Generation and Characterization of *Dictyostelium* Cells Deficient in a Myosin I Heavy Chain Isoform

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Abstract. Motile activities such as chemotaxis and phagocytosis, which occur in *Dictyostelium* cells lacking myosin II, may be dependent upon myosin I. To begin to explore this possibility, we have engineered a disruption of the *Dictyostelium* myosin I heavy chain (DMIHC) gene described recently (Jung, G., C. L. Saxe III, A. R. Kimmel, and J. A. Hammer III. 1989. *Proc. Natl. Acad. Sci. USA.* 86:6186–6190). The double-crossover, gene disruption event that occurred resulted in replacement of the middle approximate one-third of the gene with the neomycin resistance marker. The resulting cells are devoid of both the 3.6-kb DMIHC gene transcript and the 124-kD DMIHC polypeptide. DMIHC⁻ cells are capable of chemotactic streaming and aggregation, but these processes are delayed. Furthermore, the rate of phagocytosis by DMIHC⁻ cells is reduced, as assessed by growth rate on lawns of heat-killed bacteria and on the initial rate of uptake of FITC-labeled bacteria. Therefore, this *Dictyostelium* myosin I isoform appears to play a role in supporting chemotaxis and phagocytosis, but it is clearly not required for these processes to occur. Using a portion of the DMIHC gene as a probe, we have cloned three additional *Dictyostelium* small myosin heavy chain genes. Comparison of these four genes with three genes described recently by Titus et al. (Titus, M. A., H. M. Warrick, and J. A. Spudich. 1989. *Cell Reg.* 1:55–63) indicates that there are at least five small myosin heavy chain genes in *Dictyostelium*. The probability that there is considerable overlap of function between these small myosin isoforms indicates that multiple gene disruptions within a single cell may be necessary to generate a more striking myosin I⁻ phenotype.

WHEN grown in suspension *Dictyostelium* cells that lack conventional-type myosin (myosin II) show a profound defect in cytokinesis (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Manstein et al., 1989). When grown on a surface, however, these cells are able to divide, albeit not by conventional cytokinesis (Spudich et al., 1989). Furthermore, they retain the ability to locomote, chemotax, extend pseudopods and lamellopods, and phagocytose. While myosin II appears to play a role in supporting some of these motile activities (e.g., chemotaxis and pseudopod expansion are impaired in myosin II⁻ cells [Wessels et al., 1988]), it is clearly not required for them to occur.

Myosin I, a low molecular weight, single-headed, nonfilamentous form of myosin (for reviews see Korn and Hammer, 1988, 1990; Adams and Pollard, 1989), may be responsible for the motile activities retained by myosin II⁻ cells. A myosin I isoform has been purified from *Dictyostelium* and shown to possess physical and enzymatic properties characteristic of the *Acanthamoeba* myosins I (Coté et al., 1985). Furthermore, immunofluorescence microscopy reveals that myosin I is concentrated in the lamellopodial and pseudopodial projections at the front of locomoting *Dictyostelium* ameba (Fukui et al., 1989). This finding, together with the fact that actomyosin I is known to be capable of producing

movement in vitro (Albanesi et al., 1985; Fujisaki et al., 1985; Adams and Pollard, 1986), suggests that actomyosin I contributes to the forces that cause extension at the leading edge of motile cells. If so, then myosin I almost certainly plays a key role (perhaps an essential role) in the extension of pseudopods and lamellopods, cell locomotion, and chemotaxis. In addition, myosin I may play an important role in the process of phagocytosis, since it is also concentrated just beneath the plasma membrane at sites of particle ingestion (Fukui et al., 1989).

We recently cloned a *Dictyostelium* myosin I heavy chain (DMIHC)[1] gene (Jung et al., 1989a) and showed that its entire deduced amino acid sequence is very similar to the heavy chain sequences of both *Acanthamoeba* myosin IB (Jung et al., 1989b) and IC (Jung et al., 1987). The primary goal of the current study was to block the expression of the DMIHC gene, using homologous recombination to render the gene nonfunctional, and to analyze the phenotype of the DMIHC⁻ cells in an effort to identify the in vivo function(s) of this particular myosin I isoform. In addition, an effort was made to identify additional myosin I heavy chain genes in *Dictyostelium*. The results obtained, together with data re-

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1. *Abbreviation used in this paper:* DMIHC, *Dictyostelium* myosin I heavy chain.

ported recently by Titus et al. (1989), indicate that *Dictyostelium* contain a minimum of five small myosin heavy chain genes.

Materials and Methods

Preparation of RNA and DNA

Dictyostelium genomic DNA was prepared by the miniprep procedure of Nellen et al. (1987), except that the number of cells per sample was increased to 15 × 10^{6}, the final concentrations of SDS and proteinase K were increased to 1.5% and 0.4 mg/ml, respectively, the temperature was decreased to 55°C, and the final pellet was resuspended in ~200 μl. Total RNA was prepared from mid-log phase cells by extraction in guanidine isothiocyanate and pelleting through a cesium chloride cushion, exactly as described by Maniatis et al. (1982).

