Antisense RNA Inactivation of Myosin Heavy Chain Gene Expression in *Dictyostelium discoideum*

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The role of myosin in the contraction of striated muscle cells is well known, but its importance in nonmuscle cells is not yet clear. The function of myosin in *Dictyostelium discoideum* has been investigated by isolating cells which specifically lack myosin heavy chain (MHC A) protein. Cells were transformed with a vector encoding RNA complementary to *mhc*A messenger RNA (antisense RNA). Stable transformants have a dramatic reduction in the amount of MHC A protein, grow slowly, and generate giant multinucleated progeny, indicating an impairment in cytokinesis. Surprisingly, the cells adhere to surfaces, extend pseudopods and are capable of ameboid locomotion. The developmental sequence that is initiated by starving cells is severely impaired by the lack of myosin. The cells are unable to form multicellular aggregates normally and do not undergo subsequent morphogenesis. By changing the food source from liquid medium to bacteria, expression of the endogenous *mhc*A messenger RNA can be increased relative to expression of antisense RNA. When grown in this way, the transformed cells accumulate MHC A protein, remain mononucleate, and proceed through development normally.

Dictyostelium discoideum CELLS ARE HIGHLY MOTILE EUkaryotic amebae that divide by binary fission. When deprived of a food source the amebae move chemotactically toward aggregation centers in response to rhythmic pulses of cyclic adenosine monophosphate (AMP). The multicellular aggregates then undergo a dramatic series of morphological changes leading to the formation of fruiting bodies that consist of differentiated spore and stalk cells. The growing amebae contain both actin and myosin in abundance, and it is presumed that these molecules play a key role in motility as well as other cellular functions (*1, 2*). In vitro, myosin is required for the contraction of cell cortices in response to adenosine triphosphate (ATP) and calcium ions (*3*). Myosin has been implicated in cytokinesis because of the detection of parallel thick filaments in the cleavage furrow of dividing cells (*4*).

The major *Dictyostelium* myosin consists of two 243,000-dalton (243-kD) heavy chains plus one pair each of 18-kD and 16-kD light chains (*5*). This complex assembles into thick filaments, interacts with actin filaments, and possesses an actin-activated adenosinetriphosphatase (ATPase) activity. A minor myosin-like molecule, containing a single 117-kD heavy chain has also been purified from *Dictyostelium* on the basis of its actin-activated ATPase activity (*6, 7*). The function of myosin is controlled at least in part by phosphorylation of the light and heavy chains. Heavy chain phosphorylation inhibits the assembly of thick filaments, whereas phosphorylation of the light chains regulates the actin-activated ATPase activity (*8, 9*). The phosphorylations are in turn regulated by cyclic AMP pulses during development (*10–12*). Although the interaction of all the components is not yet clear, the evidence points to a complex regulatory network for controlling the assembly of myosin thick filaments and their interaction with actin filaments.

The role of myosin in nonmuscle cell physiology might be better understood if wild-type cells could be compared to mutant cells with an altered myosin molecule, but such mutants have not been isolated. A new approach to generating mutants is to introduce into cells a cloned sequence in which the noncoding strand of a gene is transcribed to produce antisense RNA molecules. This technique was first successfully carried out in mouse cells with a thymidine

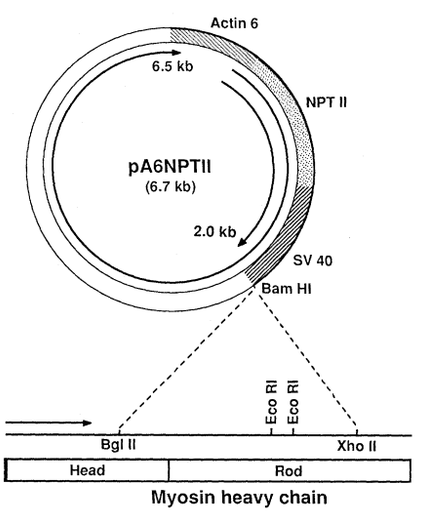

Fig. 1. Antisense myosin heavy chain transformation vector. The vector pA6NPTII (*20*) consists of the *Dictyostelium* actin-6 promoter fused to the TN5 neomycin phosphotransferase gene. This vector confers resistance to the antibiotic G418 on *Dictyostelium* cells. The transcription termination and polyadenylation sites from SV40 are downstream of the NPT gene. Since this terminator functions inefficiently in *Dictyostelium*, both 6.5- and 2.0-kb transcripts (as indicated) are found in cells transformed with this vector. Also shown is the general structure of the myosin heavy chain gene (*mhc*A). The 3.7-kb Bgl II–Xho II fragment was inserted at the Bam HI site of pA6NPTII in the antisense orientation relative to the actin promoter. The resulting plasmid is called pA6mhc6. The RNA transcripts from this plasmid are expected to be 2.0 kb and 10 kb. *Dictyostelium* AX4 cells (*20*) were transformed according to Nellen *et al.* (*21*), except that after the initial 5-day selection in HL5 medium containing G418 at 10 μg/ml (Geneticin, Sigma Inc.), the media in the petri dish were replaced with fresh HL5 without drug but including a suspension of autoclaved *Escherichia coli* B/r (*20*). When colonies of cells that had survived the first selection became visible, they were returned to selective conditions until stable transformants began to grow. Unlike the parental strain, AX4, and cells transformed with pA6NPTII, cells transformed with pA6mhc6 are not able to grow in suspension and therefore must be grown in petri dishes throughout the selection.

