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Myogenic Differentiation of L6 Rat Myoblasts: Evidence for Pleiotropic Effects on Myogenesis by RNA Polymerase II Mutations to α-Amanitin Resistance

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To assess the functional role of RNA polymerase II in the regulation of transcription during muscle differentiation, we isolated and characterized a large number of independent α-amanitin-resistant (Ama^R) mutants of L6 rat myoblasts that express both wild-type and altered RNA polymerase II activities. We also examined their myogenic (Myo) phenotype by determining their ability to develop into mature myotubes, to express elevated levels of muscle creatine kinase, and to synthesize muscle-characteristic proteins as detected by two-dimensional polyacrylamide gel electrophoresis. We found a two- to threefold increase in the frequency of clones with a myogenic-defective phenotype in the Ama^R (RNA polymerase II) mutants as compared to control ethyl methane sulfonate-induced, 6-thioguanine-resistant (hypoxanthine, guanine phosphoribosyl transferase) mutants or to unselected survivors also exposed to ethyl methane sulfonate. Subsequent analysis showed that about half of these myogenic-defective Ama^R mutants had a conditional Myo(ama) phenotype; when cultured in the presence of amanitin, they exhibited a Myo^- phenotype; in its absence they exhibited a Myo^+ phenotype. This conditional Myo(ama) phenotype is presumably caused by the inactivation by amanitin of the wild-type amanitin-sensitive RNA polymerase II activity and the subsequent rise in the level of mutant amanitin-resistant RNA polymerase II activity. In these Myo(ama) mutants, the wild-type RNA polymerase II is normally dominant with respect to the Myo^+ phenotype, whereas the mutant RNA polymerase II is recessive and results in a Myo^- phenotype only when the wild-type enzyme is inactivated. These findings suggest that certain mutations in the *ama*^{R} structural gene for the amanitin-binding subunit of RNA polymerase II can selectively impair the transcription of genes specific for myogenic differentiation but not those specific for myoblast proliferation.

The L6 rat myoblast cell line isolated by Yaffe (45) forms multinucleate myotubes and synthesizes elevated levels of several muscle-characteristic proteins when grown to confluence in physiological levels of calcium (10, 19, 30, 31, 35–37, 43). Analysis of mRNAs specific for various muscle-characteristic proteins indicates that the primary control for the expression of developmentally regulated genes in this system lies at the level of transcription. For example, the level of myosin heavy-chain mRNA, assayed by its ability to stimulate myosin heavy chain translation in vitro, increases during terminal myogenic differentiation (2). More recent molecular hybridization experiments have shown definitively that development is correlated with changes in the steady-state mRNA levels for myosin heavy chains, myosin light chains, and α-actin (3, 38), and with changes in the rate of rRNA synthesis (26). Similar changes in the levels of mRNA coding for muscle-characteristic proteins have been attributed to transcriptional control in chicken (34) and quail primary muscle cultures (4, 20). (For reviews, see reference 35a.)

In view of the probable complexity of the detailed molecular mechanisms responsible for the orderly transcription of muscle genes during myogenic differentiation, we decided to introduce mutations into the regulatory elements that control transcription specificity in this system. We focused our attention on mutations that affect RNA polymerase II because studies in procaryotes have shown that mutant RNA polymerases are sometimes defective in transcrip-

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tion specific for certain "developmental" programs such as those involved in bacterial sporulation (11, 41, 42) or bacteriophage growth (13, 14, 27). L6 mutant cell lines containing altered forms of RNA polymerase II can be obtained by selecting mutants able to grow in the presence of the mushroom toxin α-amanitin (8, 39), since this drug is a specific inhibitor of RNA polymerase II (25, 28). Most of these L6 myoblast α-amanitin-resistant (Ama^R) mutants, like those isolated in other mammalian cell lines (1, 5–7, 21–23, 44), contain altered RNA polymerase II activities. In the myoblast Ama^R mutants isolated from diploid and tetraploid parent lines, both wild-type and mutant forms of the enzyme are expressed, indicating that the *ama*^{R} mutation is dominant in L6 cells (8, 40), as it is in CHO cell hybrids (29). Consequently, growth of such mutant cells in amanitin is not significantly impaired, although the sensitive wild-type activity of the enzyme is lost. In amanitin, there is an increase in the concentration of the resistant mutant form of RNA polymerase II that compensates for the loss of the wild-type form (9, 17, 18, 22, 39). These properties allowed us to modulate experimentally the intracellular activity levels of the mutant and wild-type form of RNA polymerase II in these Ama^R mutant cell lines by adjusting the concentration of amanitin in the growth medium.

