interestingly, we also found that these levels were up-regulated in p43-overexpressing myoblasts (Fig. 6, B and D). Moreover, using different clones overexpressing different amounts of the mitochondrial T3 receptor, we observed a positive relationship between p43 expression and myogenin mRNA and protein levels (6.4- and 2.1- (mRNA) and 9.5 and 4.0-fold increase relative to control values (protein), respectively, for clones expressing high and moderate levels of p43;  $p < 0.01$ ; Fig. 6, B and D). These data strongly support the hypothesis of a regulation of myogenin expression by mitochondrial activity.

**Myogenin Expression Is a Target of Mitochondrial Activity**—To assess more accurately the relationships between modifications of myogenin mRNA levels and mitochondrial activity,

we used two other treatments known to inhibit other aspects of this activity. Treatment of myoblasts either with FCCP (a protonophore that integrates in the inner membrane of the organelle and dissipates mitochondrial membrane potential) or with oligomycin (an inhibitor of the complex V of the respiratory chain) led to a differentiation block (data not shown). Moreover, whereas CMD1 and *myf5* mRNA levels were not affected, myogenin mRNA induction was abolished by these treatments; *AchR $\alpha$*  mRNA levels were also depressed to a similar extent (Fig. 8).

This set of data clearly indicates that myogenin is a specific target of mitochondrial activity: its mRNA levels are indeed affected in relation to mitochondrial activity in myoblasts cultured in the presence of various drugs that inhibit the activity of the organelle or in p43-overexpressing myoblasts.

To determine the level of this regulation by mitochondrial activity, we studied the half-life of myogenin mRNA after abrogation of nuclear transcription by actinomycin D. Interestingly, chloramphenicol did not significantly affect this mRNA stability (4.1 versus 4.4 h for control myoblasts; not significant; Fig. 9), thus suggesting that mitochondrial activity could regulate myogenin expression at the transcriptional level.

**Myogenin Overexpression Did Not Restore Differentiation of Chloramphenicol-treated Myoblasts**—We then addressed the question of the importance of specific inhibition of myogenin expression by chloramphenicol on QM7 myoblast differentiation. We therefore overexpressed CMD1 or myogenin in QM7 cells, in order to determine whether this could overcome the

indicated time (A and C; proliferation (P), cell confluence (C), and terminal differentiation (D)) or isolated from confluent pIRV or pIRV1 stably transfected cells (B and D) was analyzed by Northern blot (A and B) for CMD1, *myf5*, and myogenin mRNA or by Western blot (C and D) for CMD1 and myogenin protein levels. Quantitation was performed using a STORM phosphorimager (Molecular Dynamics) and normalized in relation to S26 levels for mRNAs. Results are expressed as percentages of the control value recorded in proliferating myoblasts. Data are the mean  $\pm$  S.E. of five (A and B) or three (C and D) separate experiments. Typical blots are shown.



