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Biochemistry

Mutants altering coordinate synthesis of specific myosins during nematode muscle development

(*trans*-acting genetic control/myosin heavy chain isoenzymes/muscle differentiation)

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ABSTRACT **Mutations in the *unc-52* gene on linkage group II retard the construction of body-wall muscle sarcomeres during larval development in the nematode *Caenorhabditis elegans*. *Unc-52* mutants show decreased accumulation of myosin heavy chains relative to other polypeptides during larval development, correlating with the structural retardation. Pulse radiolabeling experiments show that decreased synthesis of specific body-wall myosin heavy chains that are encoded by the *unc-54* gene on linkage group I is responsible for the defective myosin accumulation. In the wild type, a constant ratio of the synthesis of the *unc-54*-coded myosin B to myosin A, about 2:1, is maintained during the larval stages in which the synthesis of both myosins increases exponentially and rapid sarcomere growth and addition ensues. During the first 26 hr of larval development, before any structural or behavioral effects of *unc-52* mutations are apparent, the synthesis of myosin heavy chains is also normal. By 38 hr, decreased synthesis of myosin B is detected in the *unc-52* mutant SU200, when sarcomere growth slows considerably. The effects of mutation in the *unc-52* locus are *trans* acting upon the synthesis of *unc-54*-coded myosin in a specific set of muscle cells during a defined period of larval development.**

The nematode *Caenorhabditis elegans* synthesizes at least three forms of myosin heavy chains (1). The A form, with a M_r of 210,000 in wild-type and mutant nematodes, is found in both body-wall and pharyngeal muscles. The B form, which also has a M_r of 210,000 in wild-type nematodes, is structurally altered with a M_r of 203,000 in strain E675 arising from mutation in the *unc-54* gene on linkage group I. B myosin heavy chains are located only in body-wall muscles. The C form, with a M_r of 206,000 in wild-type and mutant nematodes, is found only in pharyngeal muscles. The A and B myosin heavy chains have different peptide structures and are the products of at least two structural genes (2–6). The A and B chains assemble into myosin molecules that are homodimeric; no hybrid myosin has been detected (3, 7). In body-wall muscles, these two forms of myosin molecules, which may be termed A and B on the basis of their heavy-chain composition, are located within the same cells and in the same sarcomeres (8). A and B myosin heavy chains and myosin molecules are synthesized at a constant ratio of about 1:2 throughout larval development and early adult maturation. The relative amounts of A and B myosins that accumulate are identical to these synthetic ratios, suggesting that synthesis is the primary determinant of the relative accumulation rates of the two myosins in nematode body-wall muscle cells (9).

Two lines of evidence suggest that the synthesis of myosin heavy chains in *C. elegans* is regulated by specific genetic control mechanisms. The unique location of B myosin in the body-wall muscle cells and of C myosin in the pharynx implies differential gene expression between the two types of muscle cell. The differential synthesis of A and B myosins suggests that some regulatory mechanism must operate within body-wall muscle cells to maintain the constant ratio observed. This report describes a molecular genetic approach to understanding the control mechanisms involved in the synthesis of myosin heavy chains in nematode muscle. Mutants in the *unc-52* gene on linkage group II have retarded construction of sarcomeres in body-wall muscle cells during larval development, which leads to progressive paralysis (10). One class of *unc-52* mutants, which includes SU54 and SU200, exhibits more extreme retardation morphologically than the milder class, which includes SU250 (10). In the *unc-52* mutant, SU200, total myosin heavy chain accumulation was markedly decreased. Myosin heavy chains at M_r 210,000 (A and B) are decreased relative to the C heavy chains at M_r 206,000. The hypothesis was proposed that mutation in the *unc-52* locus on chromosome II affects, by a *trans* acting mechanism, the synthesis of B myosin heavy chains encoded by the *unc-54* gene on chromosome I. The reduced myosin synthesis would then cause the decrease in construction of muscle-cell sarcomeres. We present evidence here consistent with this proposal. *Unc-52* mutants exhibit decreased synthesis of B myosin heavy chains relative to A and C forms and to other proteins at the specific period of larval development during which structural retardation appears. The pattern of myosin synthesis in these mutants is clearly distinct from the coordinate synthesis of A and B forms during development found in the wild-type and E675 strains.