Southern and Northern Blots

Electrophoresis of DNA in agarose gels and RNA in agarose/formaldehyde gels, as well as transfers to nitrocellulose, were performed as described previously (Hammer et al., 1986). For Southern blots, hybridizations were performed either at 42°C in a solution containing 30% formamide, 5× SSPE, 2× Denhardts, 0.1% SDS, 0.2 mg/ml salmon sperm DNA, and 0.02 mg/ml polyadenine (moderate stringency conditions) or at 68°C in a solution containing 6× SSPE, 5× Denhardts, 0.35% SDS, 0.2 mg/ml salmon sperm DNA, and 0.02 mg/ml polyadenylic acid (high stringency conditions). Washing conditions used were either 0.4× SSPE, 0.1% SDS at 50°C (moderate stringency) or 0.2× SSPE, 0.1% SDS at 60°C (high stringency). Northern blots were hybridized and washed as described previously (Hammer et al., 1986). Probes were labeled with α-^{32}P-dATP using the random primer method (Amersham Corp., Arlington Heights, IL).

Antibodies

An antiserum, which is specific for the DMIHC polypeptide, was generated against a TRPE/DMIHC fusion protein. The 1.2-kb cDNA clone described previously (Jung et al., 1989a), which encodes the carboxy-terminal ~35% of the DMIHC (i.e., ~95% of the tail domain), was ligated in frame into the bacterial expression vector pATH 10. The fusion protein, which contained ~37 kD of the *Escherichia coli* protein TRPE fused to ~40 kD of the DMIHC, was produced in *E. coli* strain HB 101, exactly as described by Earnshaw et al. (1987). An insoluble, detergent-resistant pellet, which is highly enriched in the fusion protein, was prepared by differential centrifugation of bacterial lysates. This material was resolved by preparative SDS-PAGE, the fusion peptide band was visualized by KCl staining, excised from the gel, and ground up in ~2 ml of H₂O (total yield ~400 μg). A rabbit was injected subcutaneously at 10 sites with about one-third of this material, after mixing it into an emulsion with an equal volume of Freunds complete adjuvant. This injection was followed by boosts at 2 and 6 wk (using incomplete adjuvant) and the rabbit was bled at 10 wk. The antiserum generated against the heavy chain of purified *Dictyostelium* myosin I (gift from Dr. Thomas Lynch, National Heart, Lung, and Blood Institute, Bethesda, MD) was prepared as described elsewhere (Fukui et al., 1989).

Western Blots

Mid-log phase cells (4-12 × 10^{6}) were pelleted by centrifugation (3,000 g, 4°C, 5 min), resuspended completely in 200 μl of starvation buffer, added to 200 μl of 2× loading buffer (10% SDS, 125 mM Tris [pH 6.8], 15% sucrose, 10% β-mercaptoethanol, 1 mM EDTA (pH 8.0) and 0.01% bromophenol blue) preheated to 100°C, and boiled for 5 min. Samples were resolved in Laemmli minigels (7 and 3% acrylamide for separating and stacking gels, respectively) (Laemmli, 1970), and transferred to nitrocellulose using a semidry blotter (Milliblot-SDE; Millipore Continental Water Systems, Bedford, MA) (adjusted initially to 13 mA per gel and then run on constant voltage for 1 h). Blots were performed as described by Towbin et al. (1979) using either the antiserum generated against the TRPE/DMIHC fusion protein (1:1,000 dilution; Fig. 2) or an antiserum generated against the heavy chain of purified *Dictyostelium* myosin I (1:250 dilution; see text). Blots were developed using goat anti–rabbit IgG conjugated with horseradish peroxidase (Boehringer Mannheim Diagnostics Inc., Houston, TX).

Construction of the DMIHC Gene Disruption Vector

Plasmid pA6NPTII (gift of Dr. David Knecht, University of Connecticut, Storrs, CT), which carries a NEO[R] cassette composed of the *Dictyostelium* actin 6 promoter, the NEO[R] coding sequence, and an SV-40 terminator sequence (2.7 kb total), was linearized with Eco RI, converted to blunt ends with Klenow, converted to a Kpn I fragment by linker addition, and cut with Bam HI. The 2.7-kb Kpn I/Bam HI fragment containing the entire NEO[R] cassette was cloned into pUC 19, and this recombinant plasmid was linearized with Bam HI and treated with phosphatase (plasmid A). The 1.2-kb DMIHC cDNA clone fragment described previously (Jung et al., 1989a), which encodes the carboxy-terminal ~35% of the DMIHC (amino acids 719-1,111), was released from the bluescript plasmid with Eco RI, converted to blunt ends with mung bean nuclease, converted to a Bam HI fragment by linker addition, and cloned into plasmid A. A clone with the correct orientation (same orientation as that of the NEO[R] cassette) was identified, cut with Kpn I and treated with phosphatase (plasmid B). A 739-bp Ava II/Cla I fragment, corresponding to amino acids 59-306, was prepared from genomic clone λDMIHC·1 (Jung et al., 1989a), converted to blunt ends with Klenow, converted to a Kpn I fragment by linker addition, and cloned into plasmid B. A clone with the correct orientation (same as for the NEO[R] cassette) was identified, grown up in a maxiprep, purified by double banding in cesium chloride gradients (Hammer et al., 1987), and cut with Eco RI (cuts once, just 5' of the disruption fragment) and Xba I (cuts once, just 3' of the disruption fragment). For transformations, the mixture of pUC vector and linear disruption fragment were converted to blunt ends with Klenow and treated with phosphatase in order to reduce the incidence of religation in vivo (Manstein et al., 1989).