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kinase gene (*13*). Double-stranded thymidine kinase RNA was found only in the nuclei of such cells and appeared to be unable to exit to the cytoplasm (*14*). Antisense RNA has also been used to inhibit the expression of the discoidin genes of *Dictyostelium* (*15*) and the *hsp* 26 and *Krupple* genes of *Drosophila* (*16, 17*). We have examined the function of myosin in *Dictyostelium* cells by inhibiting expression of the myosin heavy chain gene with antisense RNA.

Construction of the antisense RNA vector. The *Dictyostelium* myosin heavy chain gene (*mhc*A) has recently been cloned and sequenced (*18, 19*). A single genomic copy encodes a 7.0-kilobase messenger RNA (mRNA) that is translated into a 243-kD polypeptide. The myosin antisense RNA vector was constructed from the *Dictyostelium* transformation vector pA6NPTII (*20*) (Fig. 1). The pA6NPTII vector consists of the *Dictyostelium* actin-6 promoter directing transcription of the neomycin phosphotransferase gene (NPT II) that confers resistance to the antibiotic G418. The SV40 transcription termination and polyadenylation signals are located downstream of the NPT II gene. Termination at the SV40 sequence is inefficient in *Dictyostelium* such that two NPT II RNA transcripts are produced from the actin promoter: a 2.0-kb RNA from termination in the SV40 sequence and a 6.5-kb RNA that terminates upstream of the *Dictyostelium* actin promoter region (*20*). The antisense RNA vector, pA6mhc6, was constructed by cloning the 3.7-kb Bgl II–Xho II fragment from the coding portion of the *mhc*A gene into the Bam HI site of pA6NPTII. The *mhc*A sequence is inserted in the reverse orientation with respect to the actin promoter in order to allow antisense RNA transcription. Because insertion of the *mhc*A sequence is downstream of the SV40 termination site, cells transformed with pA6mhc6 are expected to synthesize the same 2.0-kb transcript as pA6NPTII-transformed cells, as well as a 10-kb RNA transcript that includes the NPT II, antisense myosin, and pBR322 sequences of the vector. Both the selectable gene and the antisense RNA are under control of the actin promoter, so that selection for resistance to G418 simultaneously results in antisense RNA expression. An important feature of this construction is that even if the long transcript hybridizes to *mhc*A mRNA and cannot exit the nucleus or be translated, the 2.0-kb NPT II transcript will be unaffected and will maintain the drug-resistant phenotype of the transformed cells. The control cells used for these experiments were either cells transformed with the pA6NPTII vector or the untransformed AX4 cell line (*20*). The cells transformed with the *mhc*A antisense RNA vector (pA6mhc6) are referred to as $\overline{mhcA}$. We propose the use of this notation to indicate the genotype of cells that contain an antisense copy of a gene. The bar over the genetic locus is derived from the mathematical notation for the complement.