MATERIALS AND METHODS

Synchronous cultures of N2 (wild-type) and mutant strains of *C. elegans* were grown by the procedure of Garcea *et al.* (9). Hatching occurred 3–4 hr after embryos were plated for N2 and 4–8 hr for *unc-52* mutants. Developmental times start with hatching as zero time. Nematodes were counted in triplicate, with 1000 nematodes counted per sample. Radiolabeled nematodes were produced by feeding them *Escherichia coli* that had been grown on $^{35}SO_4^{2-}$ (either 62.5 mCi/mol for accumulation and 10-hr pulse studies or 625 mCi/mol for 2- and 5-hr pulse studies). Nematodes were harvested as described (9), but were sedimented in 25% (wt/vol) sucrose/0.1 M NaCl after collection on an 8-μm Millipore filter. The animals were resuspended in cold (4°C) 62.5 mM Tris·HCl (pH 6.8) and disrupted by sonication with four 11-sec pulses at output 5 on a Branson sonifier with 3–4 sec between pulses. The suspensions were contained in Brinkman microfuge tubes surrounded by ice. After sonication, equal volumes of 8% (wt/vol) $NaDodSO_4$, 20% (vol/vol) glycerol, 14 M 2-mercaptoethanol, and 62.5 mM Tris·HCl (pH 6.8) were added. The mixtures were heated at 90°C. Within 5 min the nematode suspensions cleared. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed

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according to Epstein *et al.* (1). Separating gels were 4.5% or 7.5% (wt/vol) acrylamide, containing 1 part bisacrylamide to 37.5 parts acrylamide. Alternatively, low bis 7.5% separating gels were prepared from a stock solution containing one part *N,N*-methylene-bisacrylamide to 300 parts acrylamide. Gels of 4.5% acrylamide were polymerized by the addition of 0.05% *N,N,N',N'*-tetramethylethylenediamine (Temed) (Bio-Rad) and 0.1% ammonium persulfate (J. T. Baker, Phillipsburg, NJ). Gels of 7.5% were polymerized with 0.025% Temed and 0.05% ammonium persulfate. Protein samples were either diluted to a concentration of 0.01–10 mg/ml and 0.1–200 μg was applied to each slot or they were diluted to 5,000–600,000 cpm/μl and 200,000–800,000 cpm was applied to each slot. Gels were stacked to a constant current of 15 mA and run at 25 mA until the dye front reached the bottom of the gel (≈7 hr for 4.5% and 7.5% reduced bis gels; 5 hr for 7.5% gels). Gels were stained overnight in 0.025% Coomassie brilliant blue R (Sigma), 50% (vol/vol) methanol, and 10% (vol/vol) acetic acid and were destained in 10% acetic acid with a small amount of Whatman DE22 ion-exchange cellulose added to the destaining tray. Gels were dried onto Whatman no. 1 filter paper by reduced pressure and autoradiographed by the methods of Garcea *et al.* (9). Scanning densitometry was used to quantitate band intensities as described (3). Wet gels stained with Coomassie blue were scanned at 600 nm; autoradiograms were scanned at 000 nm (white light) on an RFT densitometer (Transidyne, Ann Arbor, MI). The integrated densities between compared bands were linear with respect to concentration. Protein concentrations were measured by a modification of the method of Lowry *et al.* (11) after precipitation with cold 10% trichloroacetic acid and resolubilization with 1 M NaOH. ^{35}S-Labeled protein samples were precipitated by hot trichloroacetic acid (12). After the final trichloroacetic acid rinse, the filters were washed twice with 100% ethanol, dried, and placed in mini-scintillation vials. Upon addition of 400 μl of 0.1 M NaOH, the vials were incubated at 37°C for 60 min. After incubation, 90 μl of 1 M HCl was pipetted into each vial. Five milliliters of a modified Triton/xylene mixture containing 25% (vol/vol) Triton X-114 (Sigma) and 0.55% Permablend-3 (Packard) in xylene (Fisher) was added to each vial, and the radioactivity was determined by liquid scintillation spectrometry (13).