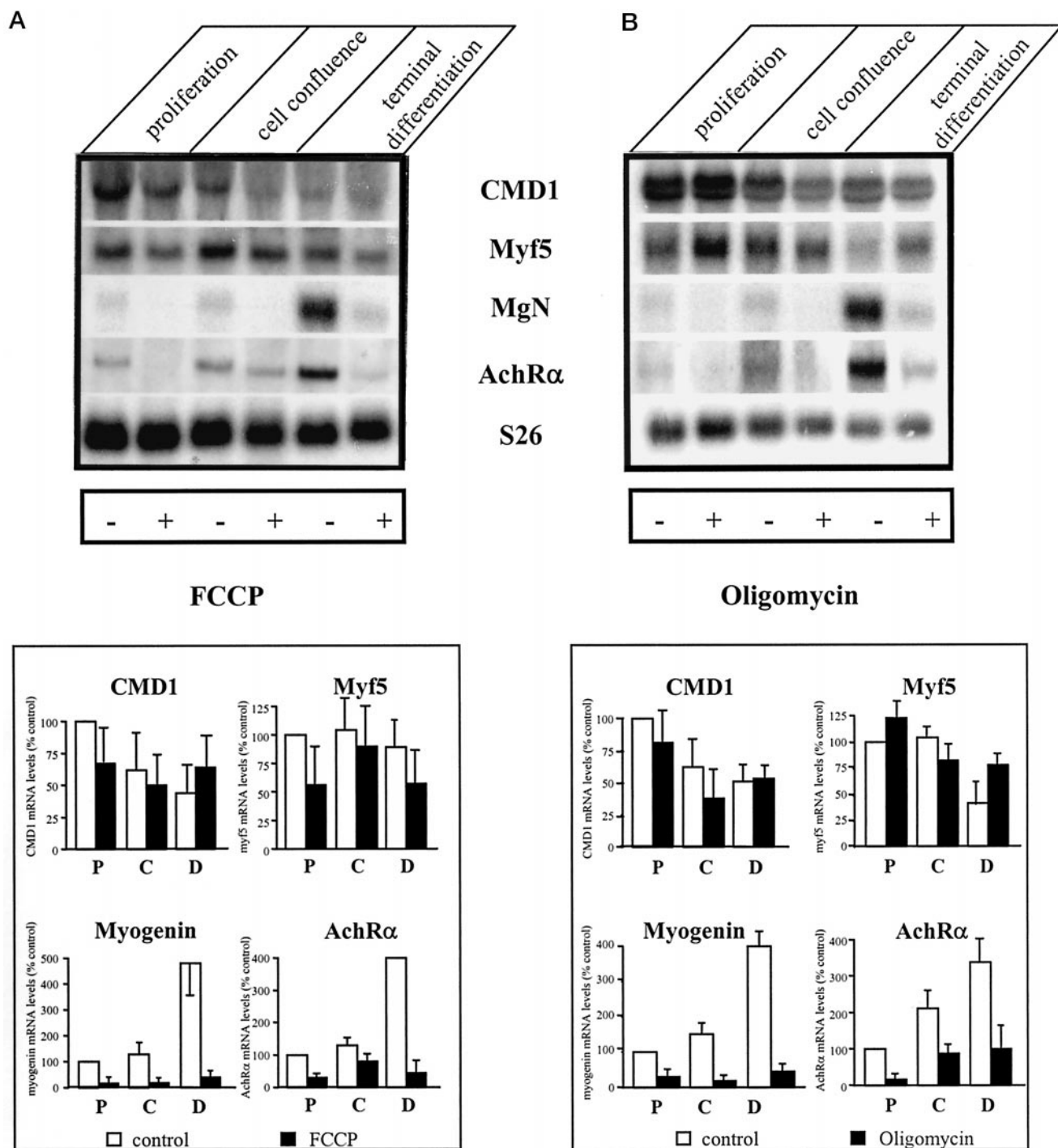


FIG. 8. Influence of FCCP or oligomycin on myogenic regulatory factors and AchR $\alpha$  mRNA levels. 15  $\mu$ g of total RNAs isolated from the control or FCCP-treated (A) or oligomycin-treated (B) myoblasts at the indicated time (proliferation (P), cell confluence (C) and terminal differentiation (D)) was analyzed by Northern blot for CMD1, myf5, myogenin, or AchR $\alpha$  mRNA. Results are expressed as percentages of the control value recorded in proliferating myoblasts. Data are the mean  $\pm$  S.E. of four separate experiments. Typical Northern blots are shown.

chloramphenicol-induced differentiation block. Chloramphenicol treatment did not influence either transfection efficiency (data not shown) or myogenin or CMD1 overexpression levels (Fig. 10).

Transient myogenin overexpression strongly stimulated QM7 myoblast differentiation, assessed either by morphological criteria and connectin accumulation ( $\times 42$  and  $\times 8$  respectively for the fusion index and number of connectin-expressing cells;  $p < 0.001$ ; Fig. 11). However, myogenin overexpression did not restore myoblast ability to differentiate under chloramphenicol exposure (Fig. 11). Nevertheless, a slight increase in

the number of connectin-expressing cells and in the fusion index was observed in myogenin-overexpressing, chloramphenicol-treated myoblasts, which remained, however, far from that observed in myogenin-overexpressing myoblasts (Fig. 11B). In addition, chloramphenicol treatment resulted in a greater impairment of fusion than connectin expression in myogenin-overexpressing myoblasts (decreased to 16 and 24% of control values, respectively). This could reflect the difference in the influence of such a treatment on fusion, on one hand, and markers of differentiation such as AchR $\alpha$  steady-state levels (Fig. 2) or creatine kinase activity (11), on the other.

