MOLECULAR AND CELLULAR BIOLOGY, Feb. 1988, p. 794–801
0270-7306/88/020794-08$02.00/0
Copyright © 1988, American Society for Microbiology
Vol. 8, No. 2

Dictyostelium discoideum Myosin: Isolation and Characterization of cDNAs Encoding the Essential Light Chain

REX L. CHISHOLM,* ALICE M. RUSHFORTH, RICHARD S. POLLENZ, EDWARD R. KUCZMARSKI, AND SHERRIE R. TAFURI

Department of Cell Biology and Anatomy, Northwestern University Medical School, 303 E. Chicago Avenue, Chicago, Illinois 60611

Received 1 May 1987/Accepted 13 November 1987

We used an antibody specific for *Dictyostelium discoideum* myosin to screen a λgt11 cDNA expression library to obtain cDNA clones which encode the *Dictyostelium* essential myosin light chain (EMLC). The amino acid sequence predicted from the sequence of the cDNA clone showed 31.5% identity with the amino acid sequence of the chicken EMLC. Comparisons of the *Dictyostelium* EMLC, a nonmuscle cell type, with EMLC sequences from similar MLCs of skeletal- and smooth-muscle origin, showed distinct regions of homology. Much of the observed homology was localized to regions corresponding to consensus Ca^{2+}-binding of E-F hand domains. Southern blot analysis suggested that the *Dictyostelium* genome contains a single gene encoding the EMLC. Examination of the pattern of EMLC mRNA expression showed that a significant increase in EMLC message levels occurred during the first few hours of development, coinciding with increased actin expression and immediately preceding the period of maximal chemotactic activity.

Myosin has been shown to play an important role in several motile processes, including cytokinesis, cell movement, and phagocytosis (1, 10, 25, 54). Native myosin consists of two copies each of three different polypeptides: (i) heavy chains, (ii) regulatory myosin light chains (also called phosphorylatable, EDTA-, or dithionitrobenzene-MLCs), and (iii) essential MLCs (EMLCs; also called alkali MLCs). Rotary-shadowed myosin molecules appear in the electron microscope as rodlike structures having two globular heads. Trypsin cleavage of myosin produces two fragments: a rodlike tail and a globular head region. The two MLCs are associated with the globular head fragment (1, 6, 10, 20, 25, 34, 35, 40), which possesses both the actin-binding site and ATPase activities. The MLCs appear to be important for actin activation of the ATPase activity, as well as for specific actin binding, and may be involved in regulating this activity. However, under certain conditions heavy chains, as well as the S1 subfragment of skeletal muscle myosin, have been shown to display ATPase activity after removal of all MLCs (50, 56). The role of the regulatory MLCs is perhaps better understood than that of the EMLCs. In several systems the regulatory MLCs appear to be involved in regulating both the actin-activated ATPase activity and myosin assembly. In contrast, the role of the EMLCs remains unclear. Using an ATP analog that can be covalently cross-linked into the ATP-binding site, Okomoto et al. (43) have recently suggested that the EMLCs participates in formation of the smooth-muscle myosin active site.

In muscle, the structure and expression of the EMLC gene are quite complex. Two different isoforms of the EMLC have been already described for mammalian and avian systems (21–23, 44), as well as for *Drosophila melanogaster* (17). All of these genes are differentially spliced to produce multiple mRNAs, which are expressed in a developmentally regulated or cell-type-specific fashion.

Comparisons of the chemical cleavage products of muscle and nonmuscle myosins show the molecules to be distinct (7). Moreover, hybridization studies involving cloned muscle myosin genes have been unable to detect the genes encoding the nonmuscle myosin in homologous cell types (39). Muscle and nonmuscle myosin systems also differ in the mechanisms by which filament formation and ATPase activity are regulated. For some myosins, such as *Acanthamoeba* myosin II, regulation occurs by phosphorylation of the heavy chain (11), whereas the activity of others, such as chicken gizzard myosin, is regulated by MLC phosphorylation (49). Scallop myosin is regulated by yet another mechanism involving the direct binding of calcium to myosin, which is mediated by one of the MLCs (27). In contrast, the activity of *Dictyostelium* myosin is regulated by phosphorylation of both heavy chains and MLCs (33).

Dictyostelium discoideum is one of the best systems available for the study of nonmuscle myosins. The cells exhibit several types of cell motility (51), including chemotaxis of the vegetative cells to folate, chemotaxis of developing cells to cAMP, phagocytosis, and cytokinesis. During chemotaxis to cAMP, the intracellular localization of myosin and its state of organization change rapidly (60). Because sufficient quantities of cells for biochemical studies can be obtained, events which occur in concert with cAMP-directed cell movement can be studied. For example, the specific but transient phosphorylation of the 18-kilodalton (kDa) regulatory MLC has been demonstrated (2). In addition, several other components of the motility apparatus from *D. discoideum* species have been purified and studied, including actin, severin (5, 59), actin-binding proteins (12), gelation and solation factors (12, 26), myosin heavy chain and MLC kinases (24, 31), and myosin phosphatases (24, 32).

The single gene encoding the *Dictyostelium* myosin heavy chain has been isolated (14), and its DNA sequence has been determined (57). The *Dictyostelium* heavy chain gene is unusual because unlike most other heavy chain genes, it consists of a single exon. Nothing is known about the structure of the *Dictyostelium* EMLC gene. Indeed, there is no information available concerning the structure of any nonmuscle MLC genes. Finally, because *Dictyostelium* spe-

* Corresponding author.

cies can be readily manipulated by molecular genetic approaches such as transformation (42) and the use of antisense RNA to establish mutant phenocopies (13), the study of the nonmuscle myosin of *Dictyostelium* species provides an important opportunity to investigate the molecular basis of cell motility.

In this report we describe the isolation and characterization of cDNAs encoding the *D. discoideum* EMLC. The DNA sequence has been determined and used to predict the amino acid sequence of the EMLC. Comparisons of this amino acid sequence with those previously reported for various skeletal- and smooth-muscle myosins are discussed. Finally, the developmental regulation of the EMLC mRNA has been examined throughout the *D. discoideum* life cycle.

