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Transcription of Muscle-Specific Genes Is Repressed by Reactivation of pp60$^{v\text{-}src}$ in Postmitotic Quail Myotubes

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Quail myogenic cells infected with temperature sensitive (*ts*) mutants of Rous sarcoma virus (RSV) exhibit a temperature-dependent transformation and block of differentiation. When the cells are allowed to differentiate at the restrictive temperature (41°C) and then shifted back to the permissive temperature (35°C), a sharp reduction in the accumulation of muscle-specific mRNAs is observed, following reactivation of the transforming protein pp60$^{v\text{-}src}$. A kinetic analysis of this down-regulation reveals that the reduction in the accumulation of muscle-specific transcripts occurs fairly rapidly within 6 to 20 h after the shift back, depending on the mRNA analyzed. Studies on transcription of endogenous muscle-specific genes and a transfected chloramphenicol acetyltransferase reporter gene under the control of muscle-specific promoters, at the different temperatures, suggest that the oncogene exerts its control mainly at the transcriptional level. On the contrary, transcription of the CMD1 gene, the avian homolog of the mouse muscle regulatory MyoD gene, is not significantly affected by the oncogene both in proliferating myoblasts and in myotubes shifted back to 35°C. These findings are consistent with the conclusion that v-*src* blocks myogenesis by controlling transcription of muscle-specific genes independently of cell proliferation. Furthermore, they suggest the existence of an alternative pathway, not requiring the silencing of CMD1 transcription, through which the oncogene exerts its effect.

Transformation of in vitro differentiating cells by Rous sarcoma virus (RSV) almost invariably leads to the inhibition of complex differentiation programs in different specialized cell types. Over the past years it was shown that the v-*src* oncogene of RSV specifically suppresses differentiation of myogenic cells (24, 25, 30), chondroblasts (4, 44), retinal melanoblasts (6), tendon cells (49), neuroretina cells (28), and erythroblasts (31). The v-*src* oncogene encodes a 60-kDa polypeptide, termed pp60$^{v\text{-}src}$, which possesses an intrinsic protein tyrosine kinase activity (14, 62). Establishment of transformation and block of differentiation depend on the expression of a functional pp60$^{v\text{-}src}$, as clearly shown by the use of conditional mutants mapping within the oncogene (62). It seems likely that a single viral gene which can produce such pleiotropic effects must code for a function that affects a key element in cellular regulation.

Among the differentiating cell systems studied, muscle cells represent a particularly attractive model because of the considerable body of information now available (47). Primary cultures derived from quail embryo breast muscles consist mainly of replicating myoblasts. Once induced to differentiate by the appropriate culture conditions, myoblasts withdraw from the cell cycle and begin to accumulate skeletal muscle-specific mRNAs. In turn, muscle-specific proteins are synthetized and assembled into myofibrils while the cells fuse into long, multinucleated myotubes (47). Primary cultures of quail myoblasts transformed by temperature-sensitive (*ts*) mutants of RSV exhibit a temperature-dependent transformation and consequent block of differentiation (reviewed in reference 3). At the permissive temperature (35°C) myoblasts remain replicating, undifferentiated cells. When shifted to the restrictive temperature (41°C), however, transformed cells revert to normality and faithfully recapitulate the myogenic program. Since in myogenic cells undergoing terminal differentiation cellular proliferation and synthesis of differentiation markers are mutually exclusive processes (42), a crucial point to clarify in this system is whether suppression of differentiation can be explained by continuous mitogenic signaling provided by pp60$^{v\text{-}src}$ or whether it is a consequence of a direct interference with expression of muscle-specific genes. Previous results obtained in our laboratory on chicken embryo myoblasts infected with a *ts* mutant of RSV and treated with mitomycin C, an inhibitor of DNA synthesis, showed that growth-arrested myoblasts kept at 35°C remained incapable of differentiating, whereas inactivation of pp60$^{v\text{-}src}$ by temperature shift was sufficient to initiate differentiation (23), thus implying that v-*src* was not affecting differentiated functions by keeping the cells cycling. Recent reports on C2 myogenic cells transformed with activated *ras* genes suggest that this oncogene inhibits differentiation through a mechanism independent of cell proliferation (43, 46).

A breakthrough in the understanding of terminal commitment events in myogenic cells was the recent isolation of the MyoD gene (17), whose ectopic expression converts a variety of nonmyogenic cell types into functional myoblasts (58). Further studies indicated that MyoD is a nuclear phosphoprotein with a region of sequence homology to *myc* oncogenes (52), consisting of a basic domain required for sequence-specific DNA binding (34) and a helix-loop-helix motif required for protein-protein interaction (39). MyoD is a member of a family of muscle regulatory genes, which also includes myogenin (21, 61), Myf-5 (8), and Myf-6 (7), that are expressed solely in skeletal muscle cells. Any of these genes can initiate myogenesis when transfected into fibroblasts, and their products share a high degree of sequence similarity. The MyoD protein has been shown to bind to the muscle creatine kinase (MCK) enhancer through its *myc* homology

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region (34), indicating that direct binding to muscle-specific regulatory regions may be one mechanism by which it activates the myogenic program.

