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Temperature-Sensitive Variants of an Established Myoblast Line

(myoblast differentiation/myosin/phosphorylase/phosphocreatine kinase/nitrosoguanidine)

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ABSTRACT Upon reaching confluency, mononucleated myoblasts fuse into multinucleated myotubes and concomitantly accumulate various characteristic muscle proteins, including myosin, actin, and several enzymes. We have approached the problem of determining the relationship between morphological and biochemical differentiation of muscle cells by isolating a series of temperature-sensitive clones from the established myoblast line, L_6.

Twelve phenotypically variant clones were isolated from mutagenized populations of myoblasts. These fell into five classes, distinguishing conditional growth variants from conditional developmental variants. The phenotype of these strains, at least for the more extensively studied ones, was stable for more than 80 generations.

Synthesis of characteristic proteins such as myosin, glycogen phosphorylase (EC 2.4.1.1), and phosphocreatine kinase (EC 2.7.3.2) has been studied in two conditional developmental mutants. One mutant, E_3, fuses into myotubes at 37° but not at 40°; the other, H_6, does not fuse into myotubes at 37° but does so at 40°. At permissive temperatures the enzymes accumulated in mutant cells with the same time course as in the parent cell line. Myosin accumulated in strain E_3 but not in strain H_6. At nonpermissive temperatures neither fusion into myotubes nor accumulation of any of the proteins occurred in the cells of these two variant lines.

The development of muscle cells *in situ* involves fusion of mononucleated myoblasts into multinucleated myotubes; accumulation of the contractile proteins, actin, myosin, tropomyosin, and troponin; and increase in specific activity of several characteristic enzymes (1). Recently, permanent cell lines were established from rat neonatal skeletal muscle that express many features of muscle development when cultured *in vitro* (2). Myoblast cells, such as those of the line used in this study (L_6E), grow exponentially until a confluent monolayer is established; thereafter many of the cells fuse to form myotubes. At about the time of onset of fusion, the specific activities of glycogen phosphorylase (EC 2.4.1.1), phosphocreatine kinase (EC 2.7.3.2), myokinase (EC 2.7.4.3), and glycogen synthetase (EC 2.4.1.11) increase significantly (refs. 3, 4, and Wahrman and Luzzati, unpublished results). Myosin also accumulates at this stage, ultimately comprising 10–40% of cytoplasmic proteins (5–7). The synthesis of each of these characteristic muscle proteins has been found to be temporally coordinated with the fusion process (ref. 3 and Wahrman and Luzzati, unpublished results). Moreover, Shainberg, Yagil, and Yaffé have shown that reducing the Ca^{++} ion concentration of the medium inhibits both fusion and the increase in enzyme activities (3). When Ca^{++} ion is added to the medium, cell fusion and the increase in enzyme specific activities are rapidly initiated. The observed coordinance suggests that the accumulation of the characteristic enzymes might be dependent upon the fusion process itself or that both enzyme accumulation and fusion might be triggered by the same developmental event.

The relationship between the different events occurring during myoblast differentiation could be determined by the isolation of conditional variants derived from one of the established myoblast lines. A biochemical and morphological analysis of these variant lines should help to elucidate the steps involved in development of myogenic cells.

We have isolated a series of variant clones from populations of myoblast line L_6E, after treatment with *N*-methyl, *N*-nitro, *N'*-nitrosoguanidine (NG). The variants fall into several classes that show temperature-sensitive properties with respect to growth and/or differentiation and have been partially described in a preliminary report (5). In this report, we describe the morphological and biochemical differentiation, which occurs under permissive and nonpermissive conditions, in two variant lines, E_3 and H_6, in which fusion is thermosensitive and thermodependent, respectively.

MATERIALS AND METHODS

Chemicals. [^{14}C]Leucine (100 μCi/mol) and ^{14}C-labeled chlorella hydrolysate (100 μCi/ml) were purchased from Commissariat de l'Energie Atomique; *N*-methyl, *N'*-nitro, *N'*-nitrosoguanidine (NG) was the product of Aldrich Chemical Co., Milwaukee, Wis.

Myoblast Cell Line L_6 was isolated several years ago from rat skeletal muscle (2, 8). Dr. David Yaffé kindly provided us with an inoculum of this line. A subclone, L_6E, derived from a single cell of line L_6 and a subsequent subclone, L_6D, derived from line L_6E, were used as our parental myoblast lines.