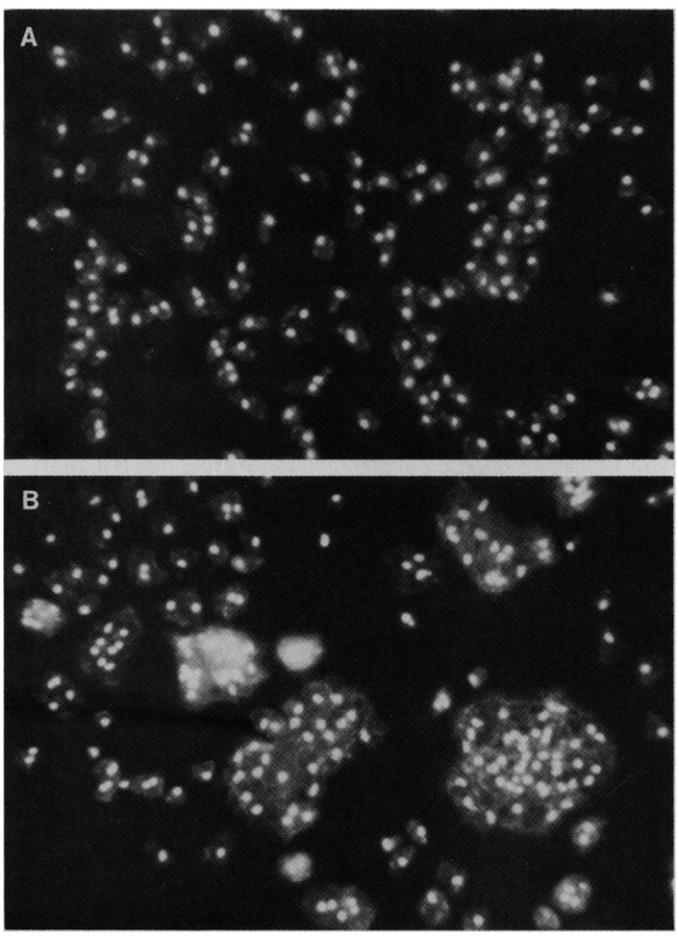

Fig. 2. Morphology of transformed cells. Cells growing on multiwell slides (Miles Scientific) were fixed for 15 minutes at room temperature in 20 m*M* 2[*N*-morpholino]ethanesulfonic acid (*p*H 6.5), 20 m*M* KCl, 5 m*M* $MgCl_2$, containing 4 percent paraformaldehyde. The slide was then incubated in 60 percent methanol for 15 minutes, rehydrated in phosphate-buffered saline (PBS) (10 m*M* sodium phosphate, *p*H 7.4, and 150 m*M* NaCl), and nuclei were stained in PBS containing the fluorescent dye Hoechst 33258 at 5 μg/ml (Calbiochem) (×100). (**A**) Control cells transformed with pA6NPTII; (**B**) cells transformed with pA6mhc6.

Cells transformed with the *mhc*A antisense RNA vector are large and multinuclear. Vector DNA was introduced into cells as a calcium phosphate precipitate and stable transformants were selected (*20, 21*). Cell lines transformed with either pA6NPTII or pA6mhc6 have multiple copies of the DNA integrated into the chromosome as a tandem array (*20*) and are able to grow under selective conditions (G418 at 10 μg/ml in HL5 medium). The $\overline{mhcA}$ cells grow slowly (~24-hour generation time) compared to control cells (10- to 12-hour generation time) and will grow only when attached to a surface, unlike the control cells that can also grow in shaken suspension. The most striking abnormality was that as many as 50 percent of the cells were substantially larger than normal, ranging up to ten times the diameter of normal cells. The proportion of giant cells has varied considerably during subsequent culturing; however, clonally derived cell lines from either giant or normal sized cells always give rise to a mixture of giant and normal sized cells.

Cells were fixed and stained with the fluorescent DNA binding dye Hoechst 33258 in order to visualize nuclei. While cells transformed with the vector alone had 1 to 2 nuclei per cell, most of the $\overline{mhcA}$ cells were multinucleate, containing up to 50 nuclei in a single cell (Fig. 2). The largest cells usually contain the most nuclei;

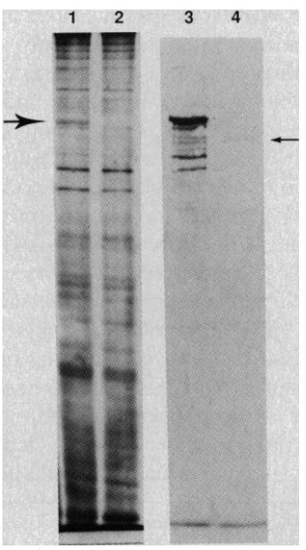

Fig. 3. Expression of myosin heavy chain protein in transformed cells. Total cell protein (10 μg) from cells transformed with either pA6NPTII (control, lanes 1 and 3) or pA6mhc6 ($\overline{mhcA}$, lanes 2 and 4) were electrophoretically separated on 6 percent SDS-polyacrylamide gels. One set of lanes was silver-stained (*31*) (lanes 1 and 2) and the other set was transferred to nitrocellulose (Western blot) and immunostained with a monoclonal antibody to *Dictyostelium* myosin heavy chain (My11) (*22*) as described (*25*) (lanes 3 and 4). Secondary antibody was goat antibody to mouse IgG conjugated with alkaline phosphatase (Tago). The large arrow on the left indicates the position of the 243-kD myosin heavy chain polypeptide in the control samples. The small arrow on the right indicates the minor 180-kD protein found in the $\overline{mhcA}$ samples.