In this study we address some questions relevant to an understanding of the molecular mechanisms responsible for the coordinate expression of differentiation genes and to an understanding of how transformation by viral oncogenes interferes with the differentiation program: is block of differentiation in replicating transformed myoblasts accompanied by lack of accumulation of skeletal muscle-specific transcripts? Does reactivation of v-*src* in postmitotic myotubes affect expression of lineage-specific genes and at what level? Is expression of muscle regulatory genes such as MyoD affected in the transformed cells?

MATERIALS AND METHODS

Cell culture and viruses. Primary cultures of quail (*Coturnix japonica*) myoblasts and fibroblasts were prepared as described previously (24, 33). Quail cells were routinely propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 10% tryptose phosphate broth, and 1% chicken serum (referred to as growth medium [GM]). Primary myoblasts were maintained proliferating in GM containing 5% quail embryo extract. Differentiation of myogenic cells was induced, as previously described (24), by substituting GM with F14 (56) supplemented with 2% fetal calf serum and 0.5 μg of insulin per ml (referred to as differentiation medium [DM]). Transformed *ts* RSV quail myoblasts were propagated at 35°C (permissive temperature) on collagen-coated dishes in GM. Myogenic differentiation was assayed at 41°C (restrictive temperature) in DM. Percentage of fusion was calculated after staining with Giemsa; approximately 1,000 nuclei were scored from randomly chosen fields for each experimental condition.

Clonal and polyclonal populations of transformed quail myoblasts were established as previously described from primary-passage cultures infected at high multiplicity with the appropriate avian transforming retrovirus (24). The origin of the viral stocks is described in reference 24.

RNA isolation and Northern (RNA) blot analysis. Total RNA was prepared by lysing the cells with 1% sodium dodecyl sulfate (SDS)–10 mM Tris-Cl (pH 7.5)–100 mM NaCl–0.1 mM EDTA on ice. Following centrifugation for 60 min at 100,000 × *g* at 10°C, the supernatant was extensively extracted with phenol and chloroform and ethanol precipitated. Aliquots (10 μg) of the obtained RNA were resolved in 0.9% agarose–2.2 M formaldehyde gels. Transfer to nitrocellulose membranes and high-stringency hybridization were carried out according to standard procedures (38). Probes were labeled with a random-primed DNA labeling kit (Amersham). For detection of muscle-specific and constitutive transcripts, inserts of the following plasmids were cut with the appropriate restriction enzymes and used as probes: cC118, containing a 590-bp quail α-actin cDNA (provided by C. Emerson); cC127, containing a 600-bp quail myosin light-chain (MLC) cDNA (provided by C. Emerson); cC128, containing a 500-bp quail myosin heavy-chain (MHC) cDNA (provided by C. Emerson); p5c5, containing a 590-bp chicken vimentin cDNA (provided by E. Lazarides); pD8, containing a 1-kb chicken desmin cDNA (9); pA1, containing a 2-kb chicken β-actin cDNA (13); pCMD1, containing a 1.5-kb chicken MyoD cDNA (36); and a plasmid containing a 1.2-kb avian glyceraldehyde-3-phosphate dehydrogenase cDNA (obtained from C. Schneider).

Immunostaining and Western immunoblot analysis. Immunofluorescence experiments were performed by using specific antibodies against skeletal muscle proteins as described earlier (24, 33). For Western analysis, proteins were resolved by electrophoresis in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane by using a transblot apparatus (Hoefer). An horseradish peroxidase conjugated anti-rabbit antiserum was used to detect proteins after incubation with affinity-purified antiphosphotyrosine antibodies (1:100) (kindly provided by P. Comoglio).

Transfections and CAT assays. Cells for transient expression of chloramphenicol acetyltransferase (CAT) reporter constructs were transfected by the *N,N-bis*(2-hydroxyethyl)-2-aminoethane-sulfonic acid-calcium phosphate precipitation technique (12). Myoblasts were plated at 10^5 per 35-mm collagen-coated dish at 35°C in GM; after cell adhesion, two sets of plates were shifted to 41°C in DM. Two days later, cells were transfected with 2 μg of the reporter plasmids per dish; after 6 to 8 h, the medium containing the precipitates was removed, and the cells were washed several times with medium containing 5% fetal calf serum and fed with fresh medium. Immediately after transfection, a set of plates transfected at 41°C was shifted back to 35°C. The following day, the plates were washed twice with phosphate-buffered saline (PBS) and frozen at −80°C. The muscle-specific constructs were plasmid α/CAT, containing the chicken α-cardiac actin promoter (22) followed by the bacterial CAT gene, and plasmid pCKCATe4 (51), containing the mouse MCK enhancer cloned upstream of the MCK basal promoter and the CAT gene. Control CAT constructs contained the chicken β-actin promoter (18) and the RSV promoter. All CAT assays were normalized to equivalent amounts of proteins. Attempts to normalize to levels of luciferase activity from a cotransfected RSV-luciferase plasmid (19) were hampered by a 10- to 50-fold-lower specific activity of the enzyme extracted from cells grown at 41°C. Activity of each CAT construct was determined in duplicate from multiple transfection experiments and with different plasmid preparations to minimize differences due to transfection variability.