MATERIALS AND METHODS

Purification of myosin and antibody production. Myosin was purified from *D. discoideum* as previously described (31). Myosin isolated in this way is greater than 95% pure as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The MLCs were separated from the heavy chains by solubilizing purified myosin in 4 M urea–2 mM EDTA. After a 30-min incubation on ice, the solution was clarified by centrifugation at 30,000 × *g* for 20 min and loaded onto a Bio-Gel A 0.5-m column (Bio-Rad Laboratories) equilibrated in 4 M urea–1 mM dithiothreitol–2 mM EDTA–25 mM triethanolamine (pH 7.5)–0.02% NaN_3–0.4 M KCl. Fractions containing the MLCs were pooled and dialyzed against 0.5 M KCl. New Zealand White rabbits were immunized by subcutaneous injection of purified MLCs emulsified in Freund complete adjuvant and boosted at 3-week intervals with native myosin emulsified in Freund incomplete adjuvant. The antibody was characterized by Western immunoblot analysis against both purified myosin and whole-cell protein extracts of *D. discoideum*.

Isolation of RNA and DNA. DNA was isolated from cells grown to stationary phase as previously described (4), except that Nonidet P-40 was substituted for Cemulsol NPT-12. RNA was prepared by extraction of cells with 4 M guanidine thiocyanate followed by centrifugation through a 5.7 M CsCl cushion. Poly(A)$^+$ RNA was isolated by chromatography on oligo(dT) cellulose as described by Maniatis et al. (36).

Construction and screening of λgt11 cDNA library. Messenger RNA isolated from cells developed for 12 h in cAMP-pulsed suspension cultures (8) was used to synthesize cDNA by priming with oligo(dT). Poly(A)$^+$ RNA (10 μg) was treated with 10 mM methyl mercury hydroxide (Alfa Products) at room temperature for 10 min. After the addition of β-mercaptoethanol to 100 mM to inactivate the MeHgOH, cDNA synthesis was carried out in 100 mM Tris hydrochloride (pH 8.3)–10 mM $MgCl_2$–10 mM dithiothreitol–1 mM deoxynucleoside triphosphates–2 μCi of [^{32}P]dATP–0.5 U of RNasin RNase inhibitor (Promega Biotec) per ml–100 μg of oligo(dT_{12-18}) per ml. After the reaction mixture was prewarmed to 42°C, AMV reverse transcriptase (Life Sciences, Inc.) was added to 600 U/ml to initiate the reaction. After 60 min, the reaction was terminated by phenol extraction, followed by ethanol precipitation. To convert the single-stranded cDNA into double-stranded cDNA, the pellet was suspended in 200 μl of 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 6.9)–10 mM $MgCl_2$–2.5 mM dithiothreitol–70 mM KCl–1 mM deoxynucleoside triphosphates. The large fragment of DNA polymerase (10 U) was added, and the reaction was incubated overnight at 15°C. After phenol extraction and ethanol precipitation, the double-stranded cDNA pellet was suspended in 200 mM NaCl–50 mM NaOAc (pH 4.5)–1 mM $ZnSO_4$–0.5% glycerol. S1 nuclease was added to give 1,000 U/ml, and the mixture was incubated at 37°C for 30 min to cleave the hairpin structures at the 5' ends of the double-stranded cDNA. The ends of the cDNA were made flush by incubation with 2 U of large-fragment DNA polymerase 1 in the presence of 1 mM deoxynucleoside-triphosphates under standard DNA polymerase reaction conditions (36). After the addition of *Eco*RI linkers, the double-stranded cDNA was ligated to *Eco*RI-digested λgt11 DNA which had been treated with 0.1 U of calf intestinal alkaline phosphatase per ml for 60 min at 37°C. The ligated DNA was packaged into λ bacteriophage particles as described by Maniatis et al. (36). The resulting library was amplified by growth in plate lysates in strain Y1090 (61).

Approximately 50,000 plaques per 150-mm dish were screened as described by Young and Davis (61), except that a peroxidase-conjugated second antibody was used in the visualization of the positive plaques. Chloronapthol (0.5 mg/ml) was used as the substrate in the peroxidase reaction.

Epitope selection. Lifts of protein produced in DdMLC-E1 plaques were used to affinity purify antibody essentially as described previously (58). Briefly, 10-cm plates containing approximately 10,000 plaques each were overlaid with nitrocellulose circles which had been soaked in isopropyl-β-D-thiogalactopyranoside and dried. After a 2-h incubation with the filters in place, the filters were removed and blocked in a solution of 5% nonfat dry milk in Tris-buffered saline for 60 min at room temperature. The filter was then incubated at room temperature for 1 h to overnight in a dilution (1:200) of antibody (in Tris-buffered saline–milk) normally used for Western blotting analysis. The filters were washed three times (10 min per wash) in Tris-buffered saline to remove unbound antibody. Specifically bound antibody was eluted from the filters by three washes (1 min each) with 5 mM glycine-HCl (pH 2.3)–150 mM NaCl–0.5% Triton X-100–100 μg of bovine serum albumin per ml. Each wash was immediately neutralized by the addition of Tris hydrochloride (pH 7.4) to a final concentration of 50 mM.

Subcloning and DNA sequencing. Subcloning of the *Eco*RI insert of DdMLC-E1 was accomplished by standard methods (36). DNA sequencing proceeded with standard chain termination protocols (48) and buffer gradient gels (3). The DNA sequence data were analyzed with the Pustell/IBI (45) and the University of Wisconsin Genetic Computer Group (16) software packages.

Northern and Southern blot analyses. Northern (RNA) blot analysis of total RNA was carried out as previously described (8). For Southern blots, agarose gels containing digested *Dictyostelium* DNA were transferred to Gene Screen Plus with 0.4 M NaOH as described elsewhere (46), prehybridized for 4 h in a hybridization solution containing 5× Denhardt solution (1× Denhardt solution is 0.02% Ficoll, 0.02% bovine serum albumin, and 0.02% polyvinyl pyrrolidone)–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–20 mM $NaPO_4$–10 μg of autoclaved salmon sperm DNA per ml–0.5% SDS. Probes were labeled with [^{32}P]dATP by nick translation (36) or random primed synthesis (19). After prehybridization, the solution was removed and replaced with the fresh hybridization buffer containing approximately 10^6 cpm of labeled probe per ml. Both hybridization and washes were at 65°C. Filters were washed with 2× SSC–0.1% SDS. Filters were exposed to Kodak X-Omat

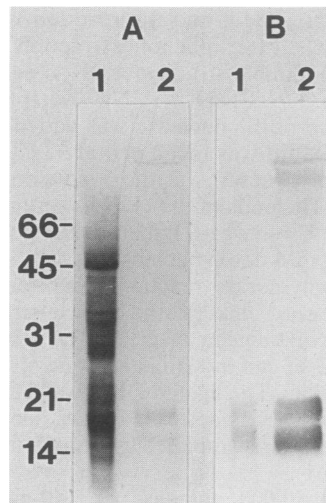


FIG. 1. NU-3 antiserum reactivity. A *Dictyostelium* whole-cell lysate (lanes 1) and purified *Dictyostelium* myosin (lanes 2) were separated on a 12% SDS-polyacrylamide gel, electrophoretically transferred to nitrocellulose, and stained with amido black to display the protein present on the blot (A) or probed with the NU-3 anti-myosin antibody (B). Molecular sizes (in kilodaltons) are shown on the left.

