Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 1122–1125, February 1984
Cell Biology

Denervated skeletal muscle displays discoordinate regulation for the synthesis of several myofibrillar proteins

(tropomyosin/troponin/myosin/protein synthesis/nerve effects)

RYOICHI MATSUDA*, DENNIS SPECTOR, AND R. C. STROHMAN†

Department of Zoology, University of California, Berkeley, CA 94720

Communicated by Howard A. Bern, October 24, 1983

ABSTRACT **Synthesis patterns of myosin heavy- and light-chain isoforms, tropomyosin and troponin, have been studied in chicken fast muscle denervated at both neonatal and adult stages. Denervated neonatal muscle does not synthesize the adult myosin heavy-chain isoform at the time of denervation, but it does synthesize the adult isoform several months after denervation. Thus, innervation does not appear to be necessary for the normal sequential replacement of embryonic and neonatal myosin heavy chain by the adult variant. Nerve is required, however, for the regulation of tropomyosin and troponin expression. Normally the pectoralis major muscle represses synthesis of both β-tropomyosin and leg-type troponin T during late embryonic development. After denervation, however, the muscle relaxes its ongoing repression of these proteins and significant amounts of both β-tropomyosin and leg-type troponin T are synthesized by the muscle. Denervation also results in an altered pattern of myosin light-chain synthesis so that the ratio of fast light-chain 3/fast light-chain 1 decreases. Similar results are found in muscle denervated at the adult stage. In denervated muscle, therefore, synthesis of these myofibrillar proteins is not coordinated: ongoing isoform shifts proceed to express an adult pattern of myosin heavy chain while tropomyosin, troponin, and myosin light-chain patterns appear to revert to embryonic configurations.**

Nerve influences on muscle physiology and on the synthesis of muscle-specific proteins have been known for some time (1–5). More recently, many workers have made extended observations on the effects of cross-reinnervation of muscle (6, 7), on denervation effects (8–12), and on effects of chronic electrical stimulation (13, 14). Most of these studies have been approached from the point of view of the ability of nerve to convert one muscle type to another and, generally speaking, muscle physiology and myosin type have been found to change in a coordinate manner under nerve influence. For the conversion of fast- to slow-muscle myosin, we know that different gene transcripts are involved for the myosin heavy chains (15, 16) and that electrical stimulation will produce changes in transcriptional activity for both myosin heavy and light chains (14). Based on the above information, the assumption has been made that the nerve effects noted above are exerted through a coordinated control of gene transcription.

It is important to note, therefore, the cases in which the muscle carries out major switches in gene expression without a requirement for input from the nervous system. Such a case evidently involves the sequential expression within selected skeletal muscles of embryonic, neonatal, and adult isoforms of myosin heavy chain. The embryonic and adult myosin heavy chains are encoded by different gene transcripts (17), all three heavy chains display different peptide maps both in the rat (18) and in the chicken (17), and it is known that in the chicken there are at least two genes for myosin heavy chain (19). If rat muscle is denervated neonatally, there is, nevertheless, uninterrupted progress in the transition the muscle makes in replacing the neonatal isoform of myosin heavy chain with the adult isoform (18). Thus the orderly switching from early to late forms of myosin heavy-chain isoforms appears to be programmed early in the muscle and, while the activation of this program could have depended on prior interaction with the nervous system, it is clear from the above studies that the nerve can be removed without significant interference in the isoform replacement process.

We now report that the response to denervation in chicken skeletal muscle is surprisingly without coordination with regard to the expression of selected myofibrillar proteins. Neonatally denervated muscle will go on to express the adult myosin heavy chain and will repress synthesis of embryonic and neonatal myosin heavy-chain isoforms. At the same time, however, the denervated muscle will fail to repress the embryonic patterns of tropomyosin and troponin and will revert to an embryonic pattern of myosin fast light-chain synthesis. Adult denervated muscle will also respond in a discoordinate manner. The denervated adult muscle displays relaxed repression of ongoing β-tropomyosin synthesis and a shift in myosin light-chain ratio toward embryonic values, but it nevertheless expresses the adult-type myosin heavy chain. Loss of nerve–muscle contact in this fast muscle is therefore without effect on the normal sequence of myosin heavy-chain isoform expression but has a profound effect on the controlled expression of the isoforms of troponin and tropomyosin and on myosin fast light-chain ratios.