RESULTS

Accumulation of Myosin Heavy Chains Is Decreased. The decreased accumulation of myosin heavy chains A and B, primarily from body-wall muscle, relative to the pharyngeal myosin heavy chain C was observed initially as a molecular correlate to the structural effects in adult *unc-52* mutants (10). Here we quantitate the amount of protein and of total myosin heavy chains that accumulated during larval development in wild-type N2 and the *unc-52* strain SU200. As in the *unc-54* structural gene mutants for myosin heavy chain, total protein accumulation of SU200 was significantly lower than in N2 (9). The total protein accumulation of the normal and mutant strains were closest to one another in the middle of larval development when the mutants were still highly mobile and had been free-living on saturating bacterial lawns (Fig. 1*A*). In early larval development and during adult maturation, the effects on general protein accumulation were more marked and correlated with the severity of the muscle structural defects in different mutants (data not shown). Similar decreases in total protein accumulation have been observed in muscle mutants of other genes, as well, and are probably a nonspecific consequence of paralysis (9).

Total accumulation of myosin heavy chain was quantitatively decreased in SU200 compared to N2 (Fig. 1*B*). The accumulation of myosin heavy chain during development in SU200 was not congruent to total protein accumulation, but there was a small but significant decrease in myosin heavy chain/total protein after 40 hr in SU200 when compared to N2. For example, at 55 hr, there was an over 6-fold difference in myosin content between the two strains and less than a 4-fold difference in total protein. Similar results were obtained in multiple experiments.

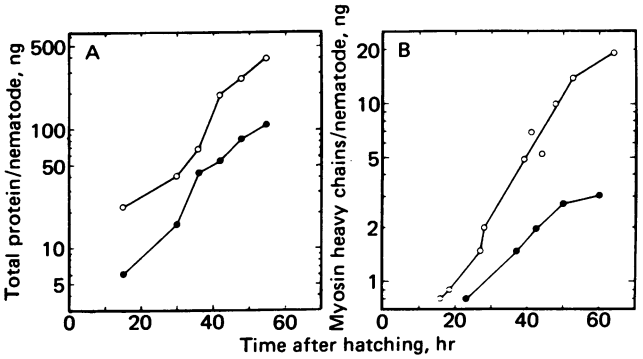

FIG. 1. Accumulation of total protein (*A*) and myosin heavy chains (*B*) in N2 (○) and SU200 (●). Synchronous populations of the nematode strains were sampled at the times indicated. Amount of protein was determined and nematodes were counted. Total myosin heavy chain content was determined by densitometry of Coomassie brilliant blue staining of bands on 4.5% NaDodSO$_4$/polyacrylamide gel electrophoretic separations of nematode homogenates.

Further experiments were performed in order to assess whether the accumulation of all three myosin heavy chains was uniformly affected in SU200 as a function of developmental time. Fig. 2 shows that the decreased accumulation of myosin heavy chains evident by adulthood affected the combined myosin A and B heavy chain band at M_r 210,000 relative to the pharyngeal myosin C band at 206,000. No difference in the relative accumulation of the different classes of myosin heavy chain was detected through at least 36 hr of development, the point at which structural alterations first became apparent in SU200.

Relative Synthesis of Myosin Heavy Chains A and B Is Decreased. Pulse studies of relative myosin synthesis in N2 and the *unc-54* mutant strain E675 have demonstrated that the ratios of accumulated A, B, and C myosins are closely similar to the ratios of the new synthesized proteins (9). We have performed analogous studies on N2 and the *unc-52* strains, SU200

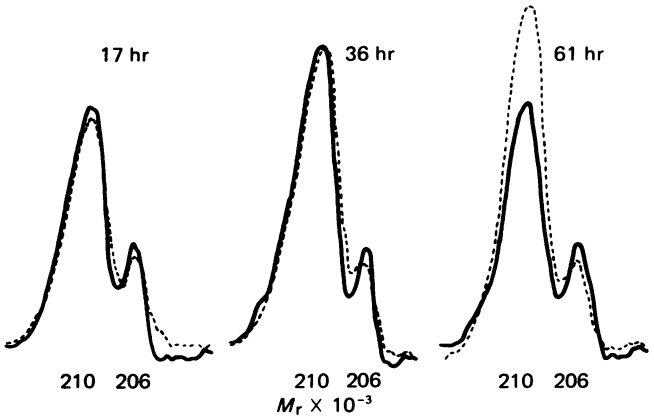

FIG. 2. Myosin heavy chain composition of N2 (- - -) and SU200 (—) during development. Densitometry of myosin heavy chain regions of autoradiogram of 4.5% NaDodSO$_4$/polyacrylamide gel electrophoretic separation of homogenized ^{35}S-labeled nematodes from synchronous populations of N2 and SU200 strains. Matched samples from each strain were run in parallel slots on slabs.

and SU250, during early adulthood, when the mutant effects upon structure and myosin accumulation are maximal but before egg production takes over protein metabolism in nematodes.