CAT activity was assayed by the method of Neumann et al. (41) in which the acetylated forms of chloramphenicol produced by CAT in the aqueous phase diffuse into the overlying scintillation fluid, thus enabling a continuous quantitative assay. Cells were harvested in 0.25 mM Tris-Cl (pH 7.8), disrupted by three freeze-thaw cycles, and then incubated at 65°C for 10 min to minimize deacylation activity. Protein concentration in the lysates was determined by using a commercially available protein assay reagent (Pierce). A 300-μl reaction mixture containing the appropriate amount of proteins, 0.1 μCi of [^{3}H]acetyl coenzyme A, 0.35 μM acetyl coenzyme A, and 1.25 mM chloramphenicol was incubated at 37°C for various periods of time. Under these conditions the reaction was linear with enzyme concentrations until the percentage of acetylated chloramphenicol, quantitated by scintillation counting, reached 50%. CAT assays on the same extracts by the endpoint method (26) gave essentially the same results.

Run-on experiments. Three sets of cells were plated at 10^6 per 150-mm collagen-coated dish at 35°C in GM, and the next day two sets were transferred to 41°C in DM. Two days later, one set was shifted back to 35°C in DM. The following day, the plates were processed for isolation of nuclei as follows. Cells were washed with ice-cold PBS, incubated in hypotonic solution (10 mM Tris-Cl [pH 7.5], 5 mM $MgCl_2$, 10 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride) on ice for 10 min, collected with a rubber policeman, homogenized in a

Dounce homogenizer fitted with a tight pestle (30 strokes) on ice, and pelleted at 500 × *g* for 10 min. The cell pellet was suspended in 400 μl of lysis buffer (10 mM Tris-Cl [pH 7.5], 5 mM $MgCl_2$, 10 mM NaCl, 0.5% Nonidet P-40), loaded on top of 4 ml of sucrose buffer (700 mM sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Tris-Cl [pH 7.5], 0.5 mM spermidine, 150 mM spermine, 2 mM EDTA, 0.5 mM EGTA, 14 mM β-mercaptoethanol, 0.1% Triton X-100), and centrifuged in the cold at 1,300 × *g* for 10 min. The nuclear pellet was then suspended in 400 μl of storage buffer (40% glycerol, 5 mM $MgCl_2$, 50 mM Tris-Cl [pH 8.0], 0.1 mM EDTA) and frozen at −80°C. Run-on transcription reactions and isolation of radiolabeled RNAs were performed as previously described (27, 55). Labeled RNAs (2×10^6 cpm/ml) were hybridized to nitrocellulose filters containing excess DNA probes (5 μg), previously linearized and denatured, in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–1% SDS at 42°C for 3 to 4 days. Filters were washed three times in 2× SSC at room temperature for 10 min and twice in 0.1× SSC–0.5% SDS at 60°C for 30 min.

RESULTS

Expression of muscle-specific genes in *ts* RSV-transformed quail myoblasts. Clonal strains of skeletal quail myoblasts transformed by *ts*LA33, a temperature-sensitive mutant of RSV, obtained as described previously (24), were used in these experiments. Clearly, the homogeneity of cloned myogenic populations is desirable to avoid interference from the presence of fibroblasts, always contaminating primary muscle cultures. At 35°C most of the transformed cells were maintained replicating and undifferentiated in GM, the incidence of differentiated cells being less than 1%. When shifted to 41°C in DM, the cells promptly withdrew from the cell cycle, assembled myofibrils, and formed multinucleated myotubes which expressed muscle-specific proteins. The proportions of nuclei in myotubes after 2 and 4 days at 41°C were 85 and 95%, respectively. If, after 2 days at 41°C, the cultures were shifted back to 35°C to reactivate the thermolabile kinase $pp60^{v\text{-}src}$, the myotubes flattened, the sarcomeres disassembled, and nuclei were confined to the central area of the sarcoplasm; concomitantly, a reduction in the accumulation of muscle-specific myosin and desmin, as detected by immunofluorescence experiments, was observed (data not shown). The fraction of nuclei within myotubes in cultures previously kept at 41°C for 2 days and shifted down to 35°C for 2 and 3 days was 90%, indicating that at the time of the shift-down there were no replicating cells and thus all mononucleated cells were postmitotic myoblasts. The changes induced by reactivation of the *src* kinase appeared to be reversible since in cultures previously shifted to 35°C for 2 days and shifted back to 41°C for 2 more days, myotubes reacquired a normal morphology with nuclei aligned along the longitudinal axis of fibers (not shown).

To confirm that $pp60^{v\text{-}src}$ kinase activity was effectively reactivated in shift-down experiments, tyrosine phosphorylation of total cellular proteins was examined by using specific antibodies reacting to phosphotyrosine. A Western blot analysis showed that extensive tyrosine phosphorylation of several cellular substrates occurred only at the permissive temperature, both in proliferating myoblasts and in myotubes allowed to differentiate at 41°C and then shifted back to 35°C, as expected (not shown).