METHODS AND MATERIALS

Denervation of the Pectoralis Major Muscle. Denervation of the pectoral muscle was carried out according to Metafora *et al.* (11) with some modification. A deep incision was made between the pectoralis major and the teres major muscles to expose the pectoral nerves. The nerves were then tied with surgical thread at two locations and the section between threads was excised to prevent regeneration. The wound was closed with a suture and the operated area painted with mercurochrome. All procedures were done under sterile conditions while the animal was under pentobarbital anesthesia.

Protein Isolation and Electrophoretic Analysis. Myosin was isolated according to procedures used previously (17). Myosin light chains and tropomyosins were analyzed from total lysates of muscle (20). Troponin was prepared by the method of Ebashi *et al.* (21) with minor modifications (22).

Two-dimensional gel electrophoresis was carried out according to O'Farrell (23) using ampholines (LKB) at 1.8%

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: MHC, myosin heavy chain.

*Present address: Department of Biology, Tokyo Metropolitan University, Setagaya-Ku, Tokyo 158, Japan.
†To whom correspondence should be addressed.

(pH 5–8) and at 0.2% (pH 3.5–10); 12.5% $NaDodSO_4$/polyacrylamide gels were used in the second dimension. For one-dimensional gel analysis of troponin, 12.5% $NaDodSO_4$/polyacrylamide gels were used throughout.

Peptide Mapping of Myosin Heavy Chains. Peptide mapping was done on myosin heavy chains that had been isolated by electrophoresis on 5% $NaDodSO_4$/polyacrylamide gels. The myosin heavy-chain bands were cut from the gels and peptide mapping was done according to procedures we have previously used to identify the various myosin heavy chains of both fast and slow muscle (15–17).

Troponin Antibody and Immunoblotting. Preparation and characterization of troponin antibody was carried out as described (24). Troponin extracts were analyzed by one-dimensional $NaDodSO_4$/PAGE. Proteins from the gel were transferred to nitrocellulose (25) and they reacted with antitroponin as described. Horseradish peroxidase-conjugated swine anti-rabbit IgG was used to stain bound antibodies (26).

RESULTS

Denervated Muscle Fails to Repress β-Tropomyosin Synthesis. The pectoralis major muscle was denervated 1 day after hatching (neonatal stage) or in the 1-year-old chicken. The results are the same with regard to effect both on overall growth and on the pattern of tropomyosin expression (Fig. 1; Table 1). Seven months after neonatal denervation or 7 weeks after adult denervation, the experimental muscle is roughly only one-half the mass of the control contralateral muscle. The normal neonatal muscle grew to a weight of 40 g, but the experimental muscle weighed 19 g after 7 months. Seven weeks after denervation of adult muscle, the denervated side weighed only 22 g; the muscle on the control side weighed 42 g. In both cases, while the posterior aspect of the control muscles consisted of white fibers exclusively, this area of the denervated muscle was heavily invaded with dark fibers that are normally seen only in the small red area of the anterior pectoralis major. In all of the biochemical experiments reported below, we used only the posterior part of the pectoralis major, which normally consists entirely of fast fibers.