The cells were grown either in 60-mm Falcon dishes coated with gelatin (9) or in 35 × 80-mm Falcon culture bottles, at 37° in an atmosphere of 5–7% CO_2 in air. Weymouth medium containing 0.5% chick embryo extract, 10% horse serum, 100 μg/ml of streptomycin per ml, and 5 μg/ml of aureomycin per ml, was used. The culture medium was replaced every 2–3 days. Under these conditions, L_6 cells grow exponentially with a division time of 20–24 hr to a density of 3×10^6 cells per plate. During subsequent incubation, 30–80% of the cells fuse into myotubes. Morphological differentiation was followed by

Abbreviation: NG, *N*-methyl, *N'*-nitro, *N'*-nitrosoguanidine.

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TABLE 1. *Frequency of variant myoblast lines*

Exp. no.	Parental line	Clones* picked	Clones analyzed	Clones with altered phenotype	Clones with parental phenotype
1	L_6E	13	4	4	0
2	L_6E	20	8	6	2
3	L_6D	23	22	2	20
Total		56	34	12	22

* Only clones that grew well at 37° were analyzed further. Several isolates were lost due to inadvertant contamination.

daily microscopic observation of the cultures and determination of the number of myotubes per unit area of the plates.

Mutagenic Treatment. Exponentially growing cells were removed from plastic dishes by treatment with phosphate-buffered saline (pH 7.6) containing 0.5 mg of trypsin per ml (9). After 10–15 min, the cells detached and were collected by centrifugation at 300 × g for 5 min. The pellet was resuspended at 10^5 cells per ml in phosphate-buffered saline (pH 6.5) containing 10 μg of NG per ml, and the suspension was incubated at 37° in a 5% CO_2 atmosphere. Treatment with NG resulted in progressive loss of viability, such that after 30 min incubation only 1–2% of the cells could give rise to viable clones. Thus, after 30 min of incubation in medium with NG, the cells were pelleted by centrifugation, resuspended in fresh medium, immediately distributed into the wells of Falcon microtest plates, at a density of about 50 cells per well, and incubated at 37° for 2 weeks without medium change. Since only about 2% of the cells were viable, about a quarter of the wells received a single viable cell.

Myosin was estimated by measurement of the amount of material in cell extracts that coelectrophoresed with authentic myosin on sodium dodecyl sulfate–acrylamide gels after an initial purification by precipitation of fibrous proteins at low ionic strength (7). The relative rate of myosin synthesis was determined by labeling cultures at 37° for 3 hr in fresh medium

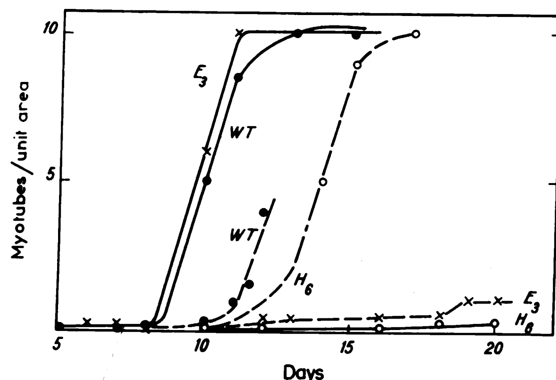

FIG. 1. Morphological differentiation of wild type and two conditional variant myoblast lines. The number of myotubes that formed upon fusion of the myoblasts was monitored microscopically upon incubation at 37° (——) or 41° (- - -) of the parental line. ●, strain L_6E (*WT*, wild type), ×, strain E_3; and O, strain H_6. A unit area is 0.785 mm^2.

TABLE 2. *Phenotypes of variant lines*

Class	No. of isolates	Growth* 37°	Growth* 41°	Differentiation† 37°	Differentiation† 41°
Wild type	22	+	+	+	+
Class I	2	+	0	+	0
Class II	2	+	0	0	0
Class III	5	+	+	+	0
Class IV	1	+	+	0	+
Class V	2	+	+	0	0

All tests were repeated at least twice, once between the 15th and the 20th generation after isolation, and again about 30 generations after isolation, for the 34 clones.

* Growth was scored as positive (+) if the culture increased exponentially with a doubling time of less than 30 hr. Growth was scored as negative (0) if the culture did not double within a week.

† Differentiation was scored as positive if the culture formed at least four myotubes per unit area. Differentiation was scored as negative if less than one myotube per unit area developed within 2 weeks after growth to confluency.

with either [^{14}C]leucine (100 μCi/mol) or ^{14}C-labeled chlorella-protein hydrolysate (1 mCi/mg). The labeled material that coelectrophoresed with authentic myosin was compared to the total amount of label incorporated into 10% trichloroacetic acid-insoluble material in cell extracts.