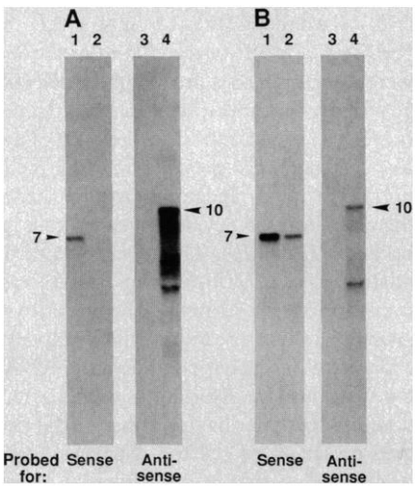

Fig. 4. Northern analysis of RNA from transformed cells. RNA was isolated by guanidinium thiocyanate extraction and CsCl gradient centrifugation; 5 μg of total cell RNA from each strain was electrophoretically separated on denaturing agarose-formaldehyde gels (*32*). The RNA was partially hydrolyzed in 50 m*M* NaOH, 10 m*M* NaCl for 45 minutes at room temperature, neutralized with 0.1*M* tris-HCl, *p*H 7.5, for 10 minutes, transferred to nitrocellulose paper (Schleicher & Schuell) in 10× SSPE (1× SSPE is 1 m*M* sodium phosphate, 1 m*M* EDTA, and 150 m*M* NaCl, *p*H 7.4), and then hybridized with strand-specific single-stranded ^{32}P-labeled RNA probes synthesized in a Gemini (Promega Biotech) bidirectional transcription system. The myosin probe was transcribed from a 400-bp Eco RI fragment (indicated in Fig. 1) cloned into the Eco RI site of pGem™-1. Sense probe was synthesized with T7 polymerase and the antisense probe with SP6 polymerase from plasmids linearized in the polylinker region. (**A**) RNA from cells grown in axenic HL-5 medium. (lanes 1 and 3) Control (pA6NPTII-transformed) cell RNA. (lanes 2 and 4) *mhcA* cell RNA. The filter with lanes 1 and 2 was hybridized with the probe that detects sense *mhc*A mRNA (7 kb), and the filter with lanes 3 and 4 was hybridized with probe that detects antisense RNA (10 kb). (**B**) Same as (A) except that the RNA was made from cells grown in association with bacteria.

however, we occasionally observe very large cells with four to eight nuclei and smaller cells with 20 to 30 nuclei. Approximately 90 percent of the total number of nuclei in a population are in giant multinucleated cells. The *mhcA* cells appear to have a defect in cytokinesis, leading to nuclear division in the absence of cytoplasmic cleavage.

Expression of myosin heavy chain is inhibited. A monoclonal antibody (My11) specific for the major myosin heavy chain protein was used to assay for the presence of this protein in *mhcA* cells (*22*). Extracts from control (pA6NPTII-transformed) and *mhcA* cells were electrophoretically separated on SDS-polyacrylamide gels, and the proteins were either silver stained or transferred to nitrocellulose (Western blot) and immunostained with My11 (Fig. 3). The monoclonal antibody detects the prominent 243-kD MHC A protein in the control cells plus several less abundant, smaller proteolytic fragments (Fig. 3, lane 3). The 243-kD protein is also easily identified on silver-stained gels because of its unusually large size and abundance (Fig. 3, lane 1). In the *mhcA* cell extract, neither the 243-kD protein nor any of its proteolytic fragments were detected by either Western blotting or silver staining (Fig. 3, lanes 2 and 4). The absence of the 243-kD band was the only obvious difference between the two samples when the silver-stained band pattern was compared. Similar results were obtained with two other monoclonal antibodies (My2 and My5) which react with other epitopes on the MHC A protein (*23*). When larger amounts of *mhcA* cell extract were loaded on the gel, a faint 243-kD band was detected by immunostaining. The amount of MHC A in *mhcA* cells was estimated to be 250-fold reduced by titrating the control extract to the point where a band of comparable intensity was detected. In the *mhcA* cells, a 180-kD band was reproducibly detected by the monoclonal antibody My11 (Fig. 3, lane 4). This band, if present in the control cells, would represent about 5 percent of the amount of the 243-kD MHC A protein and make it difficult to detect against the background of proteolytic fragments of the 243-kD polypeptide. The 180-kD polypeptide may be a previously unrecognized minor myosin-like protein.

RNA from both control and *mhcA* cells was extracted, electrophoretically separated, and transferred to nitrocellulose filters. The Northern blots were hybridized with strand-specific RNA probes capable of detecting either *mhc*A mRNA or antisense RNA (Figs. 1 and 4A). The 7.0-kb *mhc*A mRNA in the *mhcA* cells was dramatically reduced relative to the amount found in the control (Fig. 4A, lanes 1 and 2). In some RNA preparations from *mhcA* cells, a faint 7.0-kb band could be detected. However, it is not known whether this is derived from functional mRNA or an inactive double-stranded hybrid. The probe used to detect the *mhc*A mRNA was