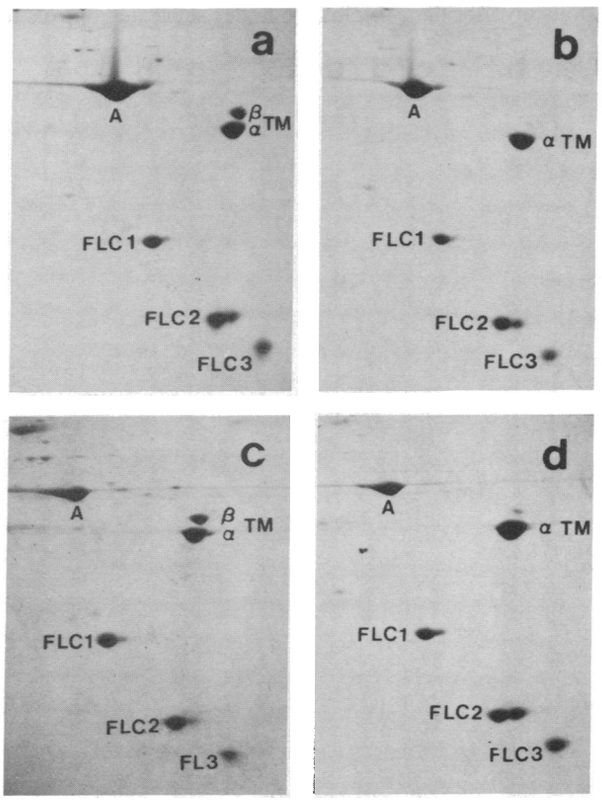

FIG. 1. Two-dimensional electrophoresis of total lysates from denervated and control pectoralis major muscles. Approximately 100 μg of total lysate protein was loaded for each gel. Only the myosin light-chain area of the gels are shown after staining with Coomassie brilliant blue. (*a*) Muscle was denervated 1 day after hatching (neonatal stage) and a lysate was prepared from the muscle 7 months later. (*b*) Lysate from contralateral control muscle of *a*. (*c*) Muscle denervated at the adult stage and lysate prepared 7 weeks later. (*d*) Lysate from contralateral control of *c*. A, actin; β TM, β-tropomyosin, α TM, α-tropomyosin; FLC1, -2, and -3, myosin fast light-chains 1, 2, and 3.

If whole cell lysates are prepared from various control and denervated muscles and subjected to two-dimensional $NaDodSO_4$/PAGE, the results are typically those shown in Fig. 1. Control muscles accumulate very little, if any, β-tropomyosin and α-fast-tropomyosin is predominant. The denervated muscles show, however, in addition to α-fast-tropomyosin, a major accumulation of β-tropomyosin. β-Tropomyosin is typical of embryonic breast muscle, but it rapidly disappears during embryonic development (20) and now may be seen (Fig. 1 *A* and *C*) to reappear after denervation. To study synthesis in addition to accumulation of these molecules, the muscles described in Fig. 1 were directly injected with [^{35}S]methonine 1 hr prior to lysate preparation. After electrophoresis of the lysates, the different spots were then cut from the gel and the radioactivity was determined by scintillation counting. The results are presented in Table 1 for one neonatally denervated muscle and for two muscles denervated in the 1-year-old adult. Synthesis of β-tropomyosin is insignificant in all control muscles. In denervated muscles, the percentage of total tropomyosin counts in β-tropomyosin rises to ≈23% in the neonatal case and to ≈35% in the adult case. In addition, there is a parallel decrease in the rate of synthesis of α-fast-tropomyosin. Synthesis of α-slow-tropomyosin appears to remain at the insignificant level in both denervated and control muscles.

The stained gels in Fig. 1 suggest that the denervated muscles may contain less myosin fast light chain 3 than controls. Table 1 shows that synthesis of fast light-chain 3 is in fact depressed in denervated muscle, and this appears to be correlated with a parallel increase in fast light-chain 1 in all cases. The denervated muscle, however, is without any significant increase in slow myosin light-chain synthesis paralleling the absence of α-slow-tropomyosin mentioned above. In the denervated muscle 6 weeks after injury, the fast light-chain 2 synthesis levels fall well below the expected 50% level. Theoretically, the fast light-chain 2, as the constitutive light chain, should not accumulate below the 50% level as the fast light-chain 1 and 3 levels vary. But our results refer to synthesis rates over short periods and do not necessarily predict levels of accumulation.