In our initial characterization of L6 Ama^R mutants, we noticed that some isolates were unable to form myotubes as readily as others (or as readily as their wild-type parent). Two explanations for this observation seemed reasonable: (i) the mutant RNA polymerase II is defective in the transcription of a function or functions resulting in a pleiotropic effect on myogenesis; or (ii) other secondary mutations induced by the mutagen treatment before the selection for amanitin resistance are responsible for the myogenic-defective phenotype. The first explanation is based on the known pleiotropic effects of RNA polymerase mutations on bacterial sporulation or phage development (cited above). The latter explanation is based on the observation that acquisition of the neoplastic "transformed" phenotype results in a loss of the myogenic-positive phenotype in the rat myoblasts (24). Since Ama102, the first L6 Ama^R mutant characterized, exhibited some properties of the transformed state, such as the loss of anchorage-dependent growth control (39), we were prejudiced in favor of the second hypothesis. To distinguish between these two models, we decided to isolate and characterize the myogenic phenotype of independent Ama^R mutants to see whether there was any correlation between the selected Ama^R and the unselected myogenic-defective phenotypes. In the course of this study, we found such a correlation. Moreover, about half of the myogenic-defective mutants exhibited a conditional amanitin-dependent Myo(ama) phenotype, i.e., the Myo^- phenotype was expressed only in the presence of amanitin. These experiments have led us to conclude that some RNA polymerase II *ama*^{R} mutations cause a pleiotropic defect in rat myoblast differentiation.

MATERIALS AND METHODS

Mutant cell lines and culture conditions. The wild-type cell line L6(D1) that was used as the parent cell line for the selection of Ama^R and thioguanine-resistant (Thg^r) mutants was a freshly cloned isolate of L6(D0) (8). It is a pseudodiploid, amanitin-sensitive (Ama^s), myogenic-positive (Myo^+) clone. All cell lines derived from L6(D1) were cultured at 37°C in 90% (vol/vol) Dulbecco modified Eagle medium and 10% vol/vol fetal calf serum (GIBCO Laboratories) as described previously (39).

Cultures of L6(D1) were divided, and portions (10^6 cells per 100-mm dish) were mutagenized separately by exposure to 500 μg of ethyl methane sulfonate (EMS; Eastman Organic Chemicals) per ml in 90% Dulbecco modified Eagle medium–10% fetal calf serum for a period of 24 h (approximately 50% survival) as described previously (8). After mutagenesis, the cells were grown in fresh medium for 4 days to permit the fixation and phenotypic expression of any induced mutations. Each independently mutagenized culture was then split into two (10^6 cells per 100-mm dish) and was challenged with either 3 μM α-amanitin (Boehringer-Mannheim Corp), or 30 μM 6-thioguanine (2-amino-6-mercaptopurine; Calbiochem). Throughout the selection, the medium was changed one to two times during a period of between 1.5 to 4 weeks. Surviving colonies were picked (a maximum of two colonies per independently mutagenized culture dish) and replated in the concentration of drug in which they were selected. The apparent mutation frequencies of $\sim 1.5 \times 10^{-6}$ for α-amanitin resistance and $\sim 2 \times 10^{-6}$ for 6-thioguanine resistance were similar to those observed previously (8). Spontaneous mutation frequencies (no EMS) were approximately 10- to 20-fold lower in both cases. A total of 43 Ama^R mutants and 55 Thg^r mutants derived from independently mutagenized cultures were obtained from these selections. Two different cultures of the wild-type parent cell line L6(D1) were also independently mutagenized by the same procedure as described above and subcloned immediately into Micro Test II tissue culture plates (Falcon Plastics). A total of 36 and 44 clonal isolates were obtained from the first and second mutagenized cultures, respectively.

RNA polymerase and creatine kinase activity assays. RNA polymerase activity was determined in cell lysates in assays dependent on either the endogenous DNA as template (in 0.4 M ammonium sulfate), or on exogenous DNA (in 0.1 M ammonium sulfate) as described previously (8, 9, 39). Creatine kinase specific activities were determined by direct fluorometric assay as described previously (31).

Cell labeling and two-dimensional polyacrylamide gel electrophoresis. Cell cultures (5×10^4 to 8×10^5 cells

per 100-mm dish) were labeled for 2 to 3 h in 90% Dulbecco modified Eagle medium–10% fetal calf serum containing 250 μCi of [^{35}S]methionine (200 μM methionine, 100 μCi/ml) by using the same medium in which the cells were growing. Cells were lysed directly in sodium dodecyl sulfate-mercaptoethanol, precipitated with trichloroacetic acid, and then precipitated with acetone, boiled for 3 min, and made up in urea-Nonidet P-40 ampholyte lysis solution by the method of O'Farrell (33). Isoelectric focusing was done on samples containing 2.5×10^5 cpm by use of a mixture of 1% vol/vol (pH 3.5 to 10) and 4% vol/vol (pH 5 to 8) ampholytes. The effective pH ranged from 4 to 8. Samples were run in the second dimension on 5 to 20% wt/vol polyacrylamide gradient gels. Marker proteins were included to establish the apparent molecular weight scale.