Enzyme Assays. The specific activities of glycogen phosphorylase (EC 2.4.1.1) and phosphocreatine kinase (EC 2.7.3.2) were determined as described elsewhere (Wahrman and Luzzati, in preparation). Protein was estimated by the method of Lowry et al. (10).

RESULTS

The isolation of variant lines from many eukaryotic cells can be greatly enhanced by treatment with NG (11–14). In order to obtain as high a proportion of variants as possible, cells of myoblast lines L_6E and L_6D were exposed to NG under conditions resulting in 97–99% killing by the mutagen. The mutagenic treatment was performed in three separate experiments (Table 1). From a total of 334 wells inoculated with NG-treated cells, 56 surviving clones derived from single cells were subcultured into 60-mm Falcon dishes and incubated for 3 weeks at 37°. 13 of these clones grew slowly at 37° and were not studied further. 34 clones that grew well at 37° were further analyzed for the presence of temperature-sensitive alterations in growth or fusion. 12 Clones with an altered phenotype were thus isolated. They fell into five classes (Table 2).

(*a*) Classes I and II: growth temperature-sensitive clones. Four isolates failed to grow at 41°. Two of these strains differentiated normally into myotubes at 37° (class I), while the other two did not differentiate even after prolonged growth at 37° (class II). When strain G_4 (class I) was inoculated at low density and incubated at 41°, the cells were seen to divide only once in the next few days and no further growth was observed during the next 3 weeks. We were thus able to isolate revertants of strain G_4 after prolongated incubation at 41° and to estimate its reversion frequency, which appears to be no greater than 1 in 10^5.

(*b*) Classes III and IV: differentiation temperature-sensitive and temperature-dependent clones. Six isolates grew

equally well at both 37° and 41°, but showed temperature-sensitive patterns of differentiation. Five of these failed to differentiate into myotubes at 41°, while differentiating normally at 37° (class **III**). The sixth isolate (H_6) also grew well at both 37° and 41° but failed to differentiate into myotubes at 37°, while differentiating normally at 41° (class **IV**).

The karyotypes of a strain (E_3) of class **III**, of a strain (H_6) of class **IV**, and of the parental strain (L_6E) were determined (15). After about 60 generations in culture, the karyotype of both variant lines was identical to that of the parental line, which contains 39 chromosomes including a characteristic large subtelocentric (the normal rat karyotype consists of 42 chromosomes). The variant phenotypes of strains E_3 and H_6 were stable for up to 80 generations after isolation but, upon subsequent growth, the cultures often gave rise to revertant cells. The characteristics of the revertants and the mechanism of reversion will be reported elsewhere.

(*c*) Class **V**: differentiation negative. Two isolates grew well at both 37° and 41°, but failed to differentiate at either temperature.

Morphological Differentiation of Conditional Developmental Variants E_3 and H_6. In order to determine whether growth at the temperature that is nonpermissive for differentiation would result in a permanent inability of the cells to differentiate into myotubes, cultures of strains E_3 and H_6 were inoculated at 1 to 2 × 10^4 cells per plate and grown at 41° and 37°, respectively, for four generations. The cultures were then shifted to the permissive conditions where another four generations ensued. Upon reaching confluency, cells of both lines fused normally into myotubes (Table 3). It is clear that growth at the developmentally nonpermissive temperatures does not result in a permanent block in differentiation.

We also investigated whether extensive growth and cell division was necessary at the developmentally permissive temperature for the acquisition of competence to differentiate. Strains E_3 and H_6 were grown to confluency at the nonpermissive temperatures before shifting the cultures to the permissive temperature (Table 3). When confluent cultures of strain H_6 were shifted to 41°, extensive fusion occurred within 2 days. Likewise, confluent cultures of strain E_3 gave rise to myotubes when shifted to 37°, but only after several days. It appears that extensive cell division is not required at the permissive temperature but that, at least for cells of strain E_3, a period of incubation at the permissive temperature during the prefusion stage is a necessary prerequisite for fusion.

It seemed possible that cells grown at the permissive temperatures might accumulate stable components, which would permit fusion even when subsequent incubation was at nonpermissive temperatures. Therefore, strains E_3 and H_6 were grown to confluency at 37° and 41°, respectively, and shifted to the nonpermissive temperatures just before the onset of fusion (Table 3). During subsequent incubation at 41°, cells of strain E_3 were found to fuse extensively within a day. When cultures of strain H_6 were grown to confluency at 41° and then shifted to 37° either before or during the fusion process, all subsequent morphological differentiation stopped immediately.