Denervated Breast Muscle Fails to Repress Leg-Type Troponin T. Normally, breast muscle contains leg and breast-type troponin molecules during embryonic development, but it represses the leg-type troponin by hatching (22). We examined troponin extracts of control and denervated muscles and found a persistent presence of small amounts of leg-type troponin in the denervated but not in the control breast muscle. Both experimental and control muscles show breast-type troponin together with troponin I. Fig. 2 shows this result (lanes a and b). To be certain that the band comigrating with leg-type troponin was in fact a troponin molecule, we ran whole cell lysates of denervated and control muscles on $NaDodSO_4$/PAGE as in Fig. 1 *A* and *B*. The proteins were then transferred to nitrocellulose and immunoblotting was done using an affinity-purified troponin-T antibody. As indicated in lanes c and d, the antibody reacts with the band comigrating with leg-type troponin found in denervated muscle in addition to the breast-type troponin band found in both

Table 1. Percentage of [^{35}S]methionine incorporation in tropomyosin and MLC

Time after denervation	Tropomyosin			MLC				
	αf	αs	β	FLC 1	FLC 2	FLC 3	SLC 1	SLC 2
7 months*	76.5	0.7	22.7	26.6	53.0	19.9	0.2	0.1
Control	97.2	0.6	2.2	13.2	62.4	23.7	0	0.5
6 weeks†	64.4	1.9	33.7	52.4	31.4	15.2	0	1.0
Control	95.6	1.8	2.5	13.4	51.3	33.3	1.1	0.7
7 weeks†	61.0	4.5	34.6	31.2	55.2	12.0	0.8	0.8
Control	95.3	0.9	3.8	24.1	50.0	24.6	0	1.3

The muscles were denervated at times indicated. The animals were then maintained for either 7 months or 6 weeks as stated and were injected *in vivo* with the isotope 1 hr prior to sacrifice. Total lysates were prepared from the muscles and electrophoresis was carried out as described in Fig. 1. The spots were cut from the gel and incubated with NCS (tissue solubilizer; Amersham/Searle) for 2 hr at 60°C. Radioactivity was determined by scintillation counting. αf, α-fast; αs, α-slow; β, β-tropomyosin. FLC1, -2, and -3, myosin fast light chains; SLC1 and -2, myosin slow light chains.
*Neonatal denervation.
†Adult denervation.

denervated and control muscles. The amounts of leg-type troponin are quite small even in normal leg muscle (22), and the immunological reaction therefore is not strong in the denervated breast muscle. The difference between denervated and control muscle with regard to leg-type troponin presence is nevertheless clear.

Neonatally Denervated Breast Muscle Synthesizes the Adult Isoform of Myosin Heavy Chain. Examination of myosin heavy chains (MHCs) synthesized by control and denervated muscles was carried out by a peptide mapping procedure that we have previously used to distinguish embryonic, neonatal, and adult MHC isoforms in developing muscle and in protein-synthesizing systems programmed with mRNA from muscles at different developmental stages (15, 17). These early studies showed that at 1 day after hatching (neonatal) the breast muscle contains the embryonic MHC together with traces of a neonatal MHC, but it does not contain the adult type MHC. Peptide maps of embryonic and adult MHCs from the breast muscle are shown in Fig. 3 (lanes a and b). When the muscle is denervated at the neonatal stage and examined 7 months later for MHC isoform content, it is clear that the denervated and control muscles are identical. That is, the denervated muscle has progressed to synthesize adult-type MHC (Fig. 3 lanes c, d, e, and f). If the muscle is denervated at the adult stage and examined 7 weeks later, the result is the same. That is, both denervated and contralateral normal muscle both contain a MHC with a peptide map identical to that of adult MHC (Fig. 3, lanes g–j). That the muscles remained denervated after surgery was determined by visual examination and weight. As discussed above, adult and neonatal denervated muscle was always 50% of the weight of the control contralateral muscle.