RESULTS

RNA polymerase II in Ama^R mutants. RNA polymerase II activities from L6 wild-type cells and the Ama^R mutants were assayed in cell lysates in varying concentrations of amanitin to ensure that the Ama^R mutants isolated after EMS mutagenesis had amanitin-resistant RNA polymerase II activities. Cells were harvested, quick-frozen, and stored at −70°C. After storage (<1 week), cell lysates (~10^7 cell equivalents per ml) were prepared by freezing and thawing four times and were assayed for RNA polymerase II activities in 0.4 M ammonium sulfate, as described previously (8, 9, 40). Under these conditions, RNA polymerase activity is dependent on the endogenous DNA template (9). RNA polymerase I activities (determined at concentrations of amanitin up to 1,000 μM and representing about 20 to 30% of the total activity) were subtracted from the total RNA polymerase activity values to yield RNA polymerase II activities. RNA polymerase III activity was <2% of total activity and was not taken into consideration. Estimates of the inhibition constant for amanitin, K_i(ama), were comparable to those determined in other assays dependent on an exogenous DNA template (data not shown). Amanitin-resistant RNA polymerase II activity was detectable in all of the 43 Ama^R mutants analyzed. There was no problem in distinguishing the mutant form of the RNA polymerase II activity from RNA polymerase I activity (which is completely resistant to amanitin), because all mutant forms of the enzyme were eventually inhibited by high concentrations of amanitin. Inhibition curves for RNA polymerase II activities from a representative series of Ama^R mutants grown in 1 μM amanitin for at least 3 days indicated that there is an almost complete inactivation of the wild-type form of RNA polymerase II (II^s) in vivo, leaving mainly the resistant form (II^R) to be titrated in vitro, as shown in Fig. 1A. The inhibition curves for RNA polymerase II determined under these conditions for all the mutants were roughly monophasic, yielding values for the inhibition constant, K_i(ama), that were ~5- to ~3,000-fold greater than that for the wild-type enzyme (1 to 2 nM amanitin). Different mutants had widely differing levels of resistance, reflecting the independent nature of their *ama*^{R} mutations.

To determine the relative proportion of the wild-type to the resistant form of the RNA polymerase II activity in these mutants, cells were also grown in the absence of amanitin for a period of at least 1 week before assay, a period that completely restores the level of the wild-type enzyme in vivo. Although determination of the exact ratio of RNA polymerase II^R to II^s activity in mutants containing the less resistant forms of enzyme (<100-fold more resistant to amanitin than the wild-type form) was difficult, we were able to determine this ratio in mutants containing more resistant forms of the enzyme (Fig. 1B). All mutants analyzed were found to express the wild-type form of the enzyme as well as the mutant form. The ratio of II^s/II^R RNA polymerase II activities was 1:1 in 22 of 25 mutants, as expected, since the L6(D1) parent contains a pseudodiploid chromosome complement (39 ± 1).

Of the remaining three Ama^R mutants that had II^s/II^R ratios greater than 1, Ama17-1 had a ratio of ~4:1, and the other two mutants, Ama21-1 and Ama28-1, yielded ratios of 2.5:1. Karyotype analysis of Ama17-1 showed that it contained a pseudotetraploid complement of chromosomes (75 ± 1) in accord with the higher ratio of the two forms of the enzyme (8). However, both Ama21-1 and Ama28-1 had pseudodiploid karyotypes (chromosome numbers of 39 ± 1 and 38 ± 1, respectively). The reason for their aberrant RNA polymerase II^s/II^R ratio is unknown; perhaps the resistant enzyme is unstable, or the mutation also affects synthesis.

Myogenic phenotypes of Ama^R mutants. (i) Myoblast fusion and muscle CKM. The myogenic properties of the Ama^R mutants were determined by observation of their ability to form myotubes after reaching confluence and by assay of their levels of muscle creatine kinase (CKM) enzyme activity. The morphological transformation accompanying wild-type L6(D1) fusion is illustrated in Fig. 2. Wild-type L6 myoblasts in confluent cultures aligned (Fig. 2A) and fused into myotubes within a few days (Fig. 2B). Many of the Ama^R mutants, in contrast, did not undergo this process. The morphology of two representative Myo$^-$ Ama^R mutants, Ama44-1 and Ama19-1, which exhibited very low levels of myotube formation even 8 days after reaching confluence, are shown in Fig. 2C and 2D. Accurate quantitation of myotube for-