Muscle-specific transcripts in both *ts* RSV-transformed myoblasts and normal myoblasts grown under the same conditions were also analyzed. In the transformed cells at 35°C, accumulation of muscle-specific MHC, MLC, and α-actin transcripts was absent or barely detectable (Fig. 1A, lane 2), compared with the high levels present in the same cells allowed to differentiate at 41°C (Fig. 1A, lane 3). Expression of the constitutive vimentin gene was unaffected, and expression of β-actin was high at 35°C and decreased in differentiated cells at 41°C, corresponding to the increase in accumulation of α-actin transcripts (Fig. 1A). β-Actin and α-actin transcripts have already been shown to be inversely regulated during myogenic differentiation (45). The next point to address was whether reactivation of $pp60^{v\text{-}src}$ in differentiated myotubes could affect accumulation of muscle-specific transcripts and, if so, with what time course this occurred. To this end, total RNAs were extracted at various times after the shift-down to 35°C and from control cultures kept at 41°C and were analyzed by Northern blot experiments (Fig. 1B). A marked down-regulation of muscle-specific transcripts in myotubes was observed. The reduction in the accumulation of the transcripts analyzed occurred fairly rapidly, depending on the mRNA species. Kinetics did not appear to be identical for all muscle transcripts analyzed, the down-regulation being faster for MHC and slower for MLC, α-actin, and desmin (Fig. 1B). In contrast, accumulation of constitutive transcripts was unaffected (vimentin) or up-regulated (β-actin). In differentiated normal myotubes, accumulation of muscle-specific transcripts was comparable to that of *ts* RSV-transformed myoblasts allowed to differ-

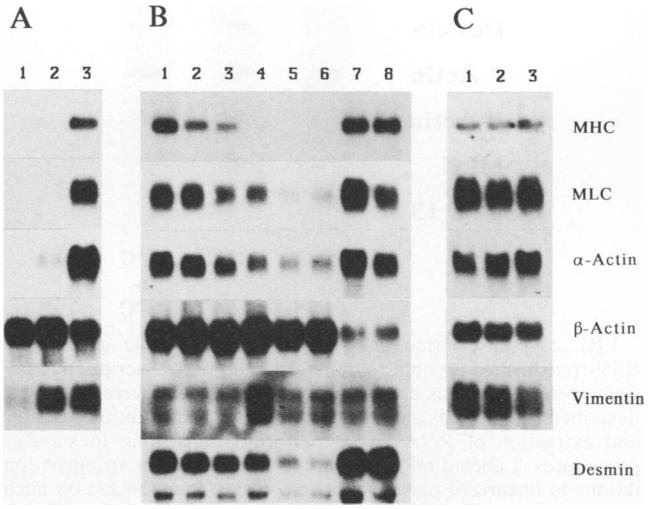

FIG. 1. Accumulation of muscle-specific mRNAs in transformed (A and B) and normal (C) quail myoblasts. (A) MC29-transformed myoblasts were cultured at 35°C in GM for 2 days (lane 1). *ts*LA33-transformed myoblasts, clone C, were grown at 35°C in GM for 2 days (lane 2) and at 41°C in DM for 2 days (lane 3). (B) Time course of down-regulation of muscle-specific mRNAs following reactivation of $pp60^{v\text{-}src}$. *ts*LA33-transformed myoblasts were cultured at 41°C in DM for 2 (lane 1), 3 (lane 7), and 4 (lane 8) days or at 41°C in DM for 2 days and shifted to 35°C for 6 h (lane 2), 12 h (lane 3), 24 h (lane 4), 36 h (lane 5), or 48 h (lane 6). (C) Normal myoblasts were cultured in DM at 41°C for 2 (lane 1) or 4 (lane 3) days and for 2 days at 41°C and shifted to 35°C for 2 days (lane 2). Total RNA was analyzed by Northern blotting (10 μg per lane), and RNAs species indicated on the right were hybridized with the DNA probes described in Materials and Methods. Ethidium bromide staining of the rRNA in parallel gels confirmed that the sample loadings were identical (not shown).

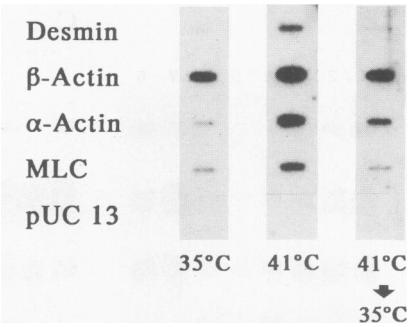

FIG. 2. Transcription analysis of muscle-specific genes in *ts* RSV-transformed myoblasts by nuclear run-on transcription assay. Cells were cultured as described for Fig. 1. Nuclei were isolated as described in Materials and Methods; nuclear transcription reactions and extraction of RNAs were performed according to standard procedures. Labeled mRNAs were hybridized under stringent conditions to linearized plasmids (5 μg per slot) immobilized on nitrocellulose filters. The plasmids indicated on the left are described in Materials and Methods. pUC13 DNA was used as a control for nonspecific hybridization.

entiate at 41°C and was not affected by temperature shifts (Fig. 1C).