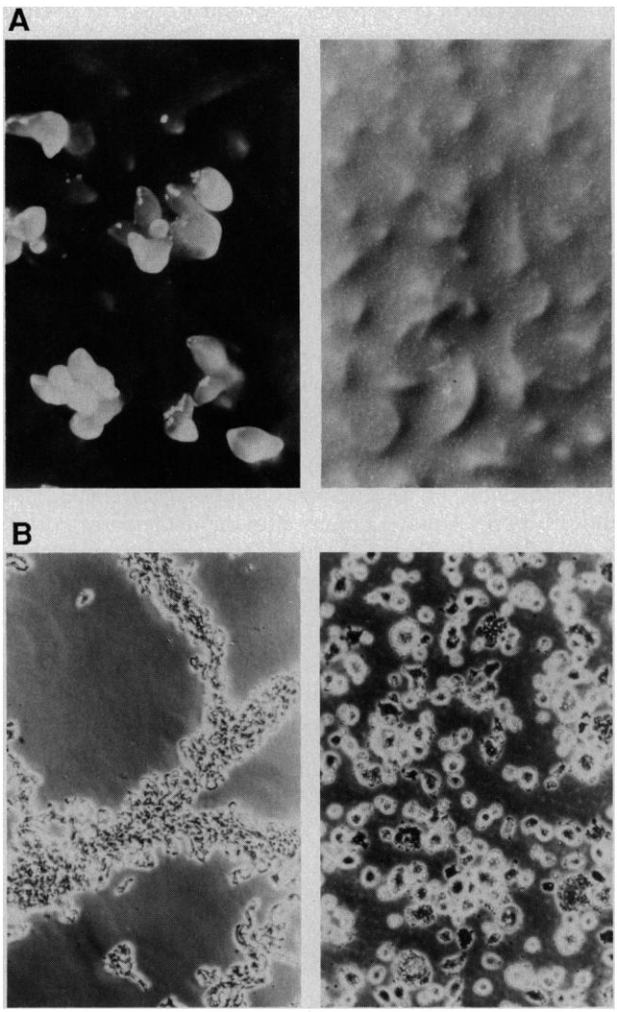

Fig. 5. Cells transformed with pA6mhc6 do not aggregate normally. (**A**) Filter development. Cells were grown in petri dishes in axenic HL5 medium containing G418 at 10 μg/ml. The cells were harvested from the dishes, washed once in PDF (20 m*M* potassium phosphate, 20 m*M* KCl, 5 m*M* $MgCl_2$, dihydrostreptomycin sulfate at 0.5 mg/ml, *p*H 6.5), and then deposited on black 47-mm nitrocellulose filters (Millipore) supported by a pad saturated with 3 ml of PDF. The filters were incubated in a humid incubator at 22°C. The filters shown were photographed at 13 hours of development. The control cells (left panel) have formed tipped aggregates and are beginning to elongate upward. The *mhcA* cells (right panel) were unable to aggregate normally. (**B**) Streaming assay. Cells were grown as above, harvested, washed in PDF, and then resuspended to 2×10^6 cells per milliliter in PDF. Portions (0.5 ml) were dispensed into 24-well tissue culture dishes and allowed to develop undisturbed in the dark until streams began to form. Progress of cells through the aggregation phase was monitored with an inverted microscope. The control cells formed aggregation streams after 9 hours (left panel). At 12 hours the *mhcA* cells had not formed streams (right panel).

from within the region that could become double-stranded RNA in the cell (Fig. 1), and it is not known whether the conditions used for denaturation of samples for electrophoresis would melt double-stranded RNA. The 10-kb RNA transcript containing the antisense RNA sequence was detected in *mhcA* cells, but not in the control cells (Fig. 4A, lanes 3 and 4). In addition, a broad range of smaller antisense RNA's was detected which might be the result of degradation of the double-stranded RNA in the cell.

Consequences of the lack of myosin on development. *Dictyostelium* cells initiate a developmental sequence when starved and spread at high density on a moist filter. In 24 hours, the cells proceed through a series of well-defined stages to produce a fruiting body composed of differentiated spore and stalk cells (*1*). Thirteen hours after the initiation of development, the control cells have completed chemotactic aggregation and formed tight aggregates with prominent tips (Fig. 5A, left panel). In contrast, the *mhcA* cells were unable to aggregate normally (Fig. 5A, right panel). Irregular ridges that appeared to be rudimentary aggregation streams were often visible on the filters, however completion of aggregation was substantially delayed. After prolonged incubation (24 to 48 hours), aggregates were eventually formed, but subsequent morphogenesis did not occur. In the control, the MHC A protein was present at a constant level throughout the normal developmental sequence (Fig. 6). In contrast, no MHC A protein was detected in the *mhcA* cells during development (Fig. 6).

When wild-type cells are spread as monolayers on plastic culture dishes and submerged in starvation buffer, they elongate and move in large streams toward chemotactic centers, eventually forming large spherical aggregates of cells (*24*). This "streaming assay" allows a very detailed assessment of the progress of cells through the aggregation phase of development. Control cells elongated and streamed between 8 and 12 hours after the initiation of starvation (Fig. 5B, left panel). The *mhcA* cells did not form streams within 12 hours (Fig. 5B, right panel). Upon prolonged incubation (24 to 48 hours), some of the cells became elongated and entered short streams. Thus in both the "streaming assay" and during development on filter supports, there is some evidence of chemotactic movement, but aggregation is not completed normally.