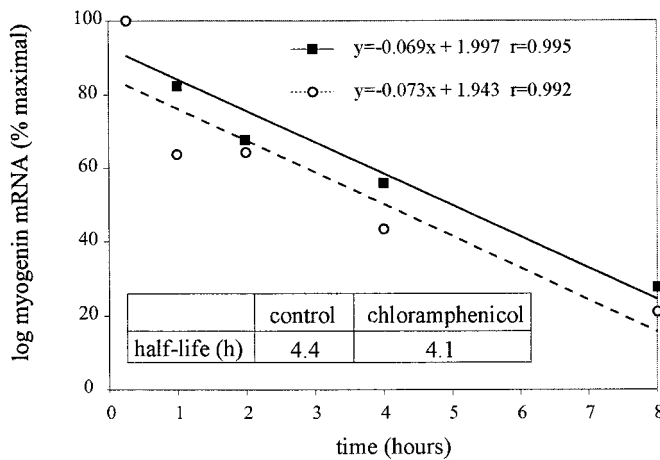


FIG. 9. Influence of chloramphenicol treatment on myogenin mRNA half-life. Myogenin mRNA half-life was determined as described under "Experimental Procedures": control (■) or chloramphenicol-treated (○) myoblasts.  $n = 2$ .

Similar results were obtained using CMD1 transient overexpression (Fig. 11) or using established cell lines constitutively overexpressing either of these myogenic factors (data not shown). It is, however, well established that expression of a single active MRF in numerous cell lines is sufficient to induce a myogenic phenotype (33). Therefore, these data demonstrate that an impairment of mitochondrial activity efficiently blocks the ability of MRF to induce myoblast differentiation.

#### DISCUSSION

Several studies have reported that impairment of mitochondrial activity inhibits differentiation in different cell types including mastocytoma cells (8), erythroblasts (7), neurons (9), and myoblasts (10–12). Although they underlined the importance of organelle function integrity for cell differentiation, these studies did not provide evidence of the occurrence of an actual regulation pathway involving the organelle. In this work, we provide several arguments establishing the existence of such a regulation: (i) inhibition of mitochondrial activity abrogates myoblast differentiation without inducing any alteration of cell viability; (ii) conversely, stimulation of organelle activity potentiates myoblast differentiation; and (iii) mitochondrial activity influences the expression of the myogenic regulatory factor myogenin but not CMD1 (chicken MyoD) or myf5.

**Mitochondrial Activity Affects Myoblast Differentiation by Interfering with a Pathway Leading to Withdrawal from the Cell Cycle**—In agreement with previous reports (10–12), in the present study, we found that inhibition of mitochondrial activity abrogates myoblast fusion and strongly inhibits connectin synthesis and  $\alpha$  subunit of acetylcholine receptor expression (Fig. 3). As other drugs (FCCP or oligomycin) used to depress some aspects of mitochondrial activity induce a similar differentiation block (Fig. 8), this influence cannot be considered as the result of a side-effect of chloramphenicol. In addition, we brought evidence that stimulation of mitochondrial activity by p43 overexpression strongly increases myoblast fusion and the expression of some differentiation markers (Fig. 5), thus demonstrating actual involvement of the organelle in the regulation of myoblast differentiation. Moreover, as p43 overexpression induces differentiation in a serum-rich medium, it appears that stimulation of mitochondrial activity is able to overcome the well established block of differentiation induced by serum. This observation clearly suggests that mitochondrial activity affects an important pathway involved in myoblast withdrawal from the cell cycle, an essential prerequisite for terminal differentiation. This result also provides new insight on our pre-

vious observations indicating that a sharp rise in mitochondrial activity occurs just before the onset of terminal differentiation (17). As this rise does not occur in differentiation-deficient myoblasts, the present data suggest that this event could take part in the induction of terminal differentiation.