AR film in the presence of Dupont Cronex intensifying screens.

RESULTS

Production of a myosin-specific antibody. *D. discoideum* cells were grown in axenic culture to stationary phase, and myosin was isolated as previously described (31). This myosin preparation, consisting of both heavy chains and MLCs, was fractionated by gel filtration in the presence of urea to obtain a preparation enriched in MLCs. This fraction was used to generate a rabbit antiserum against *Dictyostelium* MLCs. The reactivity of the resulting antiserum, NU-3, is shown in Fig. 1. NU-3 reacted with both the 16- and 18-kDa MLCs, as well as the 243-kDa myosin heavy chain when it was used to probe a nitrocellulose blot of purified myosin. When used to probe a nitrocellulose blot of a *Dictyostelium* whole-cell lysate, a very intense reaction could be seen with the 16-kDa EMLCs and 18-kDa regulatory MLCs. A significantly less intense staining of the heavy chain was seen. It was difficult to obtain myosin preparations free of at least small amounts of proteolysis, and it was not surprising that the MLC preparation used to immunize rabbits contained proteolytic fragments of the heavy chain.

Isolation of a cDNA encoding the EMLC. The NU-3 antibody was used to screen a λgt11 library constructed with *Dictyostelium* poly(A)$^+$ RNA isolated from cells developed for 12 h. More than 25 positive clones were picked and plaque purified through three rounds of rescreening. Because the NU-3 antibody used to screen the library contained antibodies reactive with the myosin heavy chain, as well as with the MLCs, it was expected to react with clones containing cDNAs for the heavy chains as well as the MLCs. To distinguish potential MLC cDNA clones from heavy-chain clones, the antibody was absorbed with an excess of purified myosin heavy chain. The heavy chain was separated from the MLCs by gel filtration in 4.0 M urea. Absorption was repeated until the antibody no longer showed heavy-chain reactivity (data not shown). The resulting antibody was then used to test spots of phages which consistently reacted with the unabsorbed antiserum. Of the 15 clones tested, 4 reacted with the absorbed antibody, suggesting that they might encode the MLCs.

To confirm the identity of any clones encoding MLCs, as well as to determine which of the two MLC types each clone represented, an epitope selection scheme was used (58). NU-3 antibody was affinity purified by absorption to filters bearing antigen produced by the putative MLC clones and subsequent elution at low pH. The antibody was then reacted with nitrocellulose blots of both purified myosin and whole-cell protein. One of the clones, DdMLC-E1, specifically selected antibodies which reacted with the 16-kDa purified MLC, as well as with a 16-kDa polypeptide in the whole-cell lysate (Fig. 2). This clone was isolated, and the DNA was prepared and used for further analysis.

DNA sequence analysis of EMLC cDNAs. To determine the DNA sequence of the DdMLC-E1 cDNA, DNA was prepared from the λgt11 clone and digested with *Eco*RI. Agarose gel analysis showed that the DdMLC-E1 phage carried a 574-base-pair *Eco*RI fragment. The DdMLC-E1 insert was then subcloned into the pUC18 plasmid vector and M13mp18 single-stranded phage vector, and its sequence was determined by the dideoxynucleotide chain termination method. DNA sequence analysis and size comparisons of the cDNA and mRNA suggested that DdMLC-E1 was not full length. The cDNA appeared to lack sequences corresponding to the 3′ end of the message. Thus, the DdMLC-E1 insert was used to rescreen the original library, as well as a second λgt11 library constructed from RNA isolated from cells developed in suspension (I. A. S. Drummond and R. L. Chisholm, unpublished data). A clone (DdMLC-E5) containing the missing 3′ sequences was identified and characterized. Fig-

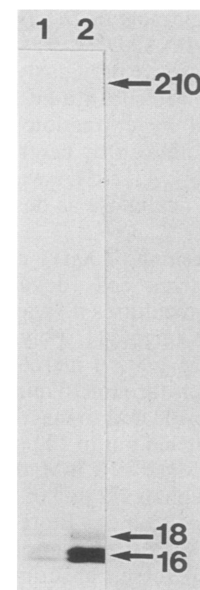


FIG. 2. Immunoblot with epitope-selected NU-3 antibody. A *Dictyostelium* whole-cell protein extract (lane 1) and purified *Dictyostelium* myosin (lane 2) were separated on a 5 to 15% gradient polyacrylamide-SDS gel, transferred to nitrocellulose, and probed with antibody affinity purified by binding and subsequent elution from proteins produced by *E. coli* cells infected with the DdMLC-E1 cDNA clone. Molecular sizes (in kilodaltons) are shown on the right.

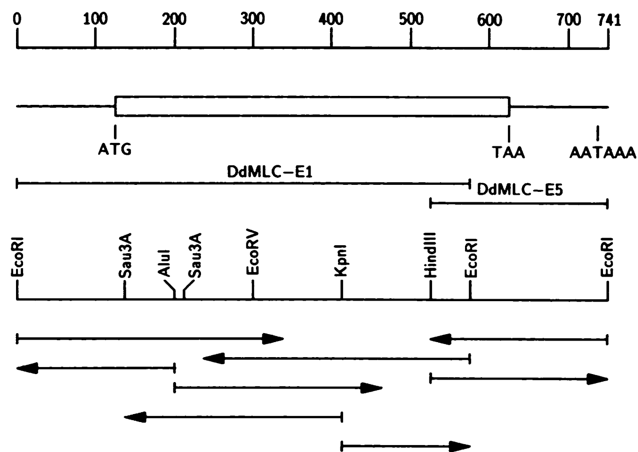


FIG. 3. Restriction map of a full-length EMLC cDNA constructed from DdMLC-E1 and DdMLC-E5. The lines to the left and right of the open box represent noncoding sequences, whereas the box itself represents coding sequences. The sequencing strategy used to determine the DNA sequence is shown by the lower portion of this diagram. The scale at the top is in bases.