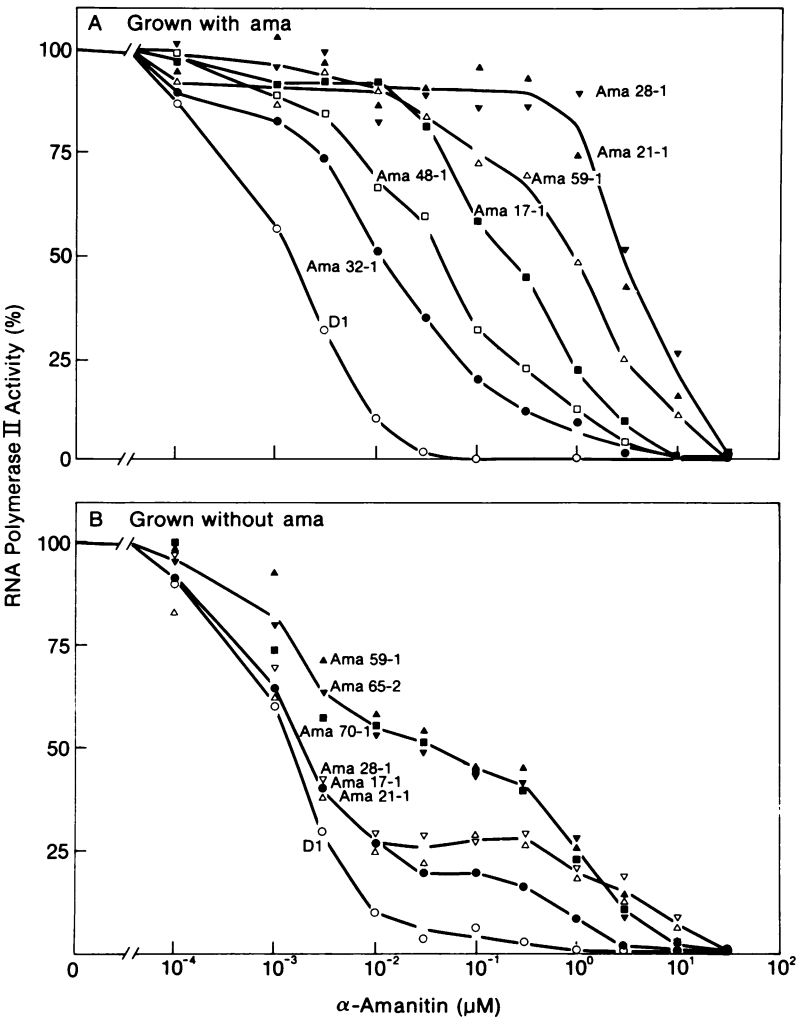

FIG. 1. Inhibition in vitro by amanitin of RNA polymerase II activities from wild type and Ama^R mutants grown in the presence and absence of amanitin. (A) RNA polymerase II activities from Ama^R mutants grown in 1 μM amanitin (ama) for at least 3 days before assay. [The wild-type cell line L6(D1) was not grown in amanitin.] Total RNA polymerase activities assayed in the absence of α-amanitin ranged from 1,000 to 7,000 cpm of [^{3}H]UMP incorporated over a background of about 100 cpm. D1, L6(D1) (wild-type parent). (B) RNA polymerase II activities from wild type and Ama^R mutants grown for at least 1 week in the absence of amanitin. Cells were harvested, frozen, and thawed four times as described for (A) and then sonicated, diluted, and centrifuged as described previously (8, 39). S-100 supernatants (~0.5×10^7 to ~1.0×10^7 cell equivalents per ml) were assayed for RNA polymerase activity in 0.1 M ammonium sulfate as previously described (8, 39). In this assay, RNA polymerase activities are dependent on an exogenous DNA template and more accurate values of the ratios of the wild-type to mutant forms of RNA polymerase II activity can be determined. RNA polymerase I activities (determined at concentrations of amanitin up to 1,000 μM) contribute about 30 to 40% to the total RNA polymerase activity assayed in the absence of amanitin and were subtracted from the total to yield RNA polymerase II activities. Total RNA polymerase activities assayed in the absence of amanitin ranged from 1,000 to 7,000 cpm of [^{3}H]UMP incorporated above a background of ~50 cpm. D1, L6(D1) (wild type).

mation in these rat myoblast cultures by measuring the fraction of nuclei in myotubes was difficult, because L6 fuses at higher cell densities than is generally the case in primary cultures of rat or chick myoblasts. Since we found that L6 myoblast fusion and CKM specific activities showed a strict temporal correlation, we used CKM specific activity measurements to quantitate differentiation, rather than determinations of myotube formation.