Taken together, these observations are consistent with the interpretation that the v-*src* oncogene interferes with muscle differentiation by directly affecting accumulation of muscle-specific transcripts and synthesis of muscle proteins in nonproliferating, terminally differentiated myotubes. Thus, the oncogene is capable of acting independently of cell proliferation.

Total RNA extracted from quail myoblasts transformed by the avian myelocytomatosis virus MC29, carrying the v-*myc* oncogene, was also analyzed for the presence of muscle-specific transcripts, as a control of cells transformed by a different oncogene. In these cells, myogenic differentiation was previously shown to be completely blocked (24). Accordingly, we found undetectable levels of muscle-specific transcripts (Fig. 1A, lane 1).

Transcription of muscle-specific genes is impaired in RSV-transformed myoblasts. The next question we asked was at what level is accumulation of muscle-specific mRNAs affected by $pp60^{v\text{-}src}$ in transformed myoblasts. To study the effect of v-*src* on transcription of muscle-specific genes, nuclear run-on experiments were performed in nuclei isolated from uncloned and clonal populations of *ts*LA33-transformed myoblasts in proliferating and differentiating conditions as well as in postmitotic myotubes shifted back to 35°C to reactivate the mutant kinase. In this assay, transcripts which are already initiated are faithfully elongated, giving an accurate measure of the level of transcription at the time of nuclear isolation (37). Radiolabeled nascent transcripts were hybridized to slot blots containing excess DNA probes specific for desmin, α-actin, MLC, and β-actin as well as a plasmid DNA used as a control for nonspecific hybridization. As shown in Fig. 2, transcription of the three muscle-specific genes in a clonal population of *ts*LA33-transformed cells, chosen as an example, was significantly lower in both proliferating myoblasts and postmitotic myotubes at 35°C than in myotubes at 41°C.

A quantitative assay of transcription was also performed in v-*src*-transformed myoblasts by transfecting constructs containing the CAT reporter gene under the control of muscle-specific or constitutive promoters and measuring

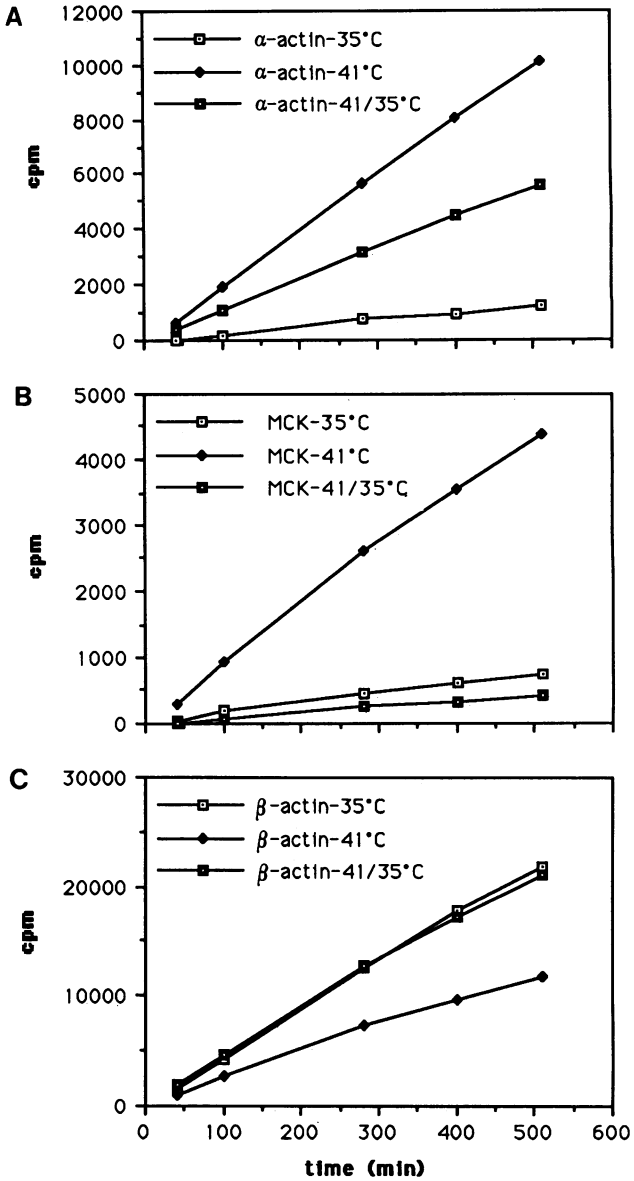

FIG. 3. Kinetic assay of CAT enzymatic activity in *ts* RSV-transformed myoblasts transfected with muscle-specific promoter-CAT constructs. Quail myoblasts transformed by *ts*LA29 RSV were transfected with α-actin–CAT (A), MCK-CAT (B) and β-actin–CAT (C) constructs as described in Materials and Methods. Cell extracts, normalized to protein content, were assayed for enzymatic activity according to the direct diffusion method described by Neumann et al. (41). Kinetics of the enzymatic reactions for each transfected CAT construct are shown.