**Inhibition of Mitochondrial Activity Does Not Affect Myoblast Differentiation by Depressing ATP Stores or Altering Cell Viability**—It has been shown that respiration-deficient cells are able to grow normally in specific culture conditions (5, 30), and energy disorders to explain differentiation deficiency have been ruled out (9, 12). In addition, growth of cultured cells has been shown not to depend on mitochondrial ATP generation in rich media (3). The present study provides various data ruling out the possibility that ATP deficiency could be responsible for the inhibition of myoblast differentiation under chloramphenicol treatment. First, we observed that anaerobic metabolism almost fully compensated for mitochondrial impairment just before and during terminal differentiation (Fig. 1C); furthermore, addition of chloramphenicol at the onset of terminal differentiation efficiently abrogated myogenesis (Figs. 3 and 4), thus suggesting that the recorded reduction of ATP stores during proliferation is not involved in the differentiation block. In agreement with this proposal, removal of the drug at this time restored myoblast fusion and muscle-specific gene expression (Figs. 3 and 4). Lastly, the involvement of ATP is not supported by the potentiation of myoblast differentiation induced by a stimulation of mitochondrial activity that does not increase intracellular ATP levels.

In addition, we may rule out the possibility that chloramphenicol could affect myoblast differentiation by a nonspecific alteration of the general cell activity, for several reasons: (i) culture observations, FACS, and trypan blue exclusion experiments led us to conclude that chloramphenicol does not influence cell viability, in short or long term culture or in proliferative or differentiating myoblasts (data not shown); (ii) several results indicate that the influence of chloramphenicol on myogenesis is independent of its effects on cell proliferation (for example, terminal differentiation is abrogated when the drug is added only at the induction of this event, whereas chloramphenicol removal results in rapid restoration of myoblast differentiation (Fig. 3)); and (iii) when the plating density of chloramphenicol-treated myoblasts is increased in order to obtain the same number of cells in control or treated dishes at cell confluence, the drug still notably inhibits terminal differentiation (Fig. 4).

**Mitochondrial Activity Regulates Myogenin Expression and MRF Activity**—To understand the myogenic influence of mitochondrial activity, we monitored MRF levels in chloramphenicol-treated or in p43-overexpressing myoblasts (Fig. 6). We found that whereas neither CMD1 (chicken homologue of MyoD) nor myf5 mRNA levels were influenced by chloramphenicol, myogenin mRNA levels were strongly down-regulated. Interestingly, FCCP or oligomycin induced a similar decrease in these levels (Fig. 8), thus underlining the importance of the integrity of mitochondrial activity for myogenin expression. Furthermore, as the half-life of myogenin mRNA remained unaffected under chloramphenicol exposure (Fig. 9), this influence appears to be exerted at the transcriptional level. Lastly, p43 overexpression increased myogenin mRNA levels positively in relation to the expression level of the transgene. This set of data, including studies of the influence of inhibition as well as stimulation of mitochondrial activity on MRF levels, demonstrates that the organelle is involved in the regulation of myogenin expression and that this gene is a specific target of mitochondria. This specific regulation of myogenin expression by mitochondrial activity was confirmed at the protein level by