Fig. 3 shows experimental comparisons between the three strains with respect to combined myosin heavy chains A and B (210,000 M_r band) and myosin heavy chain C (206,000 M_r band). Four kinds of experiments were performed. In all cases, the nematodes were labeled by feeding them ^{35}S-labeled *E. coli* for the periods indicated. As done previously, the dissolved nematodes were placed directly on NaDodSO$_4$/polyacrylamide gels and all of their proteins were separated by electrophoresis. Because the myosin heavy chains are in a region free of significant amounts of other proteins (3, 7, 9), densitometry of the autoradiographs of the gels provides a direct reading of the relative levels of the different myosin heavy chains without purification and its possible selection against any of the proteins. The differences in accumulation between N2 and the *unc-52* mutant strains were observed in the 10-hr, 5-hr, and 2-hr pulse-labeling experiments, indicating that a defect in synthesis of either myosin heavy chain A or B or both was primarily responsible for the decreased myosin accumulation. In wild-type N2 nematodes, myosin A and B synthesis or accumulation at this stage represents primarily body-wall muscle activity. Thus, the myosin synthetic and accumulation defects in SU200 and SU250 are likely to represent alterations within the body-wall muscle cells that are functionally structurally altered in contrast to other sources of myosin in unaffected structures such as the pharynx.

Fig. 4 is the result of a 10-hr pulse experiment similar to those above that compares not only the synthesis of myosin heavy chains in N2 and SU250, but also other ^{35}S-labeled polypeptides in the 50,000–200,000 M_r range. The differences between analogous nonmyosin bands or regions of the gel separations

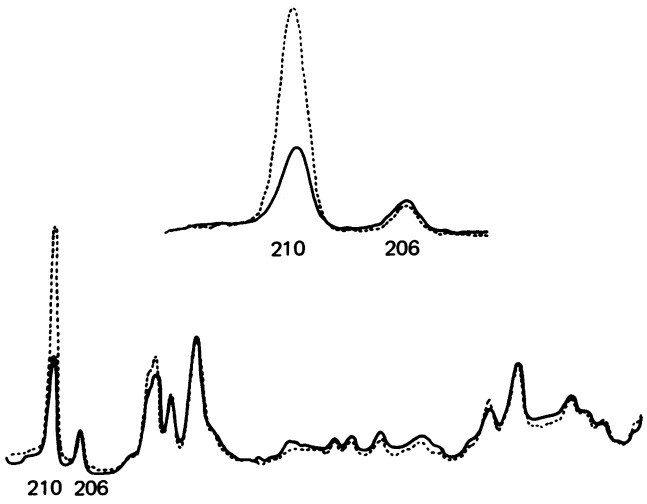

FIG. 4. Pulse labeling of proteins in SU250 (—) and N2 (· · ·). Densitometry of autoradiograms of low-bis 7.5% NaDodSO$_4$/polyacrylamide gel electrophoretic separations of matched samples of homogenized ^{35}S-labeled synchronous nematodes. Developmental period of feeding on ^{35}S-labeled *E. coli* was 41–51 hr. (*Upper*) Myosin heavy chain region; (*Lower*) entire separation. $M_r \times 10^{-3}$ are shown.

of the proteins of the two strains were small and random compared to the large difference in the combined myosin heavy chain A and B band. This result suggests that the *unc-52* mutations primarily affect the synthesis of the body-wall myosins.

Decreased Synthesis of Myosin Heavy Chain B Predominates in *unc-52* Mutants. The *unc-54*-encoded myosin heavy chain B is uniquely expressed in body-wall muscle cells of *C. elegans* (1, 8), whereas myosin A, although primarily synthe-