Transformations

Transformations were performed using axenic strain Ax3 and the calcium phosphate/DNA coprecipitation/glycerol shock method described by Nellen and Saur (1988), except that the cells were not synchronized. G418 (10 μg/ml; Gibco Laboratories, Grand Island, NY) in HL5 medium was used throughout the selection process. Primary colonies were picked with a micropipette and cloned by one or two rounds of serial dilution in 96-well plates. Pure clones were obtained from a row in which the particular dilution yielded a colony in only one or two wells out of the 12 wells in the row. Pure clones were maintained in suspension culture at 22°C in HL5 medium supplemented with antibiotics (50 μg/ml ampicillin, 12.5 μg/ml tetracycline, and 12.5 μg/ml chloramphenicol) and G418 (10 μg/ml).

Library Screening

The *Dictyostelium* random-shear genomic library in λZAP (gift of H. Ennis, Roche Institute for Molecular Biology, Nutley, NJ) was screened using standard techniques (Maniatis et al., 1982) under the moderately stringent conditions described above. Plaque purified phage were converted to bluescript plasmids using helper phage R408, as described by the manufacturer (Stratagene, La Jolla, CA).

DNA Sequencing

DNA sequencing was performed using M13 templates and the dideoxy chain termination method of Sanger et al. (1977), as described previously (Hammer et al., 1987). Nucleotide and deduced amino acid sequences were aligned with the aid of the DEC10 DNA:SEQ and PRT:ALN programs (Division of Computer Research and Technology, National Institutes of Health, Bethesda, MD).

Plaque Assay

The growth of cells on lawns of heat-killed *Klebsiella aerogenes* was performed exactly as described by Nellen et al. (1987), except that the pads and black filters, as well as the bacterial slurry, were equilibrated with starvation buffer rather than HL5 growth medium. G418 was omitted from the DMIHC⁻ cell samples since its addition caused a further reduction in the rate of plaque formation to about one-eighth that of Ax3 cells. This may be due to the fact that growth on bacteria causes a reduction in actin-6 promoter activity (Knecht and Loomis, 1987), which would make the cells increasingly sensitive to the inhibitory effects of G418 on protein synthesis.

Streaming and Filter Development Assays

For the streaming assay, 1.5 × 10^{7} mid-log phase cells were seeded on plastic tissue culture dishes (No. 3002; 60 mm; Falcon Labware, Oxnard,

CA) in HL5 medium and allowed to settle for 4 h. At time zero the HL5 medium was removed, the dish was gently rinsed with 5 ml of starvation buffer (20 mM potassium phosphate [pH 6.5], 20 mM KCl, 5 mM $MgCl_2$, and antibiotics), 2.5 ml of starvation buffer was added, and the dish was left in the dark to stream (the number of nonadherent cells was determined and, in all cases, the number of adherent DMIHC⁻ cells [~80% of the cells seeded] was at least equal to the number of adherent Ax3 cells). G418 was not included in DMIHC⁻ cell samples. The dishes were photographed with Panatomic-X32 film using a Zeiss IM 35 inverted microscope and phase-contrast optics. Filter development assays were performed as described by Knecht and Loomis (1987); ~1 × 10⁷ ameba were deposited per cm².

Phagocytosis Assay

The rate of uptake of fluorescein-labeled *E. coli* strain B/r was measured by the method of Vogel et al. (1980), as modified recently by Chia and Luna (1989), and with the following additional modifications: (*a*) experiments were performed in HL5 medium, (*b*) the concentration of amebae was increased to 5 × 10⁶/ml, (*c*) FITC-labeled bacteria were added at time zero at a ratio of 300:1, bacteria to amebae, (*d*) 5-ml samples were taken at each time point, and (*e*) centrifugation through a second polyethylene glycol 6,000 cushion was used to remove all traces of uningested bacteria. Amebae were prepared from mid-log phase cultures. Labeling of bacteria with FITC (Molecular Probes, Inc., Eugene, OR) and fluorescence measurements were done as described by Vogel et al. (1980). The relative fluorescence values obtained were corrected for the uptake of bacteria at 4°C (this amount was <2% of the values obtained at 22°C).