CAT enzymatic activity in cell extracts, according to the diffusion method described by Neumann et al. (41) (see also Materials and Methods). Quail myoblasts transformed by *ts*LA29 were transfected with α-actin–CAT, β-actin–CAT and MCK-CAT constructs either as replicating cells (35°C, GM) or differentiated myotubes (41°C, DM) and kept at the appropriate temperatures for 1 day. A set of plates transfected at 41°C was shifted back to 35°C immediately after transfection and left at 35°C for 1 day. Figure 3 shows the kinetics of CAT enzymatic activity in the various cell extracts: transcription from the MCK and α-actin muscle-

TABLE 1. Transcription of muscle-specific and constitutive promoters in *ts* and wt RSV-transformed myoblasts assayed by CAT enzymatic activity[a]

Temp (°C)	Relative CAT activity										
	*ts*LA-29				*ts*LA-33 clone C			PR-A			
	α-Actin	β-Actin	MCK	RSV	α-Actin	β-Actin	MCK	α-Actin	β-Actin	MCK	RSV
41	12	0.5	5.9	0.7	6.6	1.1	6.2	1.3	1.5	0.5	1.4
41 ➧ 35	4	0.7	0.3	0.9	2.5	0.7	0.4	1.2	1.7	0.7	1.6
35[b]	1	1	1	ND							
41[b]	10.2	0.9	5.8	ND							

[a] Quail myoblasts transformed by *ts*LA29 and wt PR-A and a clonal strain of *ts*LA33-transformed quail myoblasts were transfected with CAT reporter gene constructs under the control of muscle-specific (α-actin and MCK) and constitutive (β-actin and RSV) promoters and analyzed for CAT activity as described in Materials and Methods. Values have been normalized on the activity detected at 35°C for each sample, set at 1, and have been chosen in the linear range of the reaction. Data represent averages of two experiments. ND, Not done.

[b] Transfections were carried out at 35°C.

specific promoters was clearly suppressed in proliferating myoblasts at 35°C. Transcription from the same promoters was activated about 10-fold in differentiated myotubes at 41°C, an up-regulation comparable with that observed in normal myotubes (36). A similar activation of transcription from the MLC promoter in quail myoblasts transformed by *ts*NY68 was previously reported (50). In postmitotic myotubes shifted back to 35°C for only 24 h, transcription from the MCK promoter was again suppressed and that from the α-actin promoter was significantly inhibited (Fig. 3A and B; Table 1). In contrast, transcription from the β-actin and RSV constitutive promoters occurred at both temperatures (Fig. 3C; Table 1). Further CAT transfection experiments were performed on quail myoblasts transformed by wild-type (wt) RSV or clones of myoblasts transformed by *ts*LA33. The results obtained from three independent experiments are shown in Table 1; as expected, transcription from muscle-specific promoters was suppressed at both 35 and 41 in wt-transformed myoblasts, whereas constitutive promoters were transcribed efficiently at both temperatures. The behavior of the clonal *ts* virus-transformed myoblasts was comparable with that of the uncloned cells; moreover, no significant differences were observed between myoblasts transformed by two *ts* RSV mutants (Table 1). To test whether the conditions at which transfection experiments were performed (temperature and differentiated state of the cells) could affect transfection or transcription efficiencies, we also carried out transfections on proliferating myoblasts at 35°C and the following day shifted a set of cells to 41°C to allow differentiation. As shown in Table 1, this temperature change did not influence the transcription ability of the cells.

These data, together with the results obtained from the run-on experiments, strongly suggest that v-*src* expression leads to inhibition of transcription of muscle-specific genes.

Expression of the avian MyoD gene in v-*src* and v-*myc*-transformed myoblasts. A number of myogenic regulatory genes, such as MyoD, have recently been identified due to their ability to impose the myogenic phenotype when ectopically expressed in several different cell contexts (7, 8, 17, 61). Since the remarkably similar effect exerted by the v-*src* oncogene in different specialized cell types is suggestive of a control at the programmatic level (3), we asked whether the regulatory pathways affected by v-*src* in transformed myoblasts could involve the putative function of muscle regulatory genes. To study how the expression of the avian homolog of murine MyoD gene (36) was modulated in culture before and after transformation, RNA was extracted from primary quail myoblasts grown in GM and allowed to differentiate in DM; parallel sets of cells were infected with transformation-defective (*td*B77) and transformation-competent retroviruses carrying a *ts* or a wt *src* oncogene or a *myc* oncogene as a control. After four to five passages in culture to ensure virus spread and establishment of transformation, RNAs were extracted from the various cell types cultured either in GM at 35°C or in DM at 41°C and after shift-down to 35°C, and the levels of muscle-specific and control RNAs were analyzed. As shown in Fig. 4A, the avian homolog of the MyoD gene, referred to as CMD1 (36), was expressed at high levels in both proliferating normal myoblasts and dif-