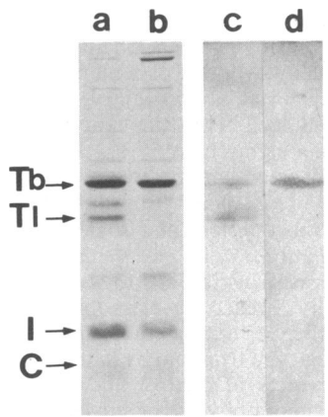

FIG. 2. Electrophoresis on 12.5% NaDodSO$_4$/polyacrylamide gel of troponin from neonatal denervated and control muscle. Approximately 10 μg of protein was loaded onto each gel slot. After electrophoresis, the gel was stained with Coomassie brilliant blue. Lane a, troponin extract from the muscle denervated at the neonatal stage. The animal was kept for 7 months and then the extract was prepared. Lane b, troponin extracted from contralateral control muscle. Lane c, immunoblot of gel shown in lane a using antibody to troponin T. Antibody binding to protein was visualized with horseradish peroxidase-conjugated anti-rabbit IgG. Lane d, immunoblot of control gel shown in lane b. Tb, breast-type troponin T; Tl, leg-type troponin T; I, troponin I; C, troponin C. Troponin bands were identified by comigration with known markers.

DISCUSSION

The main point of this communication is to point out that various myofibrillar proteins are under separate controls for

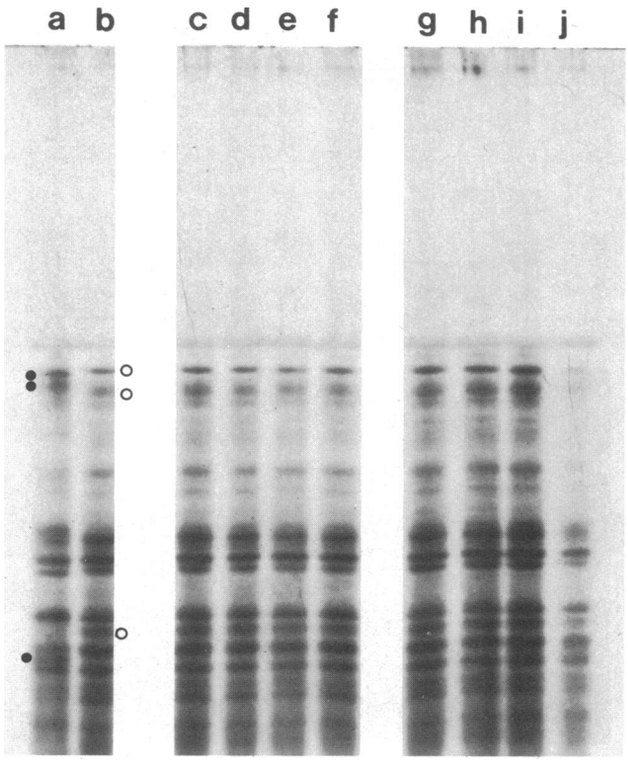

FIG. 3. Peptide maps of myosin heavy chains from denervated and control muscle. MHCs were separated from muscle extracts by electrophoresis on 5% NaDodSO$_4$/polyacrylamide gel and then subjected to peptide mapping. Lanes: a, peptide map of MHC from day 15 embryo pectoralis muscle; b, map of MHC from adult pectoralis muscle; d, map of MHC from pectoralis muscle denervated neonatally and examined 7 months later; c, contralateral control for lane d; e and f, repeats of lanes d and c on duplicate animals; h, peptide map of MHC from adult muscle denervated at the adult stage and examined 6 weeks later; g, map of MHC from the adult contralateral control; i and j, repeats of lanes g and h on duplicate animals.

their expression in denervated muscle. To the degree that these controls operate at the transcriptional level, it may also be true that coordinate gene regulation for functionally related peptides may not be the general rule in maturing skeletal muscle. At the neonatal stage of development in the chicken, we know that the breast muscle does not yet express the adult isoform of MHC but instead expresses a mixture of neonatal and embryonic MHC (17). The adult and the embryonic MHC are programmed by different mRNAs (15) and at least two genes for MHC exist in chicken muscle (19). When the neonatal muscle is deprived of its nerve, there is, nevertheless, a replacement of the early MHC isoforms by the adult variant (Fig. 3). The nerve therefore is not required either for the activation of the adult MHC gene or for the repression of the gene for embryonic MHC. Our result for denervated chicken muscle confirms the earlier report for denervated rat muscle (18).