Results

Disruption of the DMIHC Gene

The DMIHC gene disruption vector was designed to favor a double-crossover, gene replacement event over a single-crossover, gene insertion event (Fig. 1). The vector was constructed using 0.75 kb of 5′ coding sequence and 1.2 kb of 3′ coding sequence, with the neomycin-resistance gene in the middle, and was cut at the two sites shown (Fig. 1, *arrow-heads*) to create a linear disruption fragment. Constructs similar to this were used to disrupt the *Dictyostelium* myosin
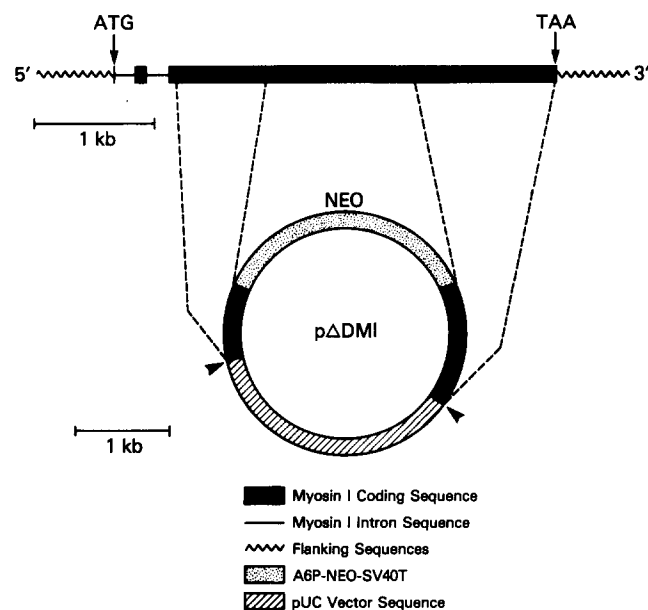

Figure 1. Structure of the DMIHC gene disruption vector. The vector was cut at the two sites shown (▲) to create a linear disruption fragment possessing two recombinogenic ends.
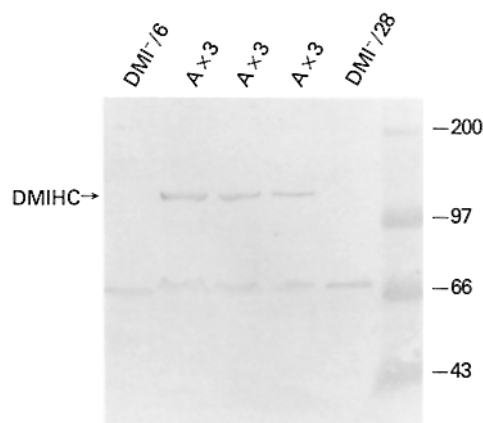

Figure 2. Western blot of whole cell extracts of strain Ax3 (0.75, 0.5, and 0.25 × 10⁶ cells, center three lanes from left to right) and two putative DMIHC⁻ cell lines, DMI⁻/6 and DMI⁻/28 (0.75 × 10⁶ cells each). The antiserum used was generated against a TRPE/DMIHC fusion peptide (see Materials and Methods). The identity of the cross-reacting peptide of ~66 kD is unknown.

II heavy chain gene (Manstein et al., 1989) and reflect the gene targeting approach used in yeast (Rothstein et al., 1983). *Dictyostelium* strain Ax3 was transformed by the method of Nellen et al. (1988) using the linear disruption fragment. About 1 in 10⁵ cells yielded a G418-resistant colony. 30 of these colonies were purified by two rounds of serial dilution in 96-well plates and then assayed by Western blot for the level of DMIHC protein. The antibody used to screen these transformants was generated against a TRPE fusion protein containing most of the DMIHC tail domain (see Materials and Methods). Fig. 2 shows a Western blot using the fusion peptide antibody. The middle three lanes of Fig. 2 are 0.75, 0.5, and 0.25 × 10⁶ cells of strain Ax3 (left to right), and the outside lanes are 0.75 × 10⁶ cells of two putative DMIHC⁻ cells, clone 6 (DMI⁻/6) and clone 28 (DMI⁻/28). Both DMIHC⁻ cell lines appear to be devoid of the ~124-kD DMIHC protein.