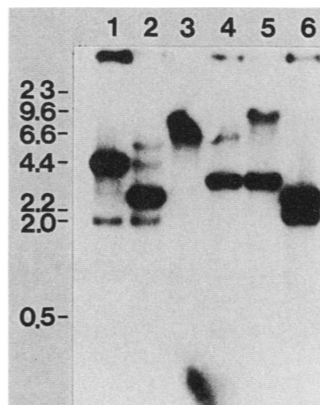


FIG. 5. Southern blot analysis of *Dictyostelium* genomic DNA digested with various restriction endonucleases and probed with the DdMLC-E1 cDNA. Lanes: 1, *Eco*RI; 2, *Eco*RI and *Hin*dIII; 3, *Hin*dIII; 4, *Hin*dIII and *Kpn*I; 5, *Kpn*I; 6, *Kpn*I and *Eco*RI. The top two bands in lane 2 are partial digestion products. Molecular sizes (in kilobases) are shown on the left.

ure 3 shows a restriction map of a full-length cDNA reconstructed from DdMLC-E1 and DdMLC-E5 as indicated. The DNA sequence of the coding region is shown in Fig. 4. The arrows at the bottom of Fig. 3 show the strategy for generating this sequence. The sequence was completely determined for both strands. It contains 130 bases of 5′ untranslated sequence, which bears stretches of A and T nucleotides common to most previoulsy sequenced *Dictyostelium* genes. In addition the ATG initiation codon is preceded by a run of 5 A's. Runs of adenosine nucleotides immediately preceding the translation initiation signal are common in *Dictyostelium* genes (28). Translation of the coding region predicts a polypeptide of 18,501 daltons with an estimated isoelectric point of 5.96. This is consistent with the observed isoelectric point of the *Dictyostelium* EMLC (unpublished data).

```
        10                            30                            50
GAATTCCAATGCAATTCCTAATGGTGATGGTAATTGATTTTTTGTTTTATAATTTTTTTT

        70                            90                           110
TTTCATTTGTAATTTTTTGTATAATATCTTGTATTATTTTTTATAACACTCAAACTATTT

       130                           150                           170
ATTAAATAAAATGTCCGCCTCAGCAGATCAAATTCAAGAATGTTTCAGTATCTTTGATAA
            MetSerAlaSerAlaAspGlnIleGlnGluCysPheSerIlePheAspLy

       190                           210                           230
AGATAATGACGGTAAGGTCTCAGTTGAAGATATTGGAGCTTGTTTAAGATCATTAGGTAA
sAspAsnAspGlyLysValSerValGluAspIleGlyAlaCysLeuArgSerLeuGlyLy

       250                           270                           290
AAGCCCAACCATGGCAGATATTGAAGCATTAAAGACTGAAATTGGTGCTAAAGAATTTGA
sSerProThrMetAlaAspIleGluAlaLeuLysThrGluIleGlyAlaLysGluPheAs

       310                           330                           350
TATCAATACATTAAAGAGCATTTACAAGAAACCAAACATTAAAACTCCACAAGAGCAACA
pIleAsnThrLeuLysSerIleTyrLysLysProAsnIleLysThrProGlnGluGlnGl

       370                           390                           410
AAAGGAAATGTTAGATGCATTCAAAGCCCTCGATAAAGAAGGCCATGGTACCATTCAAGG
nLysGluMetLeuAspAlaPheLysAlaLeuAspLysGluGlyHisGlyThrIleGlnGl

       430                           450                           470
TGCTGAATTACGTCAATTATTAACCACTCTTGGTGATTACTTATCAACCGCTGAAGTTGA
yAlaGluLeuArgGlnLeuLeuThrThrLeuGlyAspTyrLeuSerThrAlaGluValAs

       490                           510                           530
TGAATTATTTAAGGAAATCTCTGTCGATAGTACTACTGGTGCCGTTTCATATGCAAGCTT
pGluLeuPheLysGluIleSerValAspSerThrThrGlyAlaValSerTyrAlaSerLe

       550                           570                           590
AGTCAATACCATCGTTTCTGGTTATCCGGAATTCCGTCATAAATTCCAAAGTGGTTTTAG
uValAsnThrIleValSerGlyTyrProGluPheArgHisLysPheGlnSerGlyPheAr

       610                           630                           650
AGTTAAACGTGAACATTATCACCAATTTTAATATACTTTTATAAGAATAGTATAAATTTG
gValLysArgGluHisTyrHisGlnPheEnd

       670                           690                           710
AAACAAATATGTATAAAAAAAAAAAAAAAAAAAAAAATTTTGTATTTAATAAAACATCCA

       730
CCAAAAACATTAGTCTATTTATGGAATTC
```

FIG. 4. DNA sequence of the full-length *Dictyostelium* EMLC cDNA.

Southern analysis. To investigate the number of genes for the EMLC that are present in the genome of *D. discoideum*, we analyzed various restriction endonuclease digests of *Dictyostelium* DNA by blot hybridization with labeled DdMLC-E1 cDNA as a probe. The results are shown in Fig. 5. Because a single fragment reacting with the DdMLC-E1 cDNA was detected in the *Hin*dIII digest, as well as several other restriction digests (data not shown), it seems likely that there is a single gene for the EMLC. Since the DdMLC-E1 cDNA does not contain an *Eco*RI site, the presence of two hybridizing fragments suggests that the gene must at least have one intervening sequence which carries an *Eco*RI site. The presence of intervening sequences in *Dictyostelium* genes has been previously described (28). Since the EMLC gene has a relatively complicated structure in most other systems, it would not be unusual for the EMLC gene to have several intervening sequences. We are currently in the process of isolating genomic clones for the EMLC to examine this possibility and to allow detailed structural characterization of the *Dictyostelium* EMLC gene.