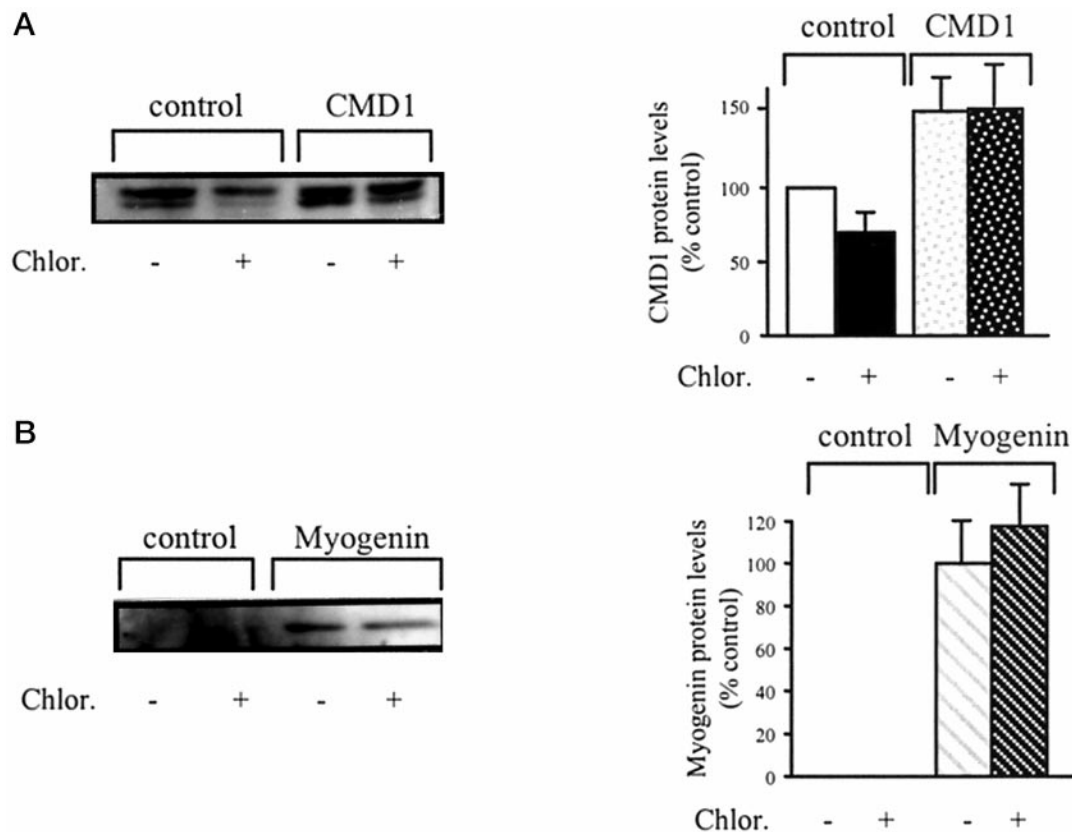


FIG. 10. **Chloramphenicol treatment (*Chlor.*) did not influence myogenin or CMD1 overexpression levels.** 50  $\mu$ g of nuclear proteins isolated from confluent, transiently transfected myoblasts was analyzed by Western blot for CMD1 (A) or myogenin (B) levels. Results are expressed as percentages of the control value (for CMD1 transient overexpression) or as percentages of the value for myogenin overexpressing myoblasts grown in the absence of chloramphenicol (for myogenin transient overexpression). Data are the mean  $\pm$  S.E. of three separate experiments. Typical Western blots are shown.