To determine if a double-crossover had occurred, the structure of the DMIHC gene in cell lines DMI⁻/6 and DMI⁻/28 was examined by Southern blot analysis (Fig. 3). In a double-crossover, the ~1.2 kb of DMIHC sequence situated between the 5′ and 3′ coding regions used in the disruption vector would be replaced by the ~2.7-kb NEOᴿ cassette. As a result, the ~5.5-kb Eco RI band encompassing the wild-type DMIHC gene should increase in size to ~7 kb in the disrupted gene and this shift should be seen with probe A. Furthermore, probe B, which corresponds to a portion of the 1.2 kb of DMIHC sequence gene that would be deleted, should not hybridize to DMIHC⁻ cell genomic DNA. Both predictions hold true for DMIHC⁻ clones 6 and 28 (Fig. 3) (see below for a discussion of the "extra" bands seen with probe A). More detailed Southern blots confirmed this result (Fig. 4). Specifically, the patterns obtained with several double digests of DMI⁻/6 genomic DNA (Fig. 4, *left*) are completely consistent with a map (Fig. 4, *right*) predicted from the data in Fig. 3. Therefore, a double-crossover, gene replacement event had occurred, which replaced approximately two-thirds of the globular head domain coding sequence with the Neoʳ gene, and that should render the DMIHC gene incapable of producing a functional protein.
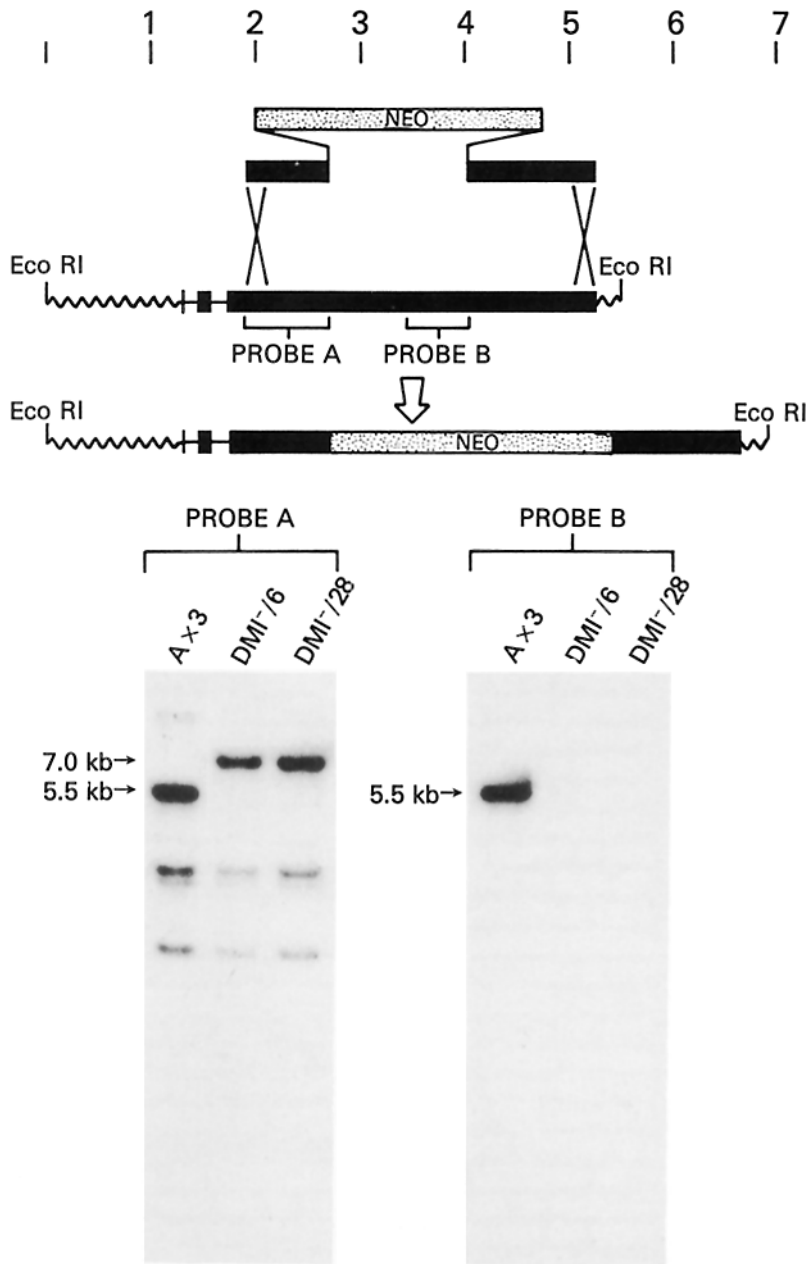
Figure 3. Southern blot analysis of cell lines DMI⁻/6 and DMI⁻/28. The upper half of the figure shows in schematic form the change that would occur in the structure of the DMIHC locus if a double-crossover, gene-replacement event occurred. The lower half of the figure shows the actual blots, which reveal that, as predicted, the ∼5.5-kb Eco RI fragment encompassing the wild-type gene increases in size to ∼7.0 kb in the disrupted gene (seen with *PROBE A, lower left* panel), and the ∼1.2 kb of coding sequence located between the 5' and 3' coding pieces used in the disruption vector has been deleted from the genome of DMI⁻/6 and DMI⁻/28 cells (seen with *PROBE B, lower right* panel). The extra bands seen with probe A (one larger and three smaller than the 5.5-kb Eco RI band corresponding to the DMIHC gene) are discussed later in the text.

This type of disruption is not subject to reversion like a single-crossover, gene insertion event (DeLozanne and Spudich, 1987). We note that 6 of the 30 G418-resistance colonies examined were devoid of DMIHC protein and showed the same Southern pattern as clones DMI⁻/6 and DMI⁻/28. Therefore, the frequency of double-crossover obtained here (∼20%) is similar to the frequency obtained by Manstein et al., 1989 (∼30%). We also note that the Southern patterns seen in Fig. 3 have not changed over several hundred generations of cell lines DMI⁻/6 and DMI⁻/28.