Developmental regulation of EMLC mRNA. During the growth phase, *D. discoideum* cells are highly motile as they seek out and engulf bacteria. After the initiation of development, two periods of active motility are seen. The first involves the chemotaxis of cells under the influence of cAMP to form the multicellular pseudoplasmodium (9). The second type of motility is seen later in development as the stalk cells migrate down through the spore mass to form the stalk. To determine when the EMLC is expressed during the developmental program, we examined the expression of the EMLC mRNA by Northern blot hybridization. Total cellular RNA was isolated from cells at 4-h intervals during the

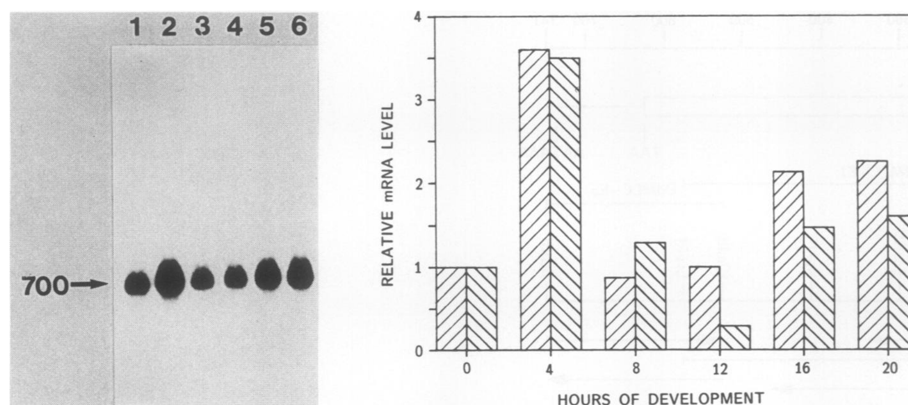


FIG. 6. (A) Northern blot analysis of *Dictyostelium* total RNA isolated from vegetative cells (lane 1) and at 4 (lane 2), 8 (lane 3), 12 (lane 4), 16 (lane 5), and 20 (lane 6) h of development and probed with labeled DdMLC-E1 insert. (B) Autoradiograms of two independent Northern blots (as in panel A) scanned with a densitometer to quantitate the relative levels of hybridization at each stage of development.

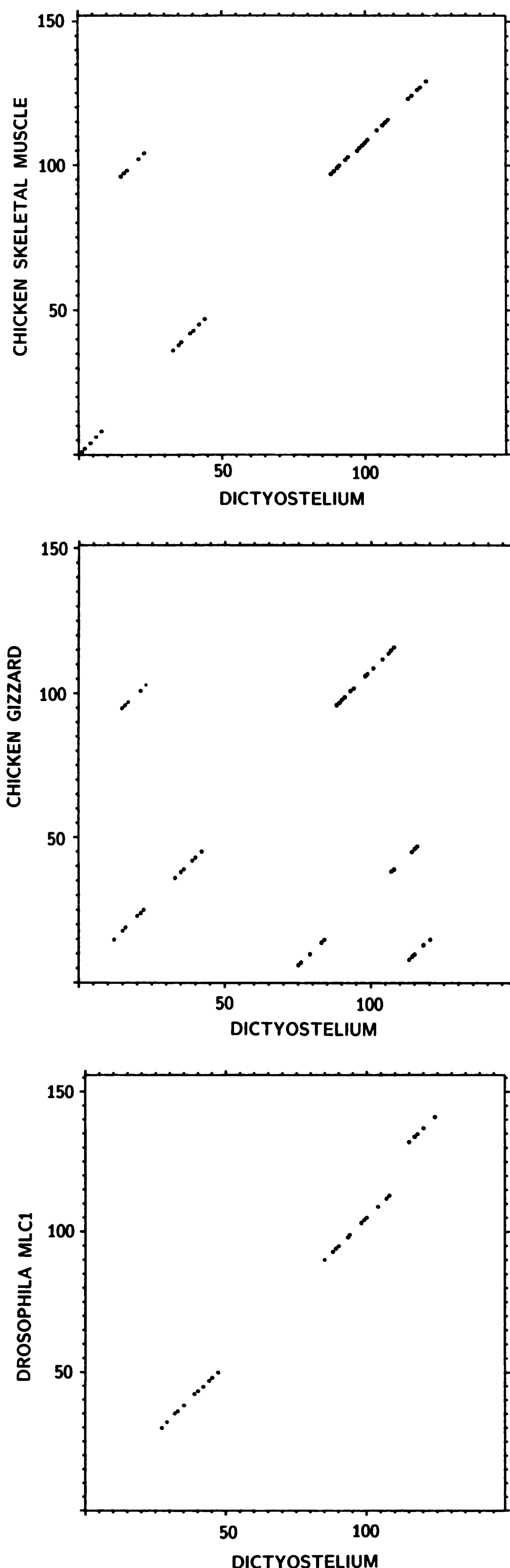
developmental program. Figure 6 shows the results of probing these RNAs with the DdMLC-E1 cDNA clone. To confirm that comparable amounts of RNA were loaded in each track, the gels were stained with ethidium bromide and the amounts of 27 and 17 S rRNAs were compared. In addition, a duplicate filter was probed with common clone CZ5 (data not shown). The level of the EMLC mRNA appeared to accumulate to significantly higher levels during the early phase of the developmental program than was seen in growing cells. Interestingly, this period of increased MLC accumulation immediately preceded the period of maximal chemotactic activity. The levels of the EMLC mRNA returned to levels comparable to those seen during growth at 8 to 12 h of development. Later in development there was a less dramatic, but reproducible, increase in EMLC mRNA accumulation. This corresponds to the period during which the stalk cells begin the morphogenetic migration which results in stalk formation.

DISCUSSION

In this report we describe the isolation and characterization of cDNAs encoding an MLC from *D. discoideum*. Several lines of evidence support the identification of these cDNAs as EMLC coding sequences. (i) Three independent polyclonal rabbit antisera specific for *Dictyostelium* myosin reacted with proteins present in cells infected with DdMLC-E1 phage. (ii) The proteins produced by DdMLC-E1-infected *Escherichia coli* cells could be used to affinity purify antibodies which reacted with the 16-kDa polypeptide present in purified myosin, as well as a polypeptide of the same molecular weight in a *Dictyostelium* whole-cell protein extract. (iii) The mRNA detected when the DdMLC-E1 cDNA was used to probe a Northern blot of *Dictyostelium* RNA had a size (approximately 750 to 800 nucleotides), consistent with that expected to encode a polypeptide of approximately 16 to 18 kDa. Finally, the amino acid sequence predicted from the DNA sequence of the cDNA encoded an 18-kDa polypeptide with an isoelectric point consistent with that observed for the EMLC.