Biochemical Differentiation of Conditional Developmental Variants E_3 and H_6. Experiments on the parent strain L_6E

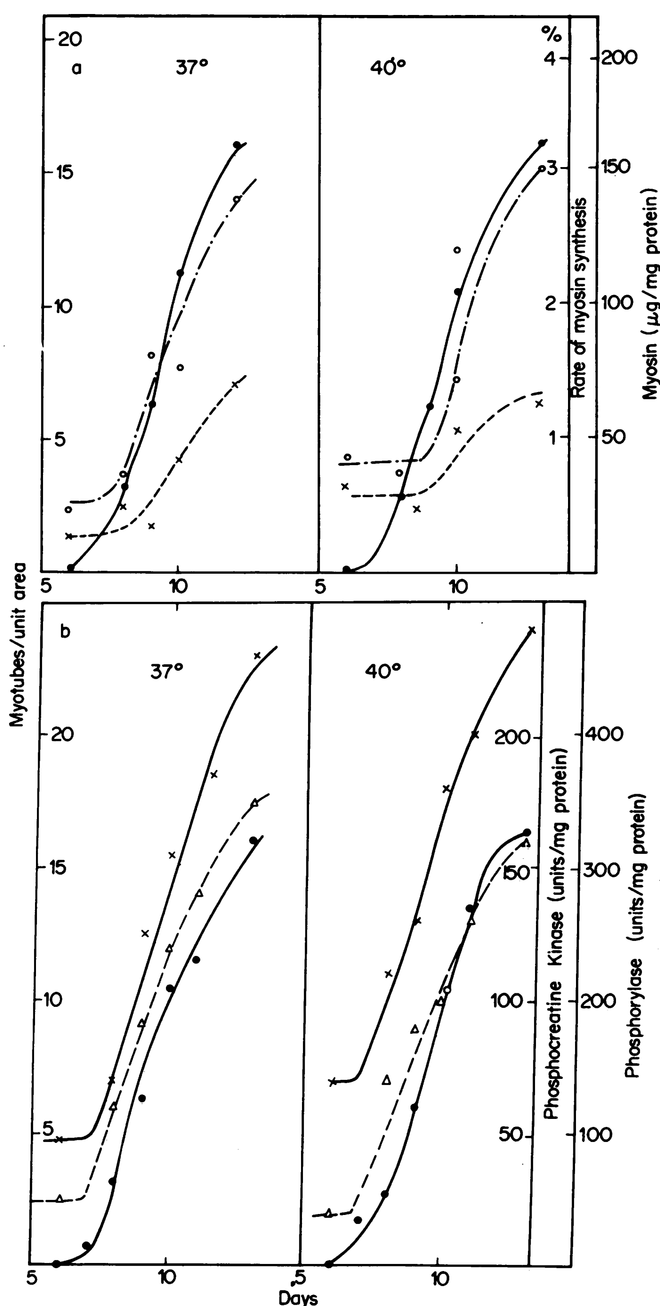

FIG. 2. Biochemical differentiation of parental line L_6E. Cultures of L_6E were initiated at 2 × 10^4 cells per plate and incubated at either 37° or 40° as indicated. (*a*) Fusion into myotubes was monitored microscropically (●——●), while the total amount of myosin (O—·—O) and the rate of myosin synthesis (X- - -X) were determined as described in *Methods*. (*b*) The specific activities of phosphorylase (X——X) and phosphocreatine kinase (Δ- - -Δ) were determined as described in *Methods*. The number of myotubes per unit area (●——●) is also presented as an indication of the timing of morphological differentiation.

incubated at 37° have shown that the onset of accumulation of all characteristic muscle proteins studied so far (myosin, glycogen phosphorylase, phosphorylase kinase, glycogen synthetase, and phosphocreatine kinase) occurs at the same time as the onset of myoblast fusion (Wahrman and Luzzati,