Northern blots of DMI⁻/6 cell RNA hybridized with probe A show no detectable 3.6-kb DMIHC gene transcript (Fig. 5). A truncated transcript of ∼1.4 kb, made off of the intact DMIHC gene promoter, is found in these cells. Controls performed with a myosin II gene probe (Fig. 5) show that DMI⁻/6 cell RNA is intact, i.e., the lack of a signal at

3.6 kb using probe A cannot be due to nonspecific degradation of high molecular weight RNA in the DMI⁻/6 sample. This control also suggests that the steady-state level of myosin II heavy chain gene transcript is not altered significantly when the DMIHC gene is disrupted.

Phenotype of DMIHC⁻ Cells

The appearance of DMIHC⁻ cells in the phase-contrast microscope is, at least superficially, normal, with many cells being polarized and possessing apparent pseudopodia and lamellopodia. Unlike myosin II⁻ cells (Spudich, 1989), DMIHC⁻ cells grow well in suspension, with an essentially normal doubling time (∼9.5 h). To determine if DMIHC⁻ cells are capable of completing development, they were seeded on a filter support at very high density in the absence
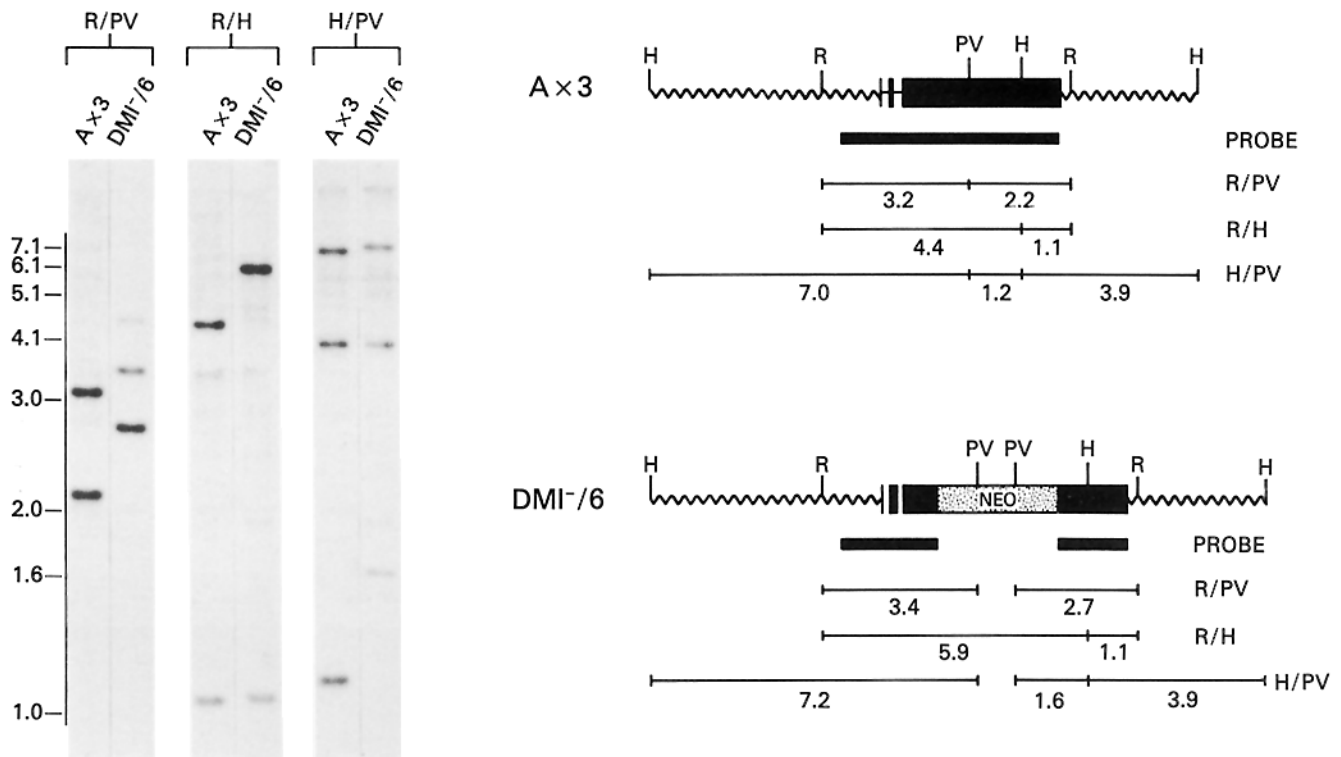
Figure 4. More detailed Southern blots of DMI⁻/6 genomic DNA confirm the results in Fig. 3 (*H*, Hind III; *R*, Eco RI; *PV*, Pvu II). In the upper right is the known map of the wild-type DMIHC locus. In the lower right is the map of the disrupted DMIHC gene, as predicted from the data in Fig. 3 and the known map of the NEO^R cassette. The heavy line immediately beneath these two maps indicates the region of homology between the 4.2-kb probe used and the genomic locus as drawn. The lighter lines (with associated numbers) immediately beneath the heavy line are the fragments that should be detected by the probe in the three different double digests. On the left are the actual Southern blot patterns obtained, which agree in every way with the predicted maps on the right.