The amino acid sequences of several EMLCs have been determined directly or predicted from cloned DNA sequences (18, 37, 41). The predicted *Dictyostelium* EMLC amino acid sequence shows significant similarity with these EMLC sequences upon comparison with those described in the PIR database with the FASTP program and the BioNet Molecular Biology Computer Resource. Because the amino acid sequence predicted from the DdMLC cDNAs represents the first EMLC sequence from a nonmuscle source, we examined the sequence relationships between it and the EMLCs from skeletal- and smooth-muscle sources. Dot matrix homology comparisons were made of the *Dictyostelium* EMLC amino acid sequence and those from chicken (skeletal- [Fig. 7A] and smooth- [Fig. 7B] muscle types) and *D. melanogaster* (a skeletal-muscle type [Fig. 7C]). The comparison between the chicken skeletal-muscle sequence and the *Dictyostelium* sequence shows four discrete regions of similarity. The last two regions show significant sequence homology to the calcium-binding consensus sequence of the E-F hand domain (30). These potential calcium-binding sites are characteristic of members of the troponin super gene family in which the MLCs have been previously classified (30). The significance of the other regions of homology is unknown. The chicken gizzard and *Drosophila* EMLC sequences show approximately the same degree of sequence homology as seen with the skeletal muscle sequence. When the chicken skeletal-muscle MLC LC_3 sequence was aligned with the *Dictyostelium* MLC sequence by the FASTP algorithm available with the BioNet system, there was 31.5% amino acid identity. When conservative amino acid substitutions are taken into consideration, the similarity between these two proteins increases to over 50%. The *Dictyostelium* EMLC appears to have approximately 20 additional amino acids beyond the end of the comparable chicken sequence. It will be interesting to learn if this additional sequence is involved in a cytoplasmic myosin-specific function.

When examining the specificity of the antibody affinity purified by binding to proteins present in plaques of the DdMLC-E1, we noted that the purified antibody reacted slightly with the regulatory MLC. This reaction could be seen with a whole-cell lysate, as well as in the purified myosin preparation. It has been noted that the regulatory MLCs of other systems have calcium-binding domains similar to those present in the *Dictyostelium* EMLC. We have recently isolated and sequenced cDNAs encoding the *Dictyostelium* regulatory MLC (manuscript in preparation). Dot matrix examination showed a distinct region of sequence similarity in a region near the 3′ end corresponding to E-H hand sequences. It is possible that the low-level cross-reactivity was due to recognition of an epitope related to the



calcium-binding site. We are currently producing antibodies against fusion proteins made by inserting the EMLC coding sequence in frame behind the β-galactosidase gene of λgt11. It will be of interest to learn whether any of the resulting antibodies also show this cross-reactivity.

The *Dictyostelium* genome has a very high adenosine and thymidine content of approximately 77%. The base composition of the EMLC coding sequence is only 64.3% A+T. In contrast, the base composition of position 1 of the codons is 52.7% A+T, whereas position 3 base composition is 75.4% A+T. Clearly *Dictyostelium* prefers to use A or T in position 3 of its codons.

Southern blot analysis suggests that *D. discoideum* most likely contains a single EMLC gene. In other systems, the EMLC genes are relatively complicated. The rat EMLC gene, for example, encodes two proteins of 21 and 16 kDa. It consists of nine exons which are differentially spliced together to form two different messages, one for each of the two isoforms (44). To add to the complexity, the gene has two different promoters (53). The chicken and mouse EMLC genes have similar structures (41, 47). Selection of one promoter over the other appears to determine which functional mRNA is produced. The two isoforms, called MLC1 and MLC3, show identical amino acid sequences over the 141 carboxy-terminal amino acid residues. The amino-terminal end of the two proteins diverge at this point, with the MLC1 isoform having 49 additional amino-terminal residues and the MLC3 isoform containing 8 amino-terminal amino acids. In contrast, *D. discoideum* appears to contain a single EMLC polypeptide. Comparison of the relative positions of restriction sites in the cDNA and the genome suggests the presence of at least one relatively large intron in the *Dictyostelium* EMLC gene (R. S. Pollenz and R. L. Chisholm, unpublished results). We are currently isolating genomic clones to further examine the structure of the EMLC gene.

In the mammalian skeletal muscle, the expression of the two EMLC isoforms is both tissue specific and developmentally regulated. In adult mouse fast skeletal muscle, the two isoforms are present in similar amounts, whereas only the MLC1 isoform is present in fetal muscle (52). Like the mammalian and avian genes, the *Drosophila* gene is differentially spliced to produce at least two different mRNAs. The two resulting messages encode polypeptides differing in amino acid sequence at their carboxy-terminal ends. The production of these mRNAs is tissue specific, with the larval and adult tissue containing both mRNA types, whereas the larval stage contains only one of the two messages. We have examined the developmental regulation of the *Dictyostelium* EMLC mRNA. Within the limits of resolution of Northern blot analysis, a single mRNA was observed throughout development. However, early in development, increased accumulation of the EMLC mRNA was observed (Fig. 6). It is intriguing that this pattern of regulation corresponds well with the previously described pattern of actin mRNA expression during the early phases of *Dictyostelium* development (38). The coincidence of increased myosin and actin mRNA accumulation just before a period of maximal cell motility (55) in response to extracellular cAMP deserves further examination.

FIG. 7. Dot matrix homology comparisons of the *Dictyostelium* EMLC amino acid sequence with chicken skeletal-muscle MLC 3 (A), the chicken gizzard EMLC (B), and the *Drosophila* alkali MLC (C). A dot was placed on the diagram when 5 amino acids showed identity within a window of 10 amino acids. Scales are shown in amino acid residues.

The isolation and characterization of the *Dictyostelium* EMLC open the doors for direct tests of the role of MLC in cell motility. Because *D. discoideum* is amenable to a variety of molecular genetic manipulations, including the reintroduction of genes (42), the use of antisense transcripts to produce phenocopies of mutations (13, 29), and perhaps even homologous disruption of enodgenous genes (15), it should be possible to examine the role of the EMLC in important cellular processes such as chemotaxis, cytokinesis, and development.

ACKNOWLEDGMENTS

The authors thank Iain A. S. Drummond for generously providing a λgt11 cDNA library and useful remarks and Y. Fukui, R. D. Goldman, and K. J. Green for their helpful comments on the manuscript.

This work was supported by Public Health Service grants from the National Institutes of Health to R.L.C. and to E.R.K. and a grant from the National Foundation—March of Dimes to R.L.C. We also acknowledge the support of the National Institutes of Health for the BioNet Molecular Biology Computer Resource.