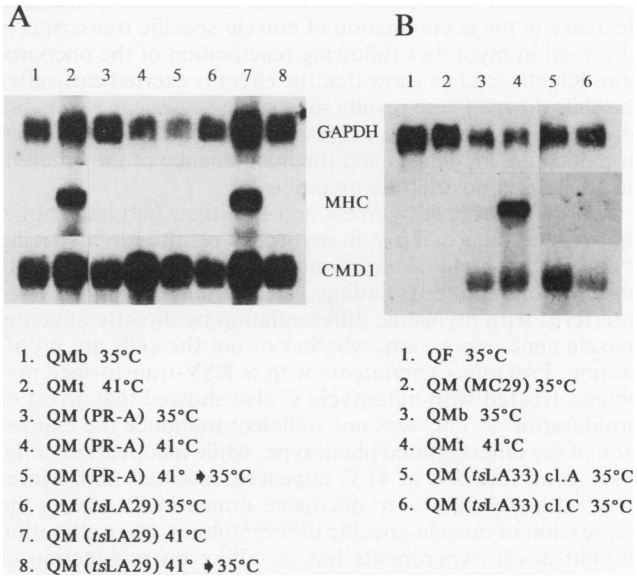

FIG. 4. Expression of avian MyoD gene in normal, v-*src*- and v-*myc*-transformed quail myoblasts. Primary quail myoblasts were cultured in GM at 35°C and in DM at 41°C; parallel sets of cells were infected with *ts*LA29, *ts*LA33, wt PR-A, and MC29 and passaged four to five times to ensure virus spread and establishment of transformation. All cells at 35°C were grown in GM, and cells kept at 41°C or shifted back to 35°C were cultured in DM. Panels A and B represent two independent experiments. Total RNA was extracted essentially as described, and the levels of muscle-specific and control mRNAs were analyzed by RNA blotting (10 μg per lane) with the probes indicated in Materials and Methods. QMb, quail myoblasts; QMt, quail myotubes; QM, transformed myoblasts; QF, quail fibroblasts; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CMD1, avian homolog of MyoD.

ferentiated myotubes, as previously reported (11, 36). Moreover, CMD1 transcript accumulation was not affected by passaging in culture or by infection with a nontransforming retrovirus (data not shown). Expression of CMD1 was not significantly down-regulated by transformation with wt or *ts src* at the permissive temperature, conditions in which expression of MHC gene was, instead, completely suppressed (Fig. 4A). In *ts* RSV-transformed myoblasts, expression of CMD1 was shown to remain high also in differentiated myotubes shifted back to 35°C in the presence of reactivated $pp60^{v\text{-}src}$ (Fig. 4A). Clonal populations of *ts src*-transformed myoblasts displayed high levels of CMD1 expression at the permissive temperature (Fig. 4B), confirming that their behavior was comparable to that of uncloned cells.

In parallel cultures of myoblasts transformed by the v-*myc* oncogene, transcript accumulation of the muscle regulatory gene was also analyzed. In contrast to v-*src*-transformed myoblasts, expression of CMD1 was undetectable in v-*myc*-transformed cells (Fig. 4B).

DISCUSSION

The studies here described focus on the mechanisms by which transformation by RSV interferes with the differentiation program of muscle cells. In RSV-transformed myoblasts, $pp60^{v\text{-}src}$ appears to prevent the accumulation of muscle-specific mRNAs. In terminally differentiated myotubes, reactivation of $pp60^{v\text{-}src}$ causes disassembly of sarcomeres and disorganization of myofibrils that can be visualized by immunostaining with antibodies against muscle-specific proteins. Concomitantly with these structural alterations, a dramatic decrease in the accumulation of muscle-specific transcripts is observed in myotubes following reactivation of the oncoprotein. Kinetic studies show that the effect is exerted early after the shift-down. These results suggest that v-*src* can reversibly affect the initiation of the expression of the myogenic program in replicating myoblasts and the maintenance of the differentiated state in postmitotic myotubes.

A previous report by West and Boettiger (60) had shown that reactivation of v-*src* in myotubes resulted in a specific reduction of eight skeletal muscle-specific proteins. This observation and our findings clearly indicate that v-*src* interferes with myogenic differentiation by directly affecting muscle gene expression, whether or not the cells are proliferating. Previous experiments with *ts* RSV-transformed myoblasts treated with mitomycin C also showed that arrest of proliferation at 35°C was not sufficient to induce the expression of the differentiated phenotype, while inactivation of the v-*src* gene function at 41°C appeared essential (23). Moreover, although the v-*src* oncogene dramatically affects the expression of muscle-specific differentiation, its reactivation in shift-down experiments has no effect on reinduction of DNA synthesis in postmitotic myotubes. Prolonged incubation of myotubes shifted down to 35°C with bromodeoxyuridine did not result in its incorporation into DNA (52a). This finding further reinforces the evidence that the effect of the oncogene on differentiation is not mediated by an action on proliferation. A similar interpretation has been recently proposed for the effect of the *ras* oncogene in preventing differentiation of C2 myogenic cell line: myoblasts transfected with activated *ras* genes exhibit normal growth properties and cease proliferating in the absence of mitogens, indicating that *ras* inhibits differentiation through a mechanism independent of cell proliferation (43, 46). In contrast, the block of differentiation observed in quail myoblasts transformed by a v-*myc* oncogene appears to be a consequence of the continuous proliferative state in which the cells are forced; if v-*myc*-transformed myoblasts are growth arrested by cocultivation with normal cells, their differentiation ability is rapidly reacquired (33).