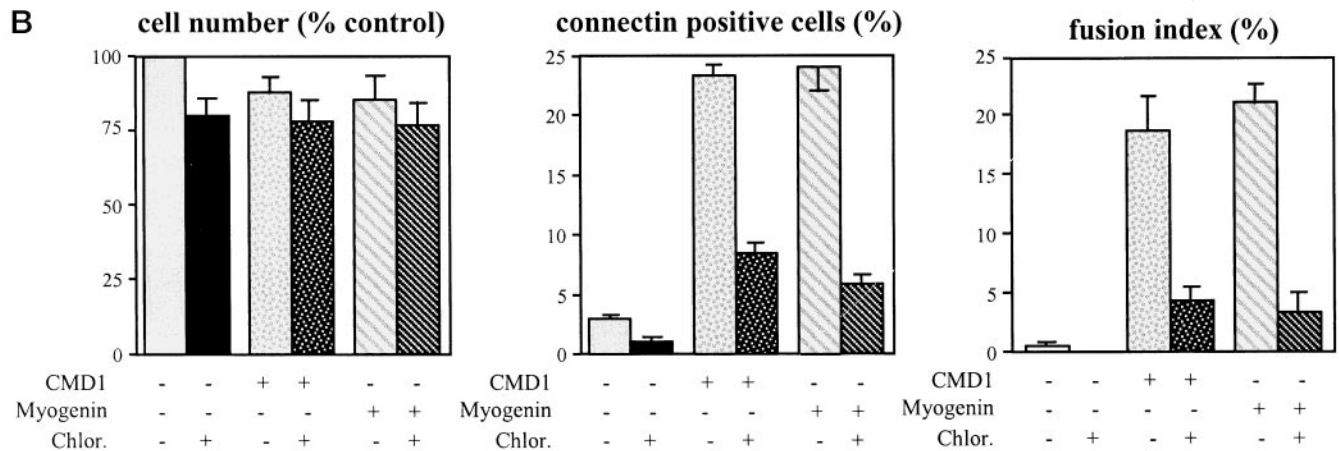
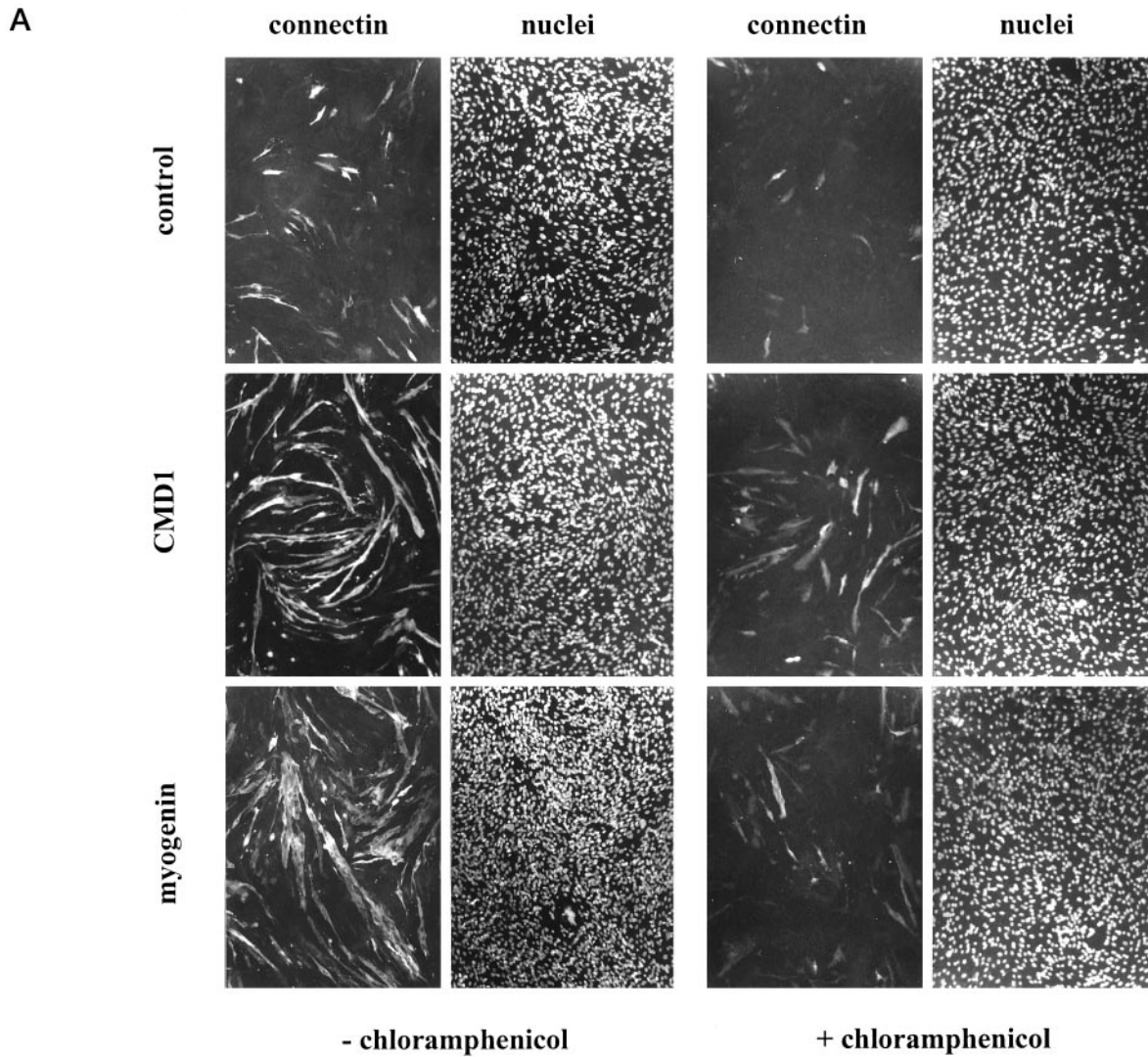
Western blot experiments. In addition, we observed that CMD1 protein levels were reduced at cell confluence by chloramphenicol treatment without any change in CMD1 mRNA level, thus indicating that the drug could affect CMD1 translation or protein stability at this stage. However, the protein level was fully restored after decreasing the serum concentration in the medium, suggesting that this posttranscriptional regulation is not a major explanation of the differentiation block induced by chloramphenicol (Fig. 6C).