The result for the adult denervated chicken muscle, also shown in Fig. 3, is the same as for the muscle denervated at the neonatal stage. In this case, however, there is ambiguity because the adult muscle at the time of denervation is already synthesizing only the adult MHC isoform (17). We also know that regenerating adult breast muscle reverts to transiently express embryonic MHC for the first several weeks after injury before it again achieves the full adult myosin phenotype (27). Our thought here was that denervated muscle might also revert to express the embryonic MHC isoform but, in the absence of nerve, fail to make the transition to the adult phenotype. It is clear from the data in Fig. 3 that if denervated muscle ever does revert to express the embryonic MHC, the absence of nerve does not interfere with the normal transition to adult MHC expression.

The nerve, however, is necessary for the normal repression that muscle exerts on the expression of embryonic forms of tropomyosin and troponin. Embryonic breast muscle synthesizes β-tropomyosin (20, 28, 29) and also synthesizes a troponin isoform normally found only in leg muscle (22). By neonatal stages of development, chicken breast muscle has completely repressed the synthesis of both β-tropomyosin and leg-type troponin (see refs. 20 and 22; Figs. 1 and 2). By removing the nerve, this repression is relaxed and both β-tropomyosin and leg-type troponin reappear in the denervated muscle. These results support recent *in vitro* experiments, which provide immunological evidence that chicken muscle cultures without nerve fail to suppress inappropriate variants of troponin T and troponin C (30). In the rat, the situation also appears to be similar because the appropriate expression of fast- or slow-type troponin I in denervated regenerating muscle appears to require the intact nerve (31).

Finally, we see that the nerve also exerts an influence on the expression of myosin light chains (Table 1). Without nerve, the breast muscle shows an increased synthesis of myosin fast light-chain 1 and a decreased synthesis of fast light-chain 3. There is evidence that fast light-chains 1 and 3 may be coded by the same gene but that variable gene transcript processing results in either one or the other light chain (32, 33). A decreased fast light-chain 3 population or an increased ratio of fast light-chain 1/fast light-chain 3 is known to be associated with early stages of breast muscle development (34). Denervation therefore results not only in the reappearance of embryonic patterns of tropomyosin and troponin but also in the reappearance of embryonic patterns of myosin light-chain synthesis.

We clearly do not have a complete description of the genetic controls operating during development of normal muscle. It appears that during terminal differentiation, many genes are expressed in a coordinate fashion (35). The rules or programs that govern muscle maturation, however, may be quite different from those governing early development (36). The results presented here suggest a lack of coordination for the control of gene expression in maturing muscle and this muscle, with or without nerve, may present new opportunities for understanding biological controls on gene activation and repression in general.

This research was supported by grants from The Muscular Dystrophy Association and from the National Institutes of Health (NS 15882 and AG 02832).