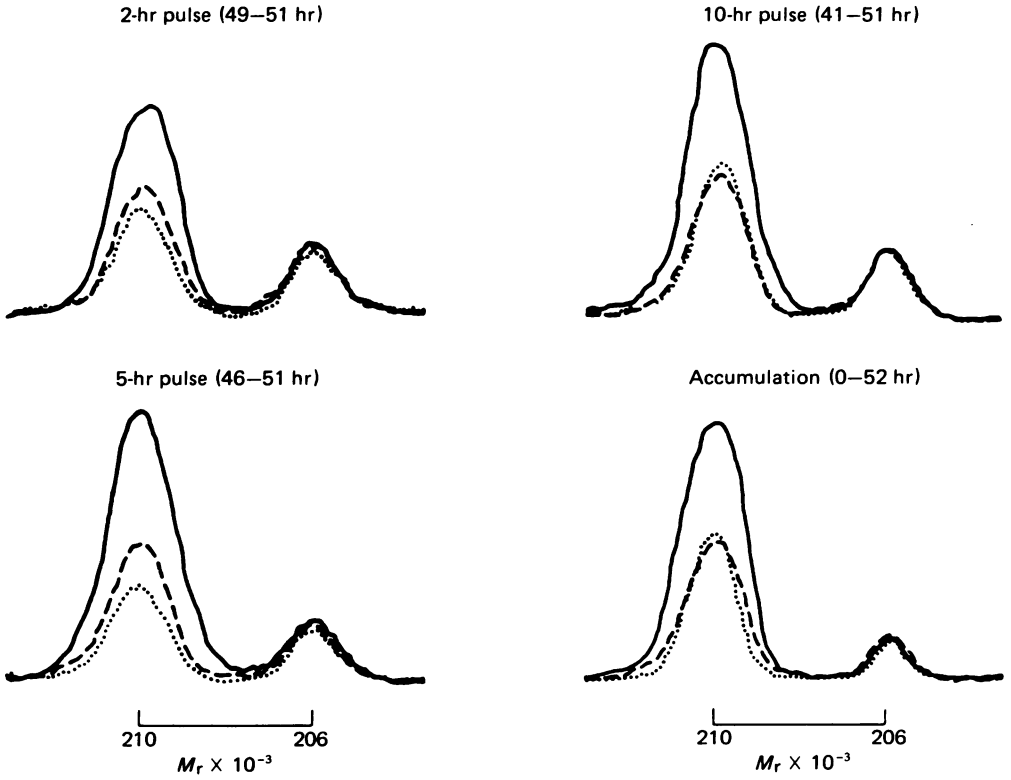

FIG. 3. Pulse labeling of myosin heavy chains in SU200 (· · ·), SU250 (- - -), and N2 (—). Densitometry of myosin heavy chain regions of autoradiograms of low-bis 7.5% NaDodSO$_4$/polyacrylamide gel electrophoretic separations of matched samples of homogenized ^{35}S-labeled synchronous nematodes. Times indicate developmental period of feeding on ^{35}S-labeled *E. coli*.

sized in body-wall muscle during late larval and adult development (9), is also present in the pharynx and probably other cell types as well (1). Because *unc-52* mutants affect sarcomere construction within only the body-wall muscle cells (10) and our studies above show that myosins A and B, but not the pharyngeal myosin C, are affected, we sought to test the hypothesis that the principal effect of the *unc-52* mutants is upon the synthesis of the body-wall muscle cell myosin B.

We have distinguished between myosin heavy chains A and B by the use of the mutant E675. This *unc-54* mutant produces a structurally altered B myosin heavy chain (1–7) that is electrophoretically unique at M_r 203,000. The synthesis and accumulation of myosin heavy chains A, B, and C relative to one another and to total protein in E675 are similar to those of the wild-type N2 strain. Therefore, the double homozygous mutant strains of E675 with the *unc-52* mutants SU250 (mild) and SU54 (severe) would be useful constructions because individual myosin heavy chains could be followed in an *unc-52* environment. In the double homozygous mutants, the behavior of myosin heavy chains A (still at 210,000 daltons) and B (now at 203,000 daltons due to the E675 molecular marker) can be compared in the presence and absence of the different *unc-52* alleles and their mutant effects. The double homozygous mutants were constructed according to established genetic methods for *C. elegans* (14).

Fig. 5 shows the relative synthesis of myosin heavy chains A, B, and C in E675, and E675-SU250 double homozygote, and the E-675-SU54 double homozygote. In E675, the ratio of *unc-54* encoded myosin heavy chain B (203,000 M_r band) to total myosin heavy chain (all three bands) synthesized during 5 hr during adulthood was 0.66. This result is similar to previous determinations in both E675 and wild-type N2 (9). In contrast, in both double homozygotes of *unc-52* mutants with the E675 marker, the synthesis of myosin B was significantly reduced. Indeed, the functionally and structurally more defective *unc-52*