of nutrients. Under these conditions, wild-type cells aggregate and undergo morphogenesis to produce within ∼24 h a stalk supporting a spore-filled sac (sorocarp). DMIHC⁻ cells (both clones 6 and 28) complete this developmental process in ∼24–28 h. However, many fewer fruits are formed, the stalks are about one-half the normal length, and the sorocarps are on average ∼50% smaller than normal. Therefore, while DMIHC⁻ cells can complete development, the morphology of the stalks and sorocarps is abnormal.

When *Dictyostelium* are seeded on plastic dishes in the absence of nutrients, they elongate and move chemotactically in large streams towards chemotactic centers, eventually forming dense round aggregates (Devreotes, 1982). This streaming assay provides a sensitive indicator of the ability of cells to locomote and chemotax. While DMIHC⁻ cells are able to form streams and aggregates of essentially normal appearance, this process is greatly delayed. For example, Fig. 6 (*top left*) shows DMIHC⁻ cells (clone DMI⁻/6) 15 h after being switched to starvation buffer. The cells are just becoming elongated and have not entered into active streaming. In contrast, Ax3 cells (and nonhomologous transformants) begin to stream at 8–10 h and by 15 h they are near the end of streaming and the formation of tight aggregates (not shown). By 18 h (Fig. 6, *top right*), DMI⁻/6 cells had finally begun to stream, and while they had formed large streams by 22 h (Fig. 6, *bottom left*), aggregation was not

near completion until 25 h (Fig. 6, *bottom right*) (similar results were seen with DMI⁻/28 cells and two out of two additional independent DMIHC⁻ isolates tested). Therefore, while DMIHC⁻ cells are clearly able to chemotax and stream, these processes are delayed by 8–10 h relative to strain Ax3.

To determine if the absence of the DMIHC isoform affected the ability of cells to phagocytose, DMIHC⁻ and Ax3 cells were seeded onto lawns of heat-killed *Klebsiella aerogenes* that were supported on filters equilibrated with a buffered salt solution, so that the only food source would be phagocytosis of the bacteria. DMI⁻/6 cells grew about one-half to one-third as fast as Ax3 cells (and nonhomologous transformants), based on their smaller plaque size (Fig. 7, *A* and *B*; similar results were seen with DMI⁻/28 cells and three out of three additional independent DMIHC⁻ isolates tested). To determine if this reduced growth rate might be due, at least in part, to an impairment of phagocytosis, we measured the rate of uptake of FITC-labeled *E. coli* B/r by DMI⁻/6 and Ax3 cells (Fig. 7 *C*). Over the three intervals of the time course (0–2.5, 2.5–5, and 5–10 min), the ratios of the rates of phagocytosis (DMI⁻/6 cells to Ax3 cells) were 0.75, 0.64, and 0.68, respectively. In measurements of DMI⁻/28 cells, the ratios of the rates were 0.79, 0.71, and 0.73 (average of two measurements). Therefore, while DMIHC⁻ cells are clearly able to phagocytose, this process is somewhat impaired (∼30%) relative to strain Ax3.

PROBE A   MII PROBE
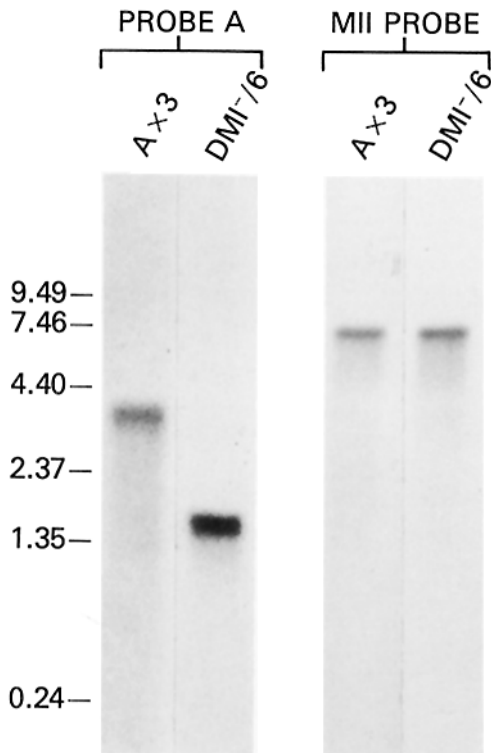
9.49—
7.46—
4.40—
2.37—
1.35—
0.24—

Figure 5. Northern blot analysis of total RNA (10 μg/lane) from vegetative Ax3 cells and vegetative cell line DMI⁻/6, using probe A (*left*) and, in duplicate lanes, a *Dictyostelium* myosin II heavy chain gene probe (*right*).