The *mhcA* cells grown on bacteria are normal. *Dictyostelium* cells of axenic strains can use either bacteria or liquid medium (HL5) as a food source (*1*). All the experiments described thus far were done with cells grown under selection in HL5 medium. When grown in association with bacteria, the cells grow two or three times faster, and the pattern of expression of a number of genes is altered. In particular, cells growing in HL5 medium prematurely express a number of genes, including actin 6, that are not expressed until after the initiation of development of cells grown in association with bacteria (*1, 21, 25*). The expression of the endogenous *mhc*A gene relative to the actin-6 gene fusion was examined in pA6NPTII transformed cells grown in the presence of bacteria in order to see if growth conditions might allow experimental modulation of the expression of sense versus antisense RNA. Cells are normally grown for 48 hours in association with bacteria and then harvested for development after having consumed most of the bacteria. To ensure the presence of excess bacteria, cells transformed with the pA6NPTII vector were harvested, washed free of bacteria, and plated on filters for development. RNA was prepared from cells at each stage of growth and during early development, and Northern blots were probed for either the endogenous *mhc*A or the actin-6 gene fusion transcripts. The *mhc*A mRNA was expressed at similar levels throughout growth and early development (Fig. 7 A). Both of the actin-6 gene fusion RNA transcripts were expressed at very low levels during vegetative growth on bacteria and induced approximately 30-fold during early development (Fig. 7B).

Since the *mhc*A and actin-6 promoters are differentially regulated, it seemed possible that MHC A protein might accumulate in *mhcA* cells during growth on bacteria. The *mhcA* cells were grown for 48 hours in association with bacteria, washed free of the residual bacteria, and then assayed for the ability to develop on filter supports, and for the presence of MHC A protein and mRNA. The vegetative cells were normal in size and almost exclusively mononucleate. The developmental program was indistinguishable from the control-transformed cells in terms of the timing and morphology of each stage, and the *mhcA* cells formed normal fruiting bodies. The vegetative cells containing the antisense vector had nearly normal amounts of the 243-kD protein, which persisted throughout development (Fig. 8). The amount of antisense RNA detected in these

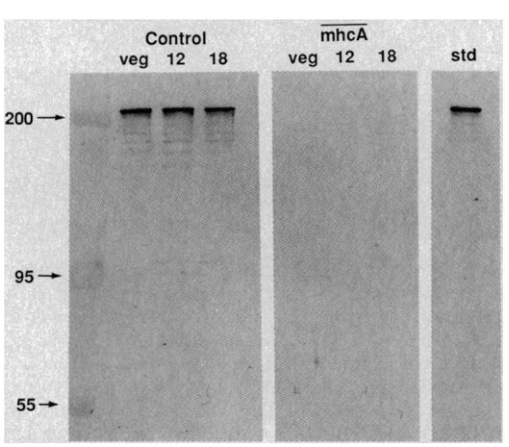

Fig. 6. Myosin heavy chain protein is not expressed during development. Cells were grown and developed as described in Fig. 5. Extracts containing 10 μg of protein from vegetative cells (veg lanes) or cells developed on filters for 12 or 18 hours were electrophoretically separated on 6 percent SDS-polyacrylamide gels, and Western blots were immunostained as described in Fig. 3. The molecular weight markers on the left are prestained rabbit skeletal myosin (200 kD), glycogen phosphorylase (95 kD), and glutamate dehydrogenase (55 kD) (Diversified Biotech). (Control) Extracts of cells transformed with pA6NPTII. (*mhcA*) Extracts of cells transformed with pA6mhc6; (std) purified *Dictyostelium* myosin heavy chain.

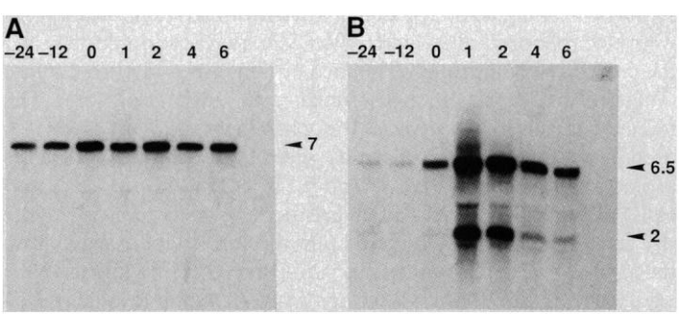

Fig. 7. Regulation of actin 6 and *mhc*A during growth in association with bacteria and subsequent development. Cells transformed with pA6NPTII were mixed with a saturated suspension of *Klebsiella aerogenes* and spread on SM agar plates (*33*). Cells were harvested after 24, 36, and 48 hours of growth, washed free of bacteria by differential centrifugation, and lysed to prepare RNA as described (Fig. 4). In addition, the cells harvested after 48 hours ($T = 0$) were plated on filters and allowed to develop for 1, 2, 4, and 6 hours before being harvested and used to prepare RNA. Equal amounts of RNA (5 μg) from each sample were electrophoretically separated on 1.2 percent agarose gels, transferred to Nytran filters, and hybridized with ^{32}P-labeled RNA probes that detect either the endogenous *mhc*A mRNA (**A**) or the NPT II sequence in the actin-6 gene-fusion RNA (**B**). The pGem-1™ construct containing the NPT II sequence used to generate the gene-fusion probe has been described (*20*). The numbers on the right side indicate the approximate size of the RNA in kilobases. The numbers on top indicate the number of hours of growth or development relative to $T = 0$ (thus 36 hours of growth = −12).