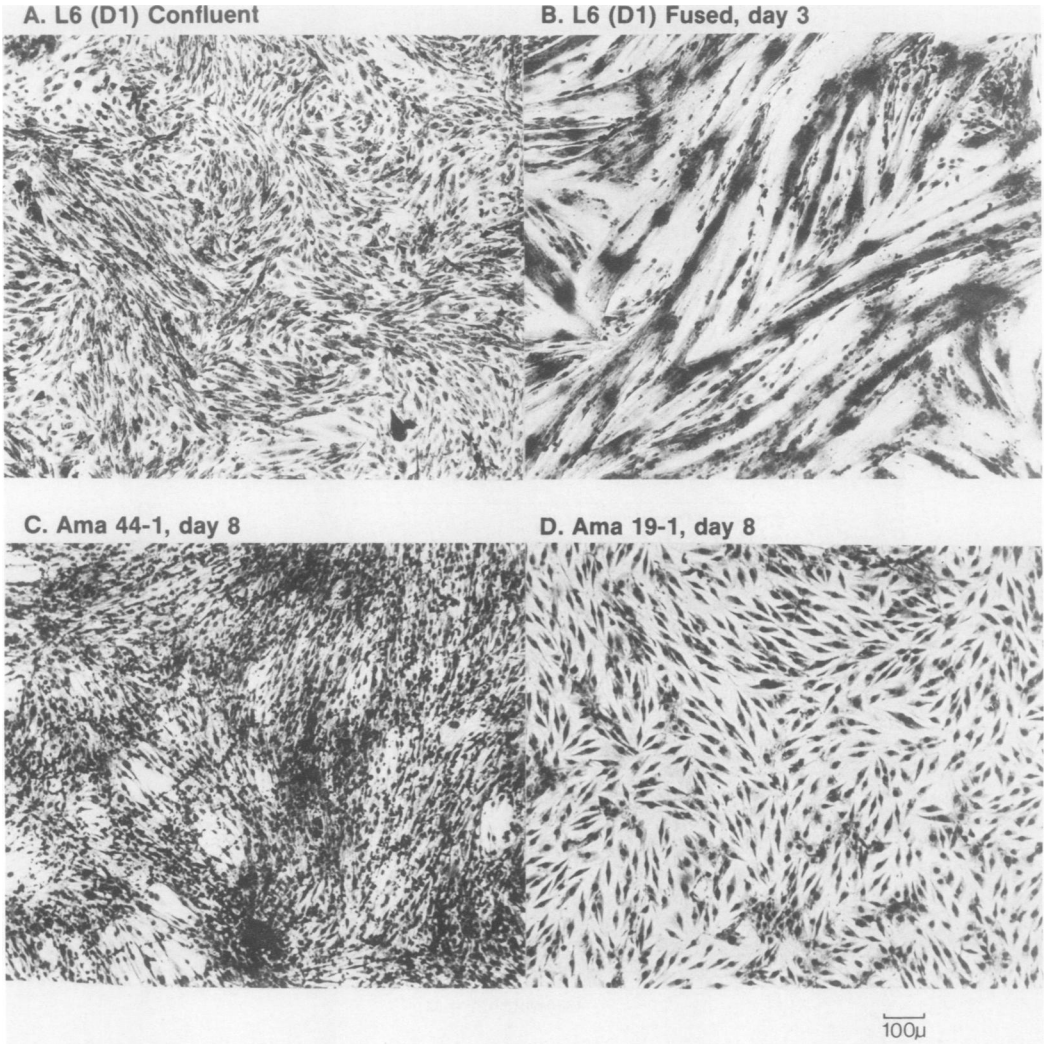

FIG. 2. Morphology of L6(D1) wild type and two representative Ama^R cultures at confluence. (A) L6(D1) myoblasts aligned at confluence. (B) L6(D1) myotubes formed by fusion of myoblasts 3 days after aligning as shown in (A). (C) Ama44-1 myoblasts 8 days after reaching confluence in 1.5 μM amanitin. (D) Ama19-1 myoblasts 8 days after reaching confluence in 1.5 μM amanitin. Photographs are of Giemsa-stained cultures.

Using this criterion, we found that the myogenic phenotype of the Ama^R mutant cell lines, when grown in amanitin, could be conveniently grouped into two classes, myogenic positive and myogenic defective. (These assays were initially performed on cells grown in amanitin to reduce the effect of the wild-type enzyme on cell physiology and to emphasize that of the mutant enzyme). Myogenic-positive clones were arbitrarily defined as those capable of fusing extensively into multinucleate myotubes and also of increasing their CKM levels a minimum of 5- to 10-fold (>0.10 U/mg of protein) within 1 to 4 days after reaching confluence. Myogenic-defective clones were defined as those that showed a decreased ability to fuse into multinucleate myotubes and exhibited an increase in CKM of <0.04 U/mg of protein by day 4.

The myogenic-defective mutants were further subdivided into two categories based on their myogenic phenotypes in the absence of amanitin, a condition under which the wild-type form of RNA polymerase II is also active in the mutant cell. Cells in one category (Myo^-) showed no alteration in myogenesis and remained myogenic defective independent of amanitin. Cells in the other category showed an altered myogenic phenotype in the absence of