Identification of Three Additional Small Myosin Heavy Chain Genes in Dictyostelium

While the DMIHC isoform targeted for disruption in this study appears to play a role in supporting chemotaxis and phagocytosis, it is clearly not solely responsible for these motile functions. If this gene were the only form of small myosin in *Dictyostelium*, as suggested by previous Southern blot analyses (Jung et al., 1989a), then it would have to be concluded that myosin I is not absolutely required for processes such as chemotaxis and phagocytosis. However, using a different approach we have now identified three additional *Dictyostelium* small myosin heavy chain genes. Specifically, we decided to investigate the possibility that the extra bands seen in genomic Southern blots probed at moderate stringency using the DMIHC gene ATP binding-site probe (probe A; see Fig. 3) represented additional myosin I heavy chain genes. Of the four extra Eco RI bands seen with probe A (Fig. 8), the 3.2-kb band corresponds to the myosin II heavy chain gene based on its size and on the fact that it hybridizes at high stringency with a myosin II-specific gene probe (see below). Efforts were directed, therefore, at cloning the genes corresponding to the 2.4-, 3.4-, and ∼20-kb Eco RI bands. We screened a random-shear *Dictyostelium* genomic library in λZAP with probe A, then discarded the clones which were also positive with probe B (see Fig. 3), which is specific for the DMIHC gene already characterized. Also eliminated were clones that were positive with a myosin II-specific probe. In the end, three nonoverlapping genomic clones were obtained, clones 2, 12, and 21. A portion of the inserts from these clones and the myosin II clone were used to probe Eco RI

digests of *Dictyostelium* genomic DNA at high stringency (Fig. 8). Clones 2, 12, and 21 hybridized to Eco RI bands that correspond, respectively, with the ∼20-, 3.4- and 2.4-kb bands seen by probe A, while the myosin II gene probe sees the 3.2-kb band, as expected. Northern blots of DMIHC⁻ cell RNA probed at high stringency with the inserts of clones 2 and 12 reveal a ∼3.4-kb transcript, while the clone 21 insert hybridizes to a slightly larger transcript of ∼3.8 kb (Fig. 9). These transcript sizes are consistent with mRNAs encoding small myosin heavy chain isoforms (i.e., ∼120–130 kD). Partial DNA sequence analysis reveals (a) that all three clones possess typical myosin globular head-like sequences, including the highly conserved ATP binding site region, and (b) that all three clones are different genes (Jung, G., R. Urrutia, and J. A. Hammer III, manuscript in preparation). Using a polyclonal antiserum generated against the whole heavy chain of *Dictyostelium* myosin I, a band of ∼125 kD is seen in Western blots of DMIHC⁻ cell extracts (data not shown). This result indicates that at least one other small myosin isoform is being expressed in DMIHC⁻ cells, and that the fusion peptide antibody used in Fig. 2 must be specific for the DMIHC isoform.

Discussion

The Disruption Event

The promoter for the disrupted DMIHC gene remains functional, generating a truncated ∼1.4-kb transcript. Myosin, as well as other motor proteins, can be fragmented into smaller, active molecules. However, no evidence was found in Western blots of DMIHC⁻ cell extracts of a peptide made from this truncated transcript. Furthermore, it is highly unlikely that such a peptide, even if it were stable, would be a functional molecule, since it would contain only the first about one-third of the globular head domain.

Gene Number

Using a portion of the DMIHC gene as a probe, we have identified three additional small myosin heavy chain genes in *Dictyostelium* (clones 2, 12, and 21). Recently, Titus et al. (1989) reported the complete sequence of a low molecular weight myosin heavy chain gene in *Dictyostelium*, termed abm a for actin-based motor a. Also reported were the partial sequences of two additional small myosin heavy chain genes, abm b and abm c. Like clones 2, 12, and 21, these abm clones were identified using an ATP-binding site probe, although the heterologous probe was from the myosin II gene rather than the DMIHC gene used here. Sequence comparisons (data not shown; Titus, M., personal communication) reveal that (a) the DMIHC gene and abm b are identical, (b) clone 12 and abm a are identical, and (c) clones 2, 21, and abm c are distinct genes. Therefore, there are at least five small myosin heavy chain genes in this haploid organism, in addition to the apparently single-copy myosin II heavy chain gene (DeLozanne et al., 1985).[2] If additional myosin genes

2. In subsequent studies we will refer to these five *Dictyostelium* small myosin heavy chain isoforms as follows: *Dictyostelium* myosin IA (corresponds to abm a and clone 12), *Dictyostelium* myosin IB (corresponds to abm b and DMIHC), *Dictyostelium* myosin IC (corresponds to abm c), *Dictyostelium* myosin ID (corresponds to clone 21), and *Dictyostelium* myosin IE (corresponds to clone 2). This nomenclature is not, however, meant to imply a direct correlation with *Acanthamoeba* myosins IA, IB, and IC.