The present studies on transcription of endogenous muscle-specific genes and transfected CAT reporter gene under the control of muscle-specific promoters in RSV-transformed myoblasts show that the oncogene exerts its control on differentiation mainly by affecting transcription. However, further mechanisms operating at the posttranscriptional level, for example on mRNA stability, cannot be excluded. An action of v-*src* on mRNA stability would account for the rapid effect exerted by the oncogene on the down-regulation of some of the muscle transcripts analyzed, particularly of the MHC transcript, which in a mouse muscle cell line has been reported to have a half-life of 55 h (40).

The action of v-*src* on transcription of muscle-specific genes represents one aspect of the pleiotropic effects exerted on a variety of cell systems. In most differentiating cells analyzed so far, v-*src* interferes with expression of the differentiated phenotype (3). Indeed, v-*src* specifically suppresses tissue-specific genes or functions such as melanin synthesis in retinal melanoblasts (6), type II collagen synthesis in chondroblasts (1), type I collagen synthesis and sensitivity to ascorbate in tendon cells (49), and neurotransmitter uptake and surface markers in embryonic neuroretina cells (10, 15). Paradoxically, the oncogene is capable of inducing differentiation toward a neuronal phenotype in PC12 cells (2) and in avian sympathetic neurons (29). In a variety of differentiating cell systems, one level of action of v-*src* is the selective regulation of transcription: expression of *src* in chicken fibroblasts represses transcription of type I procollagen (48) and fibronectin (53) genes, whereas it activates the expression of the same genes in chondroblasts concomitantly with suppression of type II procollagen (1). The recent findings that the tyrosine kinase activity of the oncogene increases the amount of a CCAAT-binding factor present in the nucleus of infected rat 3Y1 fibroblasts (20) and causes a marked and rapid increase in AP-1 DNA-binding activity in infected Rat-1 cells (59) further indicate that this cytoplasmic oncogene is capable of influencing nuclear events that may, in turn, affect the transcriptional activity of genes downstream.

Given the potential ability of the v-*src* oncogene to modulate the activity of nuclear transcription factors, we studied the effect of v-*src* transformation on the expression of CMD1, the avian homolog of the recently discovered muscle regulatory MyoD gene, coding for a muscle-specific transcription factor. A simple way to explain the inhibitory effect of v-*src* on transcription of a number of different muscle-specific genes would be via the control of myogenic regulatory genes, as reported for *ras*- and *fos*-transformed C2 cells (32, 35), as well as shown here for v-*myc*-transformed quail myoblasts. However, we found that transcription of CMD1 gene was not significantly affected by v-*src*-transformation, neither in proliferating myoblasts at 35°C nor upon $pp60^{v\text{-}src}$ reactivation in postmitotic myotubes. Therefore, it appears that $pp60^{v\text{-}src}$-induced block of differentiation and down-regulation of muscle-specific genes in myotubes do not require silencing of CMD1 gene transcription. Hence, in our system the v-*src* oncogene might inhibit differentiation through an alternative pathway, similar to that of growth factors in the mouse C2 cell line; in these cells, fibroblast growth factor and transforming growth factor beta inhibit

muscle-specific gene expression even in the presence of high levels of exogenous MyoD (54).

The finding that inhibition of differentiation in v-*src*-transformed cells is not achieved by transcriptional suppression of CMD1 contrasts in some way with the recent reports that transformation and block of differentiation of C2 myoblasts by activated *ras* or *fos* correlates with suppression of MyoD transcription (32, 35). It should be noted, however, that C2 myoblasts are an established cell line (63), whereas we used quail primary myoblasts infected with acutely transforming retroviruses to maintain the cells as close as possible to the in vivo phenotype. Thus, it is possible that regulation of MyoD expression varies according to the cellular context analyzed.

Our findings do not exclude the possibility that the v-*src* oncogene is affecting CMD1 function by interfering with its posttranscriptional regulation or its interaction with other proteins. Experiments to test the transactivating function of CMD1 in the presence of active $pp60^{v\text{-}src}$ have been attempted by transfecting the 4R-CAT construct (57), containing four MyoD-binding sites, into *ts* RSV-transformed quail myoblasts. Preliminary results indicate that significant expression of plasmid 4R-CAT is detectable only at the restrictive temperature (not shown), thus suggesting that the CMD1 transactivating function is inhibited at the permissive temperature, although the gene is actively transcribed. Other possible targets of the oncogene are positive and negative factors interacting with MyoD. The MyoD protein can form heterodimers in vitro with other nuclear factors such as E12 and Id through its helix-loop-helix domain (5, 16), and heterodimeric complexes can either enhance (16) or attenuate (5) sequence-specific DNA-binding activity of MyoD. Thus, E12 nuclear factors would behave as positive regulators of myogenesis, and Id factors would behave as negative regulators.

The apparent discrepancy observed in the regulation of MyoD transcription in different transformed cells and the discovery of other myogenic regulatory genes (7, 8, 21, 61) suggest the existence of alternative pathways involved in the suppression of muscle-specific gene expression by oncogenes. It is expected that the availability of avian-specific reagents and the analysis of how expression of the newly discovered nuclear factors and their interactions are regulated in *ts src*-transformed myoblasts will help to identify the molecular targets of the transforming protein.

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