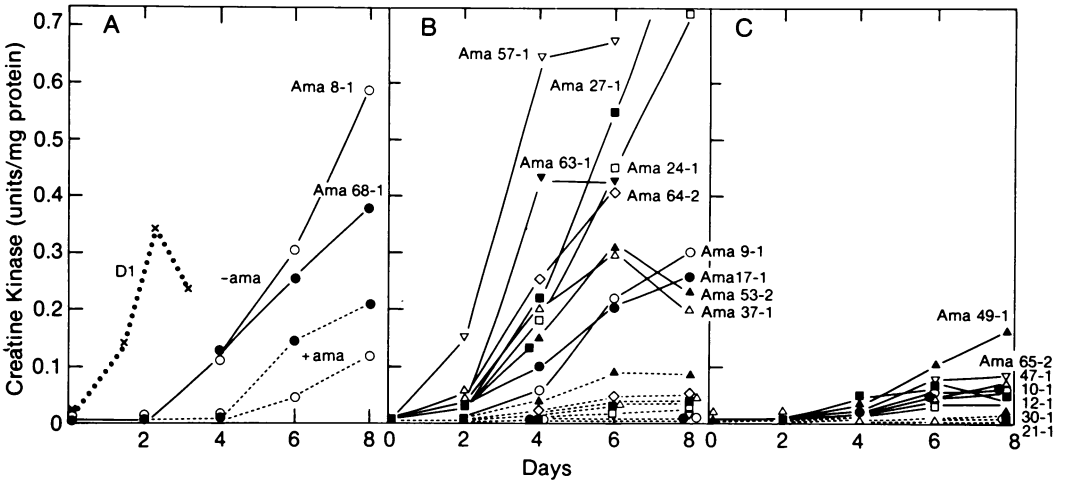

FIG. 3. CKM specific activities as a function of time in culture for Ama^R mutants that exhibit a conditional Myo(ama) phenotype. Cell lines that had been grown either in the absence of drug (for at least 1 week) or in the presence of 1.5 μM amanitin (for at least 3 days) were trypsinized and seeded into dishes at a density of 2×10^5 cells per 35-mm dish in the absence or presence of 1.5 μM amanitin, respectively. At various times after reaching confluence (in most cases, 2 days after seeding), cultures were harvested, and extracts were made corresponding to $\sim 7 \times 10^6$ cell equivalents per ml for assays of CKM activity and total protein ($\sim$3 mg of protein per 10^7 cells). CKM activities ranged from <4 to 500 nmol of creatine produced per 20 min, above a background of $\sim$8 nmol of creatine. Day 0 corresponds to the day the culture reached confluence. The solid and broken lines represent mutants grown in the absence and presence of amanitin, respectively. (A) Ama^R mutants that exhibit a highly Myo$^+$ phenotype when cultured in the absence of amanitin and a reduced Myo$^-$ phenotype in the presence of amanitin. (× · · · · ×) L6(D1) parent. (B) Ama^R mutants that show a highly Myo$^-$ phenotype when grown in the absence of amanitin, but exhibit a much more Myo$^-$ phenotype in the presence of the drug. (C) Ama^R mutants that have a greatly reduced Myo$^-$ phenotype when cultured in the absence of amanitin and a totally Myo$^-$ phenotype in the presence of amanitin. These arbitrary groupings give some indication of the variation in the Myo phenotype seen in independent Ama^R mutants.

amanitin, exhibiting a more myogenic-positive phenotype. This category is said to have a conditional myogenic-defective phenotype, Myo(ama), which appears to be correlated with the relative activity level of the mutant form of RNA polymerase II.

The CKM profiles for a representative set of these Myo(ama) mutants at various times after being grown to confluence are shown in Fig. 3. The variation in the Myo phenotype represented here indicates that different *ama*^{R} mutations can lead to quite different extents of developmental defectiveness. It also reconfirms the independent nature of the mutations.

The frequency of appearance of each of the Myo$^+$, Myo$^-$ and Myo(ama) phenotypes among the 43 independent Ama^R mutants is shown in Table 1. For comparison, the frequencies of the Myo$^+$ and Myo$^-$ phenotypes for parallel EMS-treated Thg^r mutant clones and unselected EMS survivors are also listed. The 55 independent Thg^r mutants were used as a basis for comparison with the Ama^R mutants on the assumption that mutations in hypoxanthine, guanine phosphoribosyl transferase would not lead to pleiotropic effects on myogenesis and hence would serve as a control for the influence of second-site mutations. The 80 unselected clones surviving EMS mutagenesis would provide an added control for the effect of mutagen treatment on the frequency of appearance of the Myo$^-$ phenotype. The frequency of the myogenic-defective phenotype was two- to threefold higher in the Ama^R class than in either of the control populations. These data suggest that mutation to the Ama^R phenotype more frequently leads to a loss of the capacity to differentiate normally in L6 myoblasts than do mutations to the Thg^r phenotype.

(ii) Patterns of polypeptide synthesis in the Ama^R mutants. Using two-dimensional isoelectric focusing and polyacrylamide gel electrophoresis (12), we further analyzed the myogenic phenotype of individual Ama^R mutants by examining the patterns of [^{35}S]methionine-labeled polypeptides synthesized in exponentially growing and postconfluent cultures, maintained in the absence and presence of amanitin. Autoradio-

TABLE 1. Frequency of myogenic-defective phenotypes in mutagenized L6(D1) cell cultures selected for amanitin resistance and thioguanine resistance

Selected phenotype (no. of clones)	Myogenic phenotype (%)		
	Defective		Positive
	Myo$^-$	Myo(ama)	Myo$^+$
Ama^R (43)	32	33	35
Thg^r (55)	25	NA[a]	75
Control[b] (80)	19	NA[a]	81

[a] NA, Not applicable. None of the Thg^r clones showed any influence of thioguanine on myogenesis.

[b] Control cultures were EMS-treated, unselected L6(D1) cells (see text).

grams showing these patterns for three mutants representative of the Myo$^+$, Myo$^-$ and Myo(ama) phenotypes are shown in Fig. 4.

The myoblast and myotube polypeptide patterns for the Myo$^+$ mutant, Ama67-1, grown in amanitin (Fig. 4A and B) were the same as those for the L6(D1) parent grown in the absence of the drug (data not shown). (No obvious differences in polypeptide amount or shifts in electrophoretic mobility were seen that might indicate that the EMS treatment used to generate Ama67-1 caused any mutations detectable by this method of analysis.) Mutants of the Myo$^-$ category do not synthesize muscle-characteristic proteins normally; for most, the polypeptide patterns at confluence are the same as during active growth.

Analysis of the polypeptides in the Myo$^-$ mutant Ama19-1 showed several particularly interesting differences (Fig. 4C and D). In the absence or presence of amanitin, the patterns for this mutant looked similar. Exponentially growing Ama19-1 myoblasts had patterns that looked similar to those for wild-type L6(D1), but confluent cultures did not. Instead, confluent cultures showed no shut-off of myoblast-characteristic proteins (fibronectin?) and an incomplete turn-on of myotube-characteristic proteins (myosin light chains LC1 and LC2 plus troponin C, as indicated by the arrows in Fig. 4D). Clearly, there is a perturbation in the regulation of the pattern of muscle-characteristic protein synthesis in this mutant.