LITERATURE CITED

1. **Adelstein, R. S., and E. Eisenberg.** 1980. Regulation and kinetics of the actin-myosin-ATP interaction. Annu. Rev. Biochem. **49:** 921–956.
2. **Berlot, C. H., J. A. Spudich, and P. N. Devreotes.** 1985. Chemoattractant-elicited increases in myosin phosphorylation in *Dictyostelium*. Cell **43:**307–314.
3. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80:**3963–3965.
4. **Blumberg, D. D., and H. F. Lodish.** 1980. Complexity of nuclear and polysomal RNAs in growing *Dictyostelium*. Dev. Biol. **78:** 268–284.
5. **Brown, S. S., K. Yamamoto, and J. A. Spudich.** 1982. A 40,000 dalton protein from *Dictyostelium discoideum* affects assembly properties of actin in a Ca^{++} dependent manner. J. Cell Biol. **93:**205–210.
6. **Burke, M. M., M. Sivaramakrishnan, and V. Kamalakannan.** 1983. On the mode of alkali light chain association to the heavy chains of myosin subfragment 1. Evidence for the involvement of the carboxyl-terminal regions of the heavy chain. Biochemistry **22:**3046–3053.
7. **Burridge, K., and D. Bray.** 1975. Purification and structural analysis of myosins from brain and other non-muscle tissues. J. Mol. Biol. **99:**1–14.
8. **Chisholm, R. L., E. Barklis, and H. F. Lodish.** 1984. Mechanism of sequential induction of cell-type specific mRNAs in *Dictyostelium discoideum*. Nature (London) **297:**67–69.
9. **Chisholm, R. L., D. Fontana, A. Theibert, H. F. Lodish, and P. Devreotes.** 1984. Development of *Dictyostelium discoideum*: chemotaxis, cell-cell adhesion, and gene expression. *In* R. Losick and L. Shapiro, (ed.), Microbial development. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Clarke, M., and J. A. Spudich.** 1977. Nonmuscle contractile proteins: the role of actin and myosin in cell motility and shape determination. Annu. Rev. Biochem. **46:**797–822.
11. **Collins, J. H., and E. D. Korn.** 1980. Actin activation of Ca^{++}-sensitive Mg^{++}-ATPase activity of Acanthamoeba myosin II is enhanced by dephosphorylation of its heavy chains. J. Biol. Chem. **255:**8011–8014.
12. **Condeelis, J., J. Salisbury, and K. Fujiwara.** 1981. A new protein that gels F actin in the cell cortex of *Dictyostelium discoideum*. Nature (London) **292:**161–163.
13. **Crowley, T. E., W. Nellen, R. H. Gomer, and R. A. Firtel.** 1985. Phenocopy of Discoidin-I minus mutants by antisense transformation in *Dictyostelium*. Cell **43:**633–641.
14. **DeLozanne, A., M. Lewis, J. A. Spudich, and L. A. Leinwand.** 1985. Cloning and characterization of a nonmuscle myosin heavy chain cDNA. Proc. Natl. Acad. Sci. USA **82:**6807–6810.
15. **DeLozanne, A., and J. A. Spudich.** 1987. Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination. Science **236:**1086–1091.
16. **Devereux, J., P. Haeberli, and O. Smithes.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
17. **Falkenthal, S., V. P. Parker, and N. Davidson.** 1985. Developmental variations in the splicing pattern of transcripts from the *Drosophila* gene encoding myosin alkali light chain result in different carboxyl-terminal amino acid sequences. Proc. Natl. Acad. Sci. USA **82:**449–453.
18. **Falkenthal, S., V. P. Parker, W. W. Mattox, and N. Davidson.** 1984. *Drosophila melanogaster* has only one myosin alkali light-chain gene which encodes a protein with considerable amino acid sequence homology to chicken myosin alkali light chains. Mol. Cell. Biol. **4:**956–965.
19. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
20. **Flicker, P., T. Wallimann, and P. Vilbert.** 1983. Electron microscopy of scallop myosin. Location of regulatory light chains. J. Mol. Biol. **109:**723–741.
21. **Garfinkle, L. I., M. Periasamy, and B. Nadal-Ginard.** 1982. Cloning and characterization of cDNA sequences corresponding to myosin light chains 1, 2, and 3, troponin-C, troponin-T, α-tropomyosin and α-actinin. J. Biol. Chem. **257:**11078–11086.
22. **Gauthier, G. F., and S. Lowey.** 1979. Distribution of myosin isoenzymes among skeletal muscle fiber types. J. Cell Biol. **81:**10–25.
23. **Gauthier, G. F., S. Lowey, P. A. Benfield, and A. W. Hobbs.** 1982. Distribution and properties of myosin isozymes in developing avian and mammalian skeletal muscle fibers. J. Cell Biol. **92:**472–484.
24. **Griffith, L. M., S. M. Downs, and J. A. Spudich.** 1987. Myosin light chain kinase and myosin light chain phosphatase from Dictyosteium: effects of reversible phosphorylation on myosin structure and function. J. Cell Biol. **104:**1309–1323.
25. **Harrington, W. F., and M. E. Rodgers.** 1984. Myosin. Annu. Rev. Biochem. **53:**35–73.
26. **Hellewell, S. G., and D. L. Taylor.** 1979. The contractile basis of ameboid movement. VI. The solation-contraction coupling hypothesis. J. Cell Biol. **83:**633–648.
27. **Kendrick-Jones, J., and J. M. Scholey.** 1981. Myosin linked regulatory systems. J. Muscle Res. Cell Motil. **2:**347–372.
28. **Kimmel, A. R., and R. A. Firtel.** 1983. Sequence organization in *Dictyostelium*: unique structure at the 5′-ends of protein coding genes. Nucleic Acids Res. **11:**541–552.
29. **Knecht, D. A., and W. F. Loomis.** 1987. Antisense RNA inactivation of myosin heavy chain gene expression in *Dictyostelium discoideum*. Science **236:**1081–1086.
30. **Kretsinger, R. H.** 1980. Structure and evolution of calcium modulated proteins. Crit. Rev. Biochem. **8:**119–174.
31. **Kuczmarski, E. R.** 1986. Partial purification of two myosin heavy chain kinases from *Dictyostelium discoideum*. J. Muscle Res. Cell Motil. **7:**501–509.
32. **Kuczmarski, E. R., and J. Pagone.** 1986. Myosin specific phosphatases isolated from *Dictyostelium discoideum*. J. Muscle Res. Cell Motil. **7:**510–516.
33. **Kuczmarski, E. R., and J. A. Spudich.** 1980. Regulation of myosin self-assembly: phosphorylation of Dictyostelium heavy chains inhibits formation of thick filaments. Proc. Natl. Acad. Sci. USA **77:**7292–7296.
34. **Kuwayama, H., and K. Yagi.** 1980. Localization of g2 light chain in the link between the heads and tail of cardiac myosin. J. Biochem. **87:**1603–1607.
35. **Labbe, J.-P., D. Mornet, P. Vandest, and R. Kassab.** 1981. Proximity of alkali light chains to 27K domain of the heavy chain in myosin subfragment 1. Biochem. Biophys. Res. Commun. **102:**466–475.
36. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Matsuda, G., T. Maita, Y. Kato, J. Chen, and T. Umegane.** 1981.