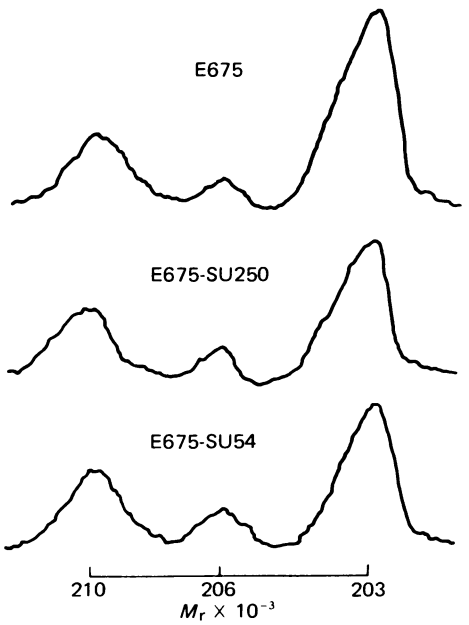

FIG. 5. Myosin heavy chain composition of pulse-labeled (5-hr pulse) E675-SU250 and E675-SU54 double homozygotes. Densitometry of myosin heavy chain regions of autoradiograms of low-bis 7.5% NaDodSO$_4$/polyacrylamide gel electrophoretic separations of matched samples of homogenized ^{35}S-labeled synchronous nematodes. Developmental period of feeding on ^{35}S-labeled *E. coli* was 51.5–56.5 hr. Ratio of 203,000 M_r myosin to total myosin: E675, 0.660; E675-SU250, 0.561; E675-SU54, 0.482.

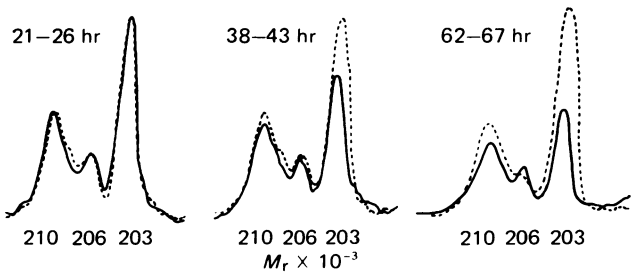

FIG. 6. Myosin heavy chain composition of pulse-labeled E675 (- - -) and E675-SU200 double homozygoate (—) during development. Densitometry of myosin heavy chain regions of autoradiograms of low-bis 7.5% NaDodSO$_4$/polyacrylamide gel electrophoretic separations of matched samples of ^{35}S-labeled synchronous nematodes. Times indicate developmental periods of feeding on ^{35}S-labeled *E. coli*.

mutant, SU54, produced a more marked reduction of myosin heavy chain B than the less defective allele, SU250 (10). The reduction of myosin heavy chain synthesis in these mutants, therefore, can be explained mainly by specific reduction in the relative synthesis of myosin heavy chain B.

Decreased Myosin B Synthesis Is Concomitant with Retarded Sarcomere Construction. Through the early stages of larval development, *unc-52* mutants produce body-wall muscle structures indistinguishable from wild-type N2. In SU200, the body-wall muscle cells are normal 24 hr after hatching, but only small increases in the sizes of the original two sarcomeres within each cell occur later in development. In wild-type N2, massive increases in the size of sarcomeres continue. Through 64 hr after hatching, most N2 body-wall muscle cells have constructed at least nine sarcomeres (10). Fig. 6 shows that the synthesis of myosin heavy chain B (203,000 M_r band) in the E675-SU200 double homozygote decreased with respect to the synthesis of the same polypeptide in E675 as larval and adult development proceeded. Significant reduction in myosin B synthesis could be measured by 38–43 hr, at which stage SU200 was abnormal, and at 62–67 hr, at which stage, when SU200 was severely retarded in body-wall muscle development and highly paralyzed, the reduction in body-wall specific myosin B was maximal. Small decreases in myosin A were also observed at this time, but further work is required to establish whether this effect is significant. These results strongly suggest that the onset and extent of decreased synthesis of the body-wall-specific myosin is concomitant with the retardation of body-wall sarcomere construction during development of SU200 and other *unc-52* mutants.