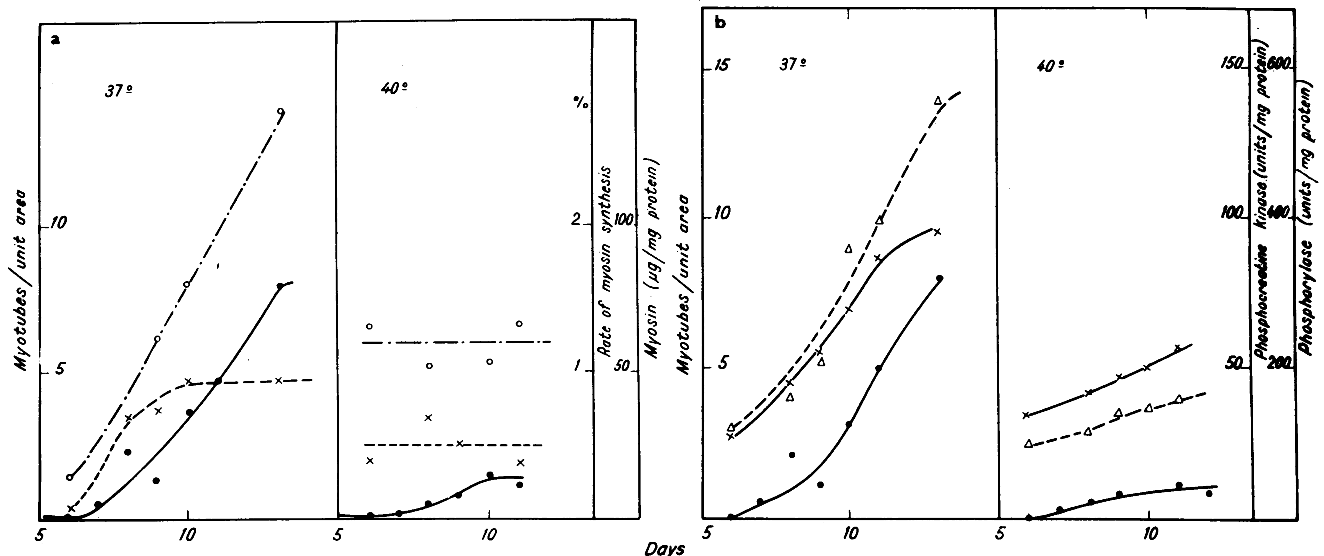

FIG. 3. Biochemical differentiation of temperature-sensitive variant line E_3. Cultures of E_3 were analyzed as described in the legend to Fig. 2. (*a*) Myotubes per unit area (●——●); myosin (○—·—○); rate of myosin synthesis, (×- - -×). (*b*) Specific activities of phosphorylase (×——×), phosphocreatine kinase (△- - -△); myotubes per unit area (●——●).

in preparation). Similar coordinance between biochemical and morphological differentiation occurred when cultures were incubated at 40°† (Fig. 2).

At the respective permissive temperatures, cultures of strains E_3 and H_6 accumulate phosphorylase and phosphocreatine kinase in a manner similar to that of the parent strain (Figs. 3 and 4). However, it was found that the rate of myosin synthesis did not increase in strain H6 although the cells fused well (Fig. 4*a*).

When the accumulation of the characteristic proteins was determined in cultures of strains of E_3 and H_6, which were incubated at the respective developmentally nonpermissive temperature, both morphological differentiation and biochemical differentiation were found to be coordinately inhibited (Figs. 3, 4).

DISCUSSION

We have isolated a series of conditional variants from mutagenized populations of myoblast cell lines L_6E and L_6D. The frequency of variants recovered from the cells surviving mutagenesis was surprisingly high (12 out of 34 clones analyzed). This may be due to the nature of the variant phenotypes for which we scored. We have observed that both growth and differentiation of the wild-type myoblast cell lines are quite sensitive to environmental conditions. This suggests that minor phenotypic changes might result in blocks to either growth or differentiation. If the number of possible mutations that can affect growth or differentiation under *in vitro* culture conditions is large, then we might expect a high proportion of heavily mutagenized cells to express aberrations in either growth or development. On the other hand, it is possible that the variants we isolated were already present in the myoblast population before mutagenesis. However, scoring for growth and developmental conditional variants in the wild-type population indicated that 3% was the upper limit of possible occurrence of spontaneous variants. Therefore, to account for the observed frequency of variants, one would have to assume that the mutagenic treatment itself somehow selected for the previously existing variants. We feel that it is more likely that the mutagen induced mutations, some of which gave rise to the observed phenotypic variations.

The variants appeared to be phenotypically stable through

† This temperature was chosen for subsequent experiments rather than 41° because the growth rate of all strains, including L_6E, is somewhat greater at 40° than at 41°. Incubation at 40° results in the same restrictions on the variant lines as incubation at 41°.