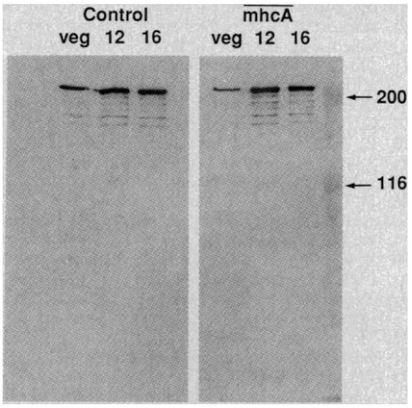

Fig. 8. Myosin heavy chain is expressed normally when cells are grown on bacteria. Cells growing in HL5 medium were harvested and spread on bacterial growth plates in association with *Klebsiella aerogenes*. After 2 days of growth, the cells were harvested, washed free of bacteria, and plated for filter development as described above. Extracts were made from vegetative cells and cells developed for 12 and 16 hours. The protein was analyzed on Western blots as described in Fig. 3. The markers on the right side are prestained rabbit myosin (200 kD) and β-galactosidase (116 kD).

cells was substantially lower than that found in cells grown in HL5 medium, and the level of *mhc*A mRNA was nearly equivalent to the control (Fig. 4B). Therefore, the regulated expression of the actin-6 promoter allows *mhcA* cells to be switched between a wild-type and mutant phenotype depending on the conditions used to grow the cells.

When cells are grown in association with bacteria, they cannot be kept under selection for G418 resistance, and conceivably might lose the vector. However, the period of growth in the absence of selection was only 2 days during which less than 12 generations occurred. Revertants, which are rare in stably transformed cells (*21*), would have had little chance to become a major component of the population. Moreover, under these conditions, the generation time of *mhcA* cells was the same as that of control cells. Since the amount of MHC A in the *mhcA* cells was equivalent to that in control cells after growth on bacteria (Fig. 8), the change in expression must have occurred in the whole population of cells, not just in a small number of rare revertants. Most importantly, when *mhcA* spores formed during development of cells grown in association with bacteria were inoculated into HL5 medium containing G418, they gave rise to amebae that grew into multinucleated cells that lacked MHC A.

The function of myosin in nonmuscle cells. We have created cells (*mhcA*) with dramatically reduced amounts of myosin heavy chain protein resulting from the inhibition of expression of the endogenous gene by antisense RNA. It is perhaps surprising that the lack of myosin was not lethal, however, we have not eliminated the possibility that small residual amounts of MHC A protein or compensatory expression of other genes allows these cells to survive.

The *mhcA* cells that lack myosin display a number of interesting phenotypes. They grow slowly in HL5 medium and have a serious defect in cytokinesis such that cell division does not keep pace with nuclear division, leading to the appearance of giant multinucleated cells. These cells can grow only when attached to a culture dish. In suspension, the cells swell without increasing in number and eventually lyse. It is likely that the defect in cytokinesis is aggravated when cells are grown in suspension. One of the most surprising properties of the *mhcA* cells that lack myosin is that they are motile. Time-lapse cinematography of growing cells indicates that they are able to extend pseudopods and to move randomly around the plate. Determining whether this movement is entirely normal will require more detailed analysis of the motion; however it appears that ameboid locomotion does not require myosin.

The phenotypic characteristics of *mhcA* cells are clearly the result of antisense RNA inactivation of the major myosin heavy chain mRNA rather than an extraneous result of the transformation procedure. Cells transformed with the vector alone or with vectors carrying other *Dictyostelium* sequences have never been found to display this phenotype, while independent transformations with the pA6mhc6 vector have produced other cell lines that lack MHC A protein and have the same phenotypes. Moreover, when *mhcA* cells are grown in association with bacteria, antisense RNA does not inhibit synthesis of MHC A protein, and the cells are phenotypically normal. Therefore, the characteristics of *mhcA* cells cannot be attributed to homologous integration of the vector into the *mhc*A gene or any other type of mutagenic effect of transformation. De Lozanne and Spudich (*26*) have isolated cells that completely lack the 243-kD MHC A protein and express only a truncated form of the polypeptide as a result of homologous recombination of their transformation vector into the endogenous *mhc*A gene. The resulting cell lines have a phenotype nearly identical to *mhcA* cells. These two independent approaches complement each other and serve to rule out many potential pitfalls of either method, thus confirming that myosin plays a crucial role in cytokinesis and development.