Finally, the patterns of polypeptides synthesized at confluence in Ama27-1, a representative of the Myo(ama) conditional category, are shown in Fig. 4E and F. These cells fused in the absence of amanitin and showed a pattern of protein synthesis characteristic of wild-type myotubes. In contrast, in the presence of amanitin these cultures did not fuse, and the pattern of proteins synthesized looked the same as that of wild-type myoblasts. Therefore, the amanitin-conditional defect in myogenesis in Ama27-1 appears to affect the global expression of muscle-characteristic genes.

The Myo$^-$ and Myo(ama) phenotypes of the Ama^R mutants of L6 could conceivably represent specific subsets of RNA polymerase II alterations affecting amanitin binding and cell physiology in some ordered fashion. Therefore, we looked for correlations between the developmental phenotype of each Ama^R mutant clone of L6(D1) and either the degree of resistance to amanitin of the mutant enzyme, as measured by the K_i(ama), or the cell doubling time. The K_i(ama) values for individual clones ranged from 5 to 20 nM up to 2,000 to 4,000 nM (Fig. 1), and the cell doubling times ranged from 15 to 29 h (data not shown). We could find no correlations between either of these factors and the myogenic phenotype of individual mutants. Also, we found no correlation between the doubling time (plus or minus amanitin) and the level of resistance of the mutant RNA polymerase II for individual mutants.

DISCUSSION

The observations reported here support the hypothesis that certain RNA polymerase II mutations to α-amanitin resistance specifically perturb myogenic differentiation in L6 rat myoblasts. This hypothesis is based on the following considerations. (i) The frequency of occurrence of the myogenic-defective phenotype is two- to threefold higher in RNA polymerase II mutant clones selected for their Ama^R phenotype than it is in control Thg^r mutants or EMS-treated unselected myoblast clones. (ii) About one-half of the myogenic-defective Ama^R mutants exhibit a Myo(ama) conditional phenotype. These mutants, when grown in amanitin, are impaired in their ability to form myotubes, to express increased levels of CKM, and to synthesize muscle-characteristic protein as determined by two-dimensional gel analysis. Since the presence of amanitin results in the inactivation of the wild-type form of the enzyme and an elevated level of the resistant form, the myogenic-defective phenotype of those Ama^R Myo(ama) mutants correlates with the relative activity level of the mutant enzyme. A trivial explanation for the Myo(ama) phenotype (that amanitin acts on some other site than RNA polymerase II to inactivate myogenesis) is eliminated by consideration of the properties of the Ama^R Myo$^+$ class of mutants in which amanitin has no effect on myogenesis. Thus, in the Myo(ama) mutants at least, the altered RNA polymerase II appears unable to activate the proper transcriptional program required for myogenesis, presumably because it is unable to interact properly with DNA control sites or