Amino acid sequences of the cardiac L-2A, L-2B, and gizzard 17,000 M light chains of chicken myosin. FEBS Lett. **135:**232–236.

38. **McKeown, M., and R. A. Firtel.** 1981. Differential expression and 5′ end mapping of actin genes in Dictyostelium. Cell **24:**799–807.
39. **Medford, R., R. Wydro, H. Nguyen, and B. Nadal-Ginard.** 1980. Cytoplasmic processing of myosin heavy chain messenger RNA: evidence provided by using a recombinant DNA plasmid. Proc. Natl. Acad. Sci. USA **77:**5749–5753.
40. **Mocz, G., E. N. A. Biro, and M. Balint.** 1982. Crosslinking by thiol interchange of 5,5′-dithiobis(2-nitrobenzoic acid)-treated light chain and heavy chain of rabbit skeletal muscle. Eur. J. Biochem. **126:**603–609.
41. **Nabeshima, Y., Y. Fujii-Kuriyama, M. Muramatsu, and K. Ogata.** 1984. Alternative transcription and two modes of splicing result in two myosin light chains from one gene. Nature (London) **308:**333–338.
42. **Nellen, W., C. Silan, and R. A. Firtel.** 1984. DNA-mediated transformation in *Dictyostelium discoideum*: regulated expression of an actin gene fusion. Mol. Cell. Biol. **4:**2890–2898.
43. **Okomoto, Y., T. Sekine, J. Grammer, and R. G. Yount.** 1986. The essential light chains constitute part of the active site of smooth muscle myosin. Nature (London) **324:**78–80.
44. **Periasamy, M., E. E. Strehler, L. T. Garfinkel, R. M. Gubits, N. Ruiz-Opazo, and B. Nadal-Ginard.** 1984. Fast skeletal muscle myosin light chain 1 and 3 are produced from a single gene by a combined process of differential RNA transcription and splicing. J. Biol. Chem. **259:**13595–13604.
45. **Pustell, J., and F. C. Kafatos.** 1984. A convenient and adaptable package of computer programs for DNA and protein sequence management, analysis, and homology determination. Nucleic Acids Res. **12:**643–655.
46. **Reed, K. C., and D. A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13:**7207–7221.
47. **Robert, B., P. Daubas, M.-A. Akimenko, A. Cohen, I. Garner, J.-L. Guenet, and M. E. Buckingham.** 1984. A single locus in the mouse encodes both myosin light chains 1 and 3, a second locus corresponds to a related pseudogene. Cell **39:**129–140.
48. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
49. **Sellers, J. R., E. Eisenberg, and R. S. Adelstein.** 1982. The binding of smooth muscle heavy meromyosin to actin in the presence of ATP. J. Biol. Chem. **257:**13880–13883.
50. **Sivaramakrishnan, M., and M. Burke.** 1982. The free heavy chain of vertebrate skeletal myosin subfragment 1 shows full enzymatic activity. J. Biol. Chem. **257:**1102–1106.
51. **Spudich, J. A., and A. Spudich.** 1982. Cell motility, p. 169–194. *In* W. F. Loomis (ed.), The development of *Dictyostelium discoideum*. Academic Press, Inc., New York.
52. **Sreter, F. A., M. Balint, and J. Gergely.** 1975. Structural and functional changes of myosin during development. Comparison with adult fast, slow and cardiac myosin. Dev. Biol. **46:**317–325.
53. **Strehler, E. E., M. Periasamy, M.-A. Strehler-Page, and B. Nadal-Ginard.** 1985. Myosin light-chain 1 and 3 gene has two structurally distinct and differentially regulated promoters evolving at different rates. Mol. Cell. Biol. **5:**3168–3182.
54. **Taylor, D. L., and J. S. Condeelis.** 1979. Cytoplasmic structure and contractility in amoeboid cells. Int. Rev. Cytol. **56:**57–144.
55. **Varnum, B., and D. R. Soll.** 1981. Chemoresponsiveness to cAMP and folic acid during growth, development and dedifferentiation in *Dictyostelium discoideum*. Differentiation **18:**151–160.
56. **Wagner, P. D., and E. Giniger.** 1981. Hydrolysis of ATP and reversible binding to F-actin by myosin heavy chains free of all light chains. Nature (London) **292:**560–562.
57. **Warrick, H. M., A. DeLozanne, L. A. Leinwand, and J. A. Spudich.** 1986. Conserved protein domains in a myosin heavy chain gene from *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. USA **83:**9433–9437.
58. **Weinberger, C., S. M. Hollenberg, E. S. Ong, J. M. Harmon, S. T. Brower, J. Cidlowski, E. B. Thompson, M. G. Rosenfeld, and R. M. Evans.** 1985. Identification of human glucocorticoid receptor cDNA clones by epitope selection. Science **228:**740–742.
59. **Yamamoto, K., J. Pardee, J. Reidler, L. Stryer, and J. A. Spudich.** 1982. The mechanism of interaction of *Dictyostelium* severin with actin filaments. J. Cell Biol. **95:**711–719.
60. **Yamura, S., and Y. Fukui.** 1985. Reversible cyclic AMP-dependent change in distribution of myosin thick filaments in *Dictyostelium*. Nature (London) **314:**184–196.
61. **Young, R. A., and R. W. Davis.** 1983. Efficient isolation of genes using antibody probes. Proc. Natl. Acad. Sci. USA **80:**1194–1198.