The defect in cytokinesis in cells lacking myosin is unprecedented, but not entirely unexpected. Myosin has been localized to the cleavage furrow in dividing *Dictyostelium* cells by means of immunofluorescence (*4*). This localization was taken as evidence that membrane movement during cell division was mediated in part by an acto-myosin complex. Moreover, Mabuchi and Okuno (*27*), and later Kiehart *et al.* (*28*), found that injection of polyclonal antibodies to myosin into dividing blastomeres blocked cytokinesis without affecting nuclear division.

An interesting question prompted by these results is how the multinuclear cells arise and why the extent of the phenotype is so variable. Time-lapse video analysis of growing *mhcA* cells indicates that the multinuclear cells may form as a result of "pseudo-cleavage." Cells that appear to be undergoing cytokinesis separate until they are joined only by a thin bridge of cytoplasm. At this point, the cells either separate to form two daughters of normal size or the two cells then rejoin to become a single, large cell. The fact that these cleavages are sometimes successful explains why the cultures contain both giant multinucleated and cells of normal size. How some of these cells divide in the absence of MHC A is unknown. It is possible that a very small amount of residual myosin is sufficient to allow cytokinesis to occur some of the time, or that another protein, such as the 117-kD myosin (*6*) or the 180-kD polypeptide detected by the monoclonal antibody to myosin in *mhcA* cells, can partially substitute for the MHC A protein.

Cells of the *mhcA* strain that lack myosin are unable to form normal multicellular aggregates during development. The expression of several actin genes increases during early aggregation and actin protein accumulates severalfold (*29*). This occurs prior to the time when an increase in overall cell movement is observed (*30*). While myosin is apparently not necessary for generalized locomotion, it appears to be necessary for some aspect of the aggregation process as well as for subsequent morphogenesis. The abnormal aggregation pattern might be the result of a defect in chemotactically directed movement or other developmental functions, such as transduction of the cyclic AMP signal, polarization of the cells, or cell-cell adhesion. Analysis of cells in which the expression of myosin heavy chain can be reversibly inactivated should further our understanding of the function of nonmuscle myosin.

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Disruption of the *Dictyostelium* Myosin Heavy Chain Gene by Homologous Recombination

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The phenomenon of homologous recombination, which allows specific gene conversion and gene insertion, can be a powerful system for the study of eukaryotic cell biology. Data are presented demonstrating that integration of a transfected plasmid by homologous recombination occurs in the motile eukaryotic cell *Dictyostelium discoideum*. A plasmid carrying a G418 resistance gene and the amino terminal half of the myosin heavy chain gene was used to transfect *Dictyostelium*. A large fraction of the resultant G418-resistant cells had the plasmid integrated into the single genomic copy of the heavy chain gene. These cells, which fail to express the native myosin but express the myosin fragment, are defective in cytokinesis and become large and multinucleate. In spite of the absence of native myosin, these cells, termed *hmm* cells, exhibit many forms of cell movement, including membrane ruffling, phagocytosis, and chemotaxis. The *hmm* cells can aggregate but are blocked at a later stage in the *Dictyostelium* developmental cycle. The *hmm* cells revert to the wild-type phenotype. Reversion of the *hmm* phenotype is due to excision and loss of the transforming plasmid. The revertant cells express native myosin, are G418 sensitive, and have a normal developmental cycle. These results constitute genetic proof that the intact myosin molecule is required for cytokinesis and not for karyokinesis.

Dictyostelium discoideum IS A EUKARYOTE THAT SERVES AS A simple model system for the study of many cellular processes (*1*). It displays many forms of cell motility found in higher eukaryotic cells. For example, it extends filopodia and pseudopodia in its direction of travel like fibroblasts or macrophages and undergoes chemotactic movements like leukocytes. During mitosis, microtubules form a typical eukaryotic spindle (*2*), and the contractile ring associated with cytokinesis has been shown to contain actin and myosin filaments (*3*). *Dictyostelium* cells exhibit extensive endocytosis and exocytosis (*4*).

Dictyostelium has a well-characterized developmental cycle (*4, 5*). When nutrients are available, either in the form of bacteria or an axenic medium, the cells grow vegetatively as amebae. In this form they can be grown in large quantities for biochemical manipulations. When *Dictyostelium* cells starve, they enter a developmental program that involves the aggregation of many cells by chemotaxis toward cyclic adenosine monophosphate (AMP) signals. The culmination of the developmental program is the formation of a fruiting body that contains two cell types, the stalk cells and the spores. The spores can then germinate to complete the cycle.

Dictyostelium grows and accomplishes its developmental program as a haploid cell, but it can be induced to form diploid cells. This allows the use of classical genetics to map genes into the seven linkage groups of the *Dictyostelium* genome (*6*). Molecular biological approaches have been extensively applied to this organism, including DNA-mediated transformation, which has been accomplished by Firtel and his co-workers (*7*). This has opened the possibility of utilizing genetic manipulation of *Dictyostelium* for many cell biological studies.

In yeast, specific gene conversions and integration of known genetic elements into specific genes by homologous recombination have been successfully used (*8*). This approach is referred to as gene targeting (*9*). Gene disruption has been used in yeast for the analysis

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