Although myogenin-deficient myoblasts are still able to differentiate *in vitro* (34), the importance of this particular MRF is well established. In particular, activation of the Ras pathway in quail myoblasts abrogates terminal differentiation by inhibiting myogenin expression (35). Consequently, we address the question of whether, as in the earlier report, myogenin complementation could restore differentiation of chloramphenicol-treated myoblasts. We found that myogenin overexpression does not overcome the differentiation block induced by this drug (Fig. 11). Similarly, CMD1 overexpression, which is enough to induce a myogenic phenotype in different cell types (33), fails to induce differentiation in the respiration-deficient myoblasts (Fig. 11). All of these data indicate that, in addition to myogenin expression, the ability of MRFs to induce terminal differentiation is a major target of the organelle. However, in transient transfection assays, we found that transcriptional efficiencies of CMD1 or myogenin are not altered by chloramphenicol, because both MRFs are able to activate the  $\alpha$ -tropomyosin or the myogenin promoter to an extent similar to that recorded in control cultures (data not shown). Interestingly, along the same lines, Kong *et al.* (36) reported that activation of the Ras pathway abrogates the CMD1-induced myogenic con-

version in CH310T1/2 fibroblasts without alteration of the transcriptional activity of this MRF. Further investigation led these authors to demonstrate that the Raf/MEK/MAP kinase pathway was partly involved in such regulations (37). Other results of Luo *et al.* (13), showing that alteration of mitochondrial activity by FCCP induced the activation of MEK1 and MEK2, may then appear of particular interest in the search for molecular mechanisms that lead from mitochondrial activity to myogenesis.

*Regulation of the Expression of a Specific Set of Nuclear Genes Is Probably an Important Element of the Mitochondria/Nucleus Cross-talk*—In addition to their interest with regard to regulation of myogenesis, the present data provide new insight into the cross-talk between mitochondria and the nucleus. Myogenesis, like other differentiation programs, is indeed the result of important changes in the expression of a set of nuclear genes; our results, together with previous works showing that inhibition of mitochondrial activity inhibits the differentiation of several cell types (7–12), also suggest that mitochondrial activity is able to influence, directly or indirectly, the regulation of such genes. Nuclear genes the expression of which is sensitive to mitochondrial activity have already been characterized in yeast (for review, see Ref. 38) and in mtDNA-less (*rho*<sup>0</sup>) chicken cells (39, 40), but no link has been established with differentiation events. A recent report demonstrated that reducing mitochondrial DNA contents induced an increase in the cytosolic calcium levels of C2C12 myoblasts, which in turn led to activation of two nuclear transcription factors, nuclear factor of activated T cells and activating transcription factor 2, whereas NF $\kappa$ B was inhibited (14). Our results strongly suggest that a restricted number of genes is under the control of mito-





**FIG. 11. Neither myogenin nor CMD1 overexpression restored differentiation of chloramphenicol-treated myoblasts.** To reach similar cell numbers at confluence, chloramphenicol-treated myoblasts (*Chlor.*) were seeded at twice the plating density of control myoblasts. 24 h after transient transfection, the medium was changed for 24 h with 10% serum. Serum concentration was then lowered to 0.5% for 3 days before cell fixation and immunofluorescence staining with a connectin antibody (A). Nuclei were stained with Hoechst 33258. Typical microphotographs are shown ( $\times 100$ ). The number of connectin-expressing cells and the fusion index were measured (B). Data are the mean  $\pm$  S.E. of four separate experiments.

chondrial activity. In our experimental model, *myogenin*, but not *myf5* or *CMD1*, is a target of the organelle.

Further analysis should lead to greater insight into the involvement of mitochondrial activity in the regulation of nuclear gene expression and into the understanding of this mitochondria-to-nucleus retrograde signaling underlying the influence of the organelle on cell growth and differentiation. In this way, the mitochondrial T3 receptor characterized in our laboratory may provide an interesting new molecular tool in addition to known inhibitors of mitochondrial activity.

**Acknowledgments**—We thank Drs. F. Pons (Montpellier, France), C. Dechenne (Montpellier, France), K. Tsim (Hong Kong), and A. Bonnieu (Montpellier, France), for the gifts of connectin antibody, probes for *CMD1* and chicken myogenin, chicken AchR $\alpha$ , and quail *myf5*, respectively. We are particularly grateful to Drs. G. Brandolin (Grenoble, France), M. Fisman (Paris, France), and D. Piquemal (Montpellier, France) for helpful discussions.

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**Mitochondrial Activity Is Involved in the Regulation of Myoblast Differentiation through Myogenin Expression and Activity of Myogenic Factors**

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*J. Biol. Chem.* 2000, 275:2733-2744.  
doi: 10.1074/jbc.275.4.2733

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