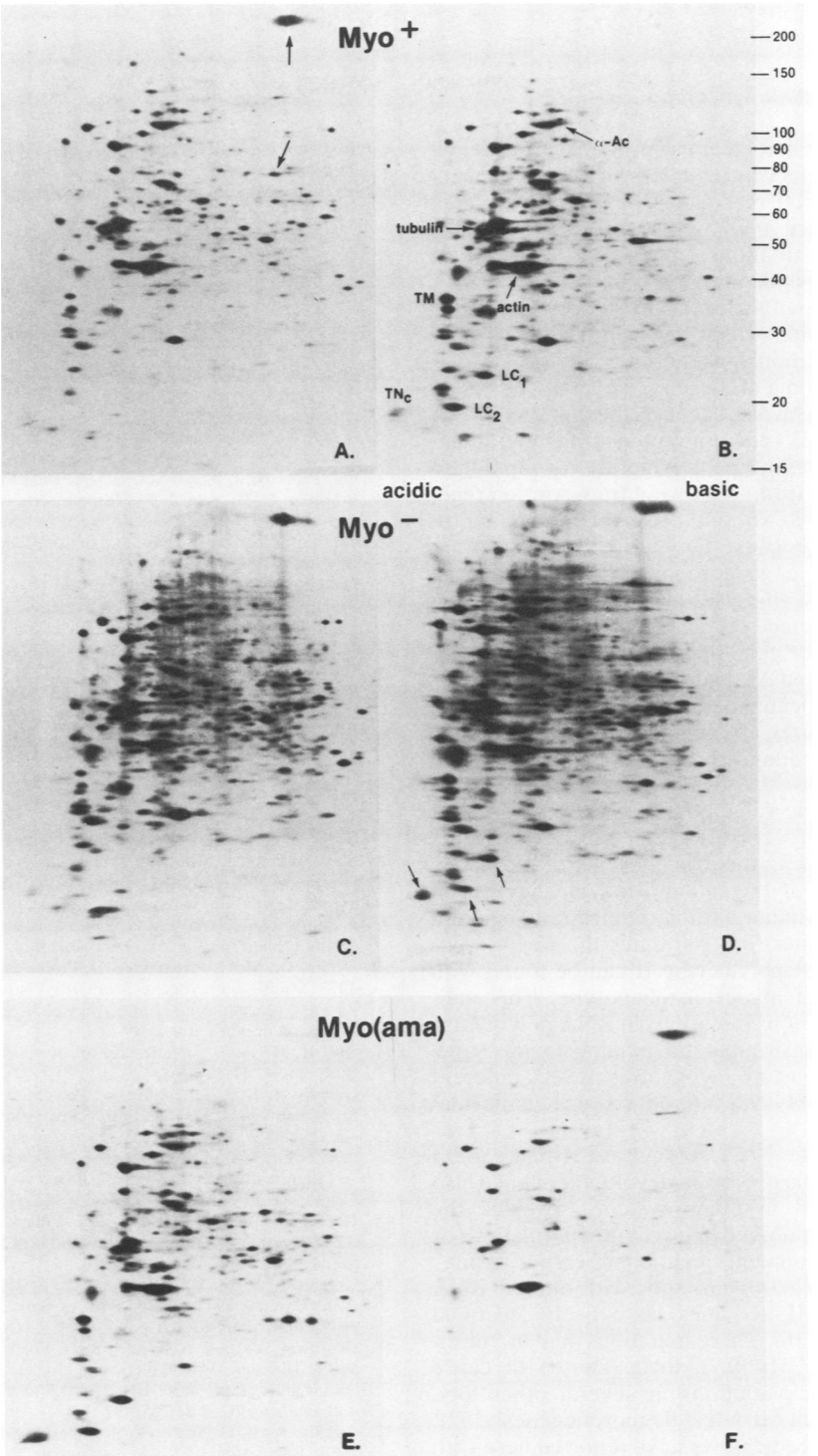

FIG. 4. Two-dimensional gel electrophoresis pattern of labeled polypeptides synthesized in Ama^R mutants exhibiting the Myo^+, Myo^-, and Myo(ama) phenotypes. Autoradiogram of polypeptides synthesized in Myo^+ Ama67-1 cells grown in the presence of 1.5 μM amanitin. Exponentially growing myoblasts (A) and confluent fused myotubes (B) were labeled with [^{35}S]methionine as described in the text. Autoradiogram of polypeptides synthesized in exponentially growing (C) and confluent unfused (D) cultures of Myo^- Ama19-1 cells grown in the presence of amanitin. Autoradiogram of polypeptides synthesized in confluent cultures of Myo(ama) Ama27-1 cells grown in the absence (E) and presence (F) of amanitin. Myotubes are formed in the absence of amanitin; fusion does not occur in its presence. The polypeptides labeled in B have mobilities similar to α-actinin (α-Ac), actin, tubulin, α- and β-tropomyosin (TM), myosin light chains (LC_1, LC_2), and troponin (TN_c). Polypeptides labeled with arrows in (A) are subject to developmental regulation during L6 myogenesis although their identity is not known. Polypeptides indicated with arrows in D appear to correspond to troponin and myosin light chains 1 and 2. The scale shows the apparent molecular weights ($\times 10^3$) for marker proteins in the sodium dodecyl sulfate dimension on 5 to 20% polyacrylamide gradient gels. The effective pH range in the isoelectric focusing dimension was roughly 5 to 8.

transcriptional regulatory proteins. The developmental defect in this class of Myo(ama) mutant is not due to secondary mutations unrelated to the primary *ama*^{R} mutations, since any such secondary mutations should not exhibit an amanitin-dependent expression.

In the case of the myogenic-defective class of Ama^R mutants, which have an absolute rather than a conditional phenotype, the situation is less clear. Some of these mutants may be defective in myogenesis because their mutant RNA polymerase II acts in a dominant fashion, competing with wild-type polymerase for binding to control sites (promoters) and transcription regulatory factors without activating them appropriately, or because they simply jam the transcription of a gene required for myogenesis. Alternatively, this class may also contain double mutants affected both in RNA polymerase II and in some other gene important for myotube formation. (Based on the high frequency of appearance of myogenic-defective phenotypes in the control cultures after mutagen treatment, such secondary mutations should be expected in roughly 20 to 25% of the Ama^R mutants compared with the value observed, 32%.)

Attempts to correlate any of the myogenic phenotypes with a particular level of resistance to amanitin of the mutant enzyme have been negative. Mutants with K_i values ranging from roughly 5 times to 3,000 times the K_i for wild-type L6 RNA polymerase II can be found with each class of myogenic phenotype. This indicates that various mutations in RNA polymerase II that diminish amanitin binding to the same extent may perturb myogenesis in very different ways, probably via altered enzyme conformations.

Mutations in RNA polymerase II that exhibit similar specific defects in development have also been isolated in other systems. Besides the bacterial mutants defective in sporulation or phage development mentioned earlier, Ama^R mutants of *Drosophila* spp. with altered RNA polymerase II activities have been described recently (15, 16). These mutations map in "ultrabithorax-like" (*ubl*), a locus known to cause allele-specific variations in several other loci, some of which can affect fly morphogenesis (32). The allele specificity we have reported here with respect to *ama*^{R} mutations and myogenesis in L6 myoblasts may be analogous to that observed in *Drosophila* spp.

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