TABLE 3. *Temperature shifts of strains H_6 and E_3*

Myoblast line	Temperature shift	No. of generations at	No. of generations at	Myotubes per unit area on 13th day*
Wild-type L_6E	41° → 37°	41°	37°	
		1 to 8	8 to 1	10
	37° → 41°	37°	41°	
		1 to 8	8 to 1	10
E_3	41° → 37°	41°	37°	
		1	8	10
		4	4	8
		8	0	0, 3†
	37° → 41°	37°	41°	
		1	8	0, 1
		5	3	3
		8	0	10
H_6	37° → 41°	37°	41°	
		1	8	10
		4	4	10
		8	0	10
	41° → 37°	41°	37°	
		1	8	0, 1
		4	4	1
		8	0 to 1	1

* In these experiments confluency was reached after eight generations.

† On the 19th day these cultures contained 10 myotubes per unit area.

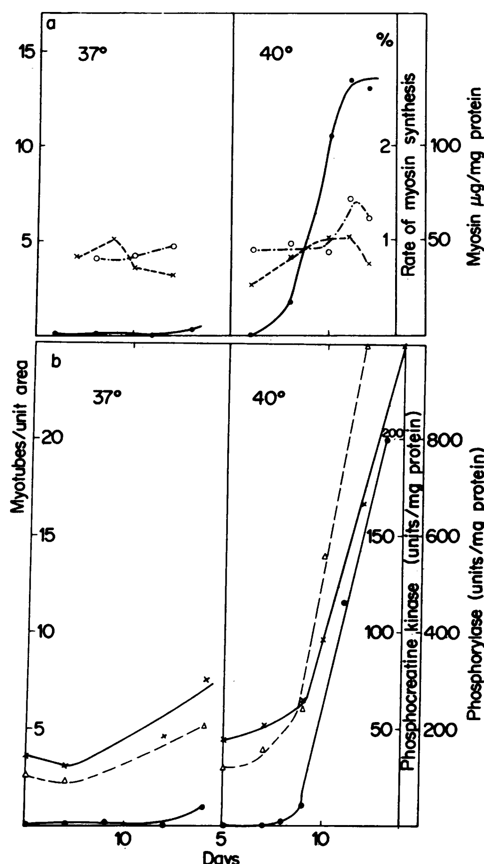

FIG. 4. Biochemical differentiation of temperature-dependent variant line H_6. Cultures of H_6 were analyzed as described in the legend to Fig. 2. (*a*) Myotubes per unit area (●——●); myosin (O—·—O); rate of myosin synthesis (X- - -X). (*b*) Specific activity of phosphorylase (X——X); phosphocreatine kinase (Δ- - -Δ); myotubes per unit area (●——●).

many generations and reverted to the wild-type phenotype at a frequency less than 1 in 10^5. The impairment of morphological differentiation at nonpermissive temperatures observed in strains E_3 and H_6 could be readily reversed by shifting the cultures to permissive temperatures at any time during the exponential growth phase. Thus, the variant phenotypes do not result from stable metabolic states but respond to the environmental conditions.

Conditions that blocked morphological differentiation in strains E_3 and H_6 also blocked the synthesis of characteristic muscle proteins, suggesting that both processes might be triggered by the same developmental event.

At permissive temperatures, cultures of strains E_3 and H_6 accumulated the characteristic muscle proteins, with the exception that myosin did not accumulate significantly in strain H_6. This strain may have suffered two independent lesions, one resulting in a specific block to myosin accumulation and the other resulting in a temperature-dependent block to all morphological and biochemical differentiation at 37°.

However, it is more likely that strain H_6 carries a single lesion that renders differentiation temperature-dependent and that accumulation of myosin is more sensitive to this lesion than is the accumulation of the other proteins characteristic of differentiation.

When cultures of strain H_6 that were in the process of fusing were shifted to the nonpermissive temperature (37°), all morphological and biochemical differentiation stopped. It would appear that the lesion in strain H_6 results in a thermolabile component necessary for fusion. However, it is also possible that the lesion results in accumulation of components that are compatible with differentiation at 41° but incompatible with differentiation at 37°.

When cultures of strain E_3, on the other hand, were shifted to the nonpermissive temperature (41°) just before or during fusion, the cells promptly fused into myotubes. The lesion in this strain appears to result in temperature-sensitive accumulation of the components necessary for fusion rather than in the accumulation of thermolabile components.

The nature of the components necessary for fusion and accumulation of characteristic muscle proteins is still unknown. The variant strains will be useful in further analysis of the biochemical changes that accompany the fusion of myoblast cells.

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