DISCUSSION

During postmitotic larval development and adult maturation, body-wall muscle cells in wild-type *C. elegans* exhibit massive growth and construction of new sarcomeres (10). Simultaneous with these structural transformations, exponential increases in the relative rates of synthesis of myosin and of total protein are observed. The synthesis of the distinct heavy chains of myosins A (non-*unc-54*-encoded myosin) and B (*unc-54*-encoded myosin) of the body-wall muscle cells increases proportionately, maintaining a constant ratio of about 2:1 (9). In this paper, we have presented biochemical results strongly suggesting that the major protein defect in all three *unc-52* mutants compared to wild-type N2 nematodes is decreased synthesis of myosin heavy chain B. This *unc-52*-affected myosin is produced only in body-wall muscle cells, the specific site of *unc-52* structural defects, in contrast to myosin A, which is produced in both body-wall and pharyngeal muscles, and myosin C, which is produced only in the pharynx (1, 8). During development, the

decreased synthesis of B myosin heavy chains occurs when body-wall sarcomere construction is first observed. Comparing SU54 and SU200 to SU250, the myosin synthetic defects correlate with the structural alterations observed in terms of severity of the specific mutant, specificity of cellular location, and appearance in developmental time.

Myosin heavy chain B, whose synthesis and accumulation are affected by mutations in the *unc-52* gene on linkage group II (10), is coded by structural gene *unc-54* on linkage group I in *C. elegans* (1–6). Some product coded by the *unc-52* gene would interact within the body-wall muscle cells to permit the normal rate of synthesis observed in the wild-type N2 strain. An altered product produced in the *unc-52* mutants fails to perform this function and leads to the decreased rates of synthesis observed in SU200, SU54, and SU250. This model is supported by the genetic observations that the *unc-52* mutations are *trans* acting, in that mutations on chromosome II specifically affect the expression of the *unc-54* gene on chromosome I, and that *unc-52* mutations are recessive, in that the mutant effects upon behavior and structure do not appear in heterozygous nematodes (10). This latter result reduces the possibility that the mutant *unc-52* product interferes with myosin synthesis whereas the normal product has no interaction with the process. We have no information about the location or mechanism of action of the *unc-52* product in myosin synthesis. The relationship between reduced myosin B synthesis and sarcomere construction defects may be the result of direct coupling between the two processes or a more indirect secondary phenomenon.

The concomitance of the retardation of body-wall sarcomere construction with the decrease in synthesis of B myosin heavy chains in *unc-52* mutants during development suggests that *unc-52* expression or the action of the *unc-52* product exhibits high developmental specificity with respect to location and time. Independent of the mechanism by which *unc-52* permits coordinate synthesis of the *unc-54* gene product, myosin B, and the other myosins of *C. elegans*, *unc-52* mutants may prove to be useful tools in dissecting the pathways by which newly synthesized contractile proteins are organized into the regular arrays of thick and thin filaments required for normal structure and function in fully differentiated muscle.

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1. Epstein, H. F., Waterston, R. H. & Brenner, S. (1974) *J. Mol. Biol.* **90,** 291–300.
2. Epstein, H. F., Schachat, F. H. & Wolff, J. A. (1977) in *Pathogenesis of Human Muscular Dystrophies*, ed. Rowland, L. P. (Excerpta Medica, Amsterdam), pp. 460–467.
3. Schachat, F. H., Harris, H. E. & Epstein, H. F. (1977) *Cell* **10,** 721–728.
4. Harris, H. E. & Epstein, H. F. (1977) *Cell* **10,** 709–719.
5. MacLeod, A. R., Waterston, R. H., Fishpool, R. M. & Brenner, S. (1977) *J. Mol. Biol.* **114,** 133–140.
6. MacLeod, A. R., Waterston, R. H. & Brenner, S. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5336–5340.
7. Schachat, F., Garcea, R. L. & Epstein, H. F. (1978) *Cell* **15,** 405–411.
8. Mackenzie, J. M., Jr., Schachat, F. & Epstein, H. F. (1978) *Cell* **15,** 413–419.
9. Garcea, R. L., Schachat, F. & Epstein, H. F. (1978) *Cell* **15,** 421–428.
10. Mackenzie, J. M., Jr., Garcea, R. L., Zengel, J. M. & Epstein, H. F. (1978) *Cell* **15,** 751–762.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
12. Schimke, R. T., Rhoads, R. E. & McKnight, G. S. (1974) *Methods Enzymol.* **30,** 694–701.
13. Anderson, L. E. & McClure, W. O. (1973) *Anal. Biochem.* **51,** 173–179.
14. Brenner, S. (1974) *Genetics* **74,** 71–94.