1. Buller, A. J., Eccles, J. C. & Eccles, R. M. (1960) *J. Physiol.* **150,** 399–416.
2. Salmons, S. & Vrbova, G. (1969) *J. Physiol.* **201,** 535–549.
3. Barany, M. & Close, R. I. (1971) *J. Physiol.* **213,** 455–474.
4. Streter, F. A., Gergely, J., Salmons, S. & Romanul, F. (1973) *Nature (London)* **241,** 17–19.
5. Weeds, A. G., Trentham, D. R., Kean, C. J. C. & Buller, A. J. (1974) *Nature (London)* **247,** 135–139.
6. Hoh, J. (1975) *Biochemistry* **14,** 742–747.
7. Amphlett, G. W., Perry, S. V., Syska, H., Brown, M. D. & Vrbova, G. (1975) *Nature (London)* **257,** 602–604.
8. Rubinstein, N. A. & Kelly, A. M. (1978) *Dev. Biol.* **62,** 473–485.
9. Ishiura, S., Nonaka, I., Sugita, H. & Mikawa, T. (1981) *Exp. Neurol.* **73,** 487–495.
10. Margreth, A., Dalla Libera, L., Silviati, G. & Ischia, N. (1980) *Muscle Nerve* **3,** 483–486.
11. Metafora, S., Felsani, A., Cotrufo, R., Tajana, G. F., Del Rio, A., De Prisco, P. P., Rutigliano, B. & Esposito, V. (1980) *Proc. R. Soc. London Ser. B* **209,** 257–273.
12. Carraro, U., Dalla Libera, L. & Catani, C. (1983) *Exp. Neurol.* **79,** 106–117.
13. Roy, R. K., Mabuchi, K., Sarkar, S., Mis, C. & Sreter, F. A. (1979) *Biochem. Biophys. Res. Commun.* **89,** 181–187.
14. Heilig, A. & Pette, D. (1983) *FEBS Letts.* **151,** 211–214.
15. Bandman, E., Matsuda, R., Micou-Eastwood & Strohman, R. C. (1981) *FEBS Letts.* **136,** 301–305.
16. Matsuda, R., Bandman, E. & Strohman, R. C. (1982) *Differentiation* **23,** 36–42.
17. Bandman, E., Matsuda, R. & Strohman, R. C. (1982) *Dev. Biol.* **93,** 508–518.
18. Butler-Browne, G. S., Bugaisky, L. B., Cuenound, S., Schwartz, K. & Whalen, R. G. (1982) *Nature (London)* **299,** 830–833.
19. Umeda, P. K., Sinha, A. M., Jakovcic, S., Merten, S., Hsu, H. J., Subramanian, K. N., Zak, R. & Rabinowitz, M. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 2843–2847.
20. Matsuda, R., Bandman, E. & Strohman, R. C. (1983) *Dev. Biol.* **95,** 484–491.
21. Ebashi, S., Wakabayashi, T. & Ebashi, F. (1971) *J. Biochem. (Tokyo)* **69,** 441–445.
22. Matsuda, R., Obinata, T. & Shimada, Y. (1981) *Dev. Biol.* **82,** 11–19.
23. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4021.
24. Obinata, T., Shimada, Y. & Matsuda, R. (1979) *J. Cell Biol.* **81,** 59–66.
25. Burnette, W. N. (1981) *Anal. Biochem.* **112,** 195–203.
26. Olden, K. & Yamada, K. M. (1977) *Anal. Biochem.* **78,** 483–490.
27. Matsuda, R., Spector, D. & Strohman, R. C. (1983) *Dev. Biol.* **100,** 478–488.
28. Roy, R. K., Sreter, F. A. & Sarkar, S. (1979) *Dev. Biol.* **69,** 15–30.
29. Montarras, D., Fiszman, M. Y. & Gros, F. (1982) *J. Biol. Chem.* **257,** 545–548.
30. Toyota, N. & Shimada, Y. (1983) *Cell* **33,** 297–304.
31. Dhoot, G. K. & Perry, S. V. (1982) *Muscle Nerve* **5,** 39–47.
32. Matsuda, G., Maita, T. & Umegane, T. (1981) *FEBS Lett.* **126,** 111–113.
33. Robert, B., Weydert, A., Caravatti, M., Minty, A., Cohen, A., Daubas, Ph., Gros, F. & Buckingham, M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2437–2441.
34. Hoh, J. F. Y. (1979) *FEBS Letts.* **98,** 267–270.
35. Devlin, R. & Emerson, C. (1979) *Dev. Biol.* **69,** 202–216.
36. Rubinstein, N. A., Pepe, F. A. & Holtzer, H. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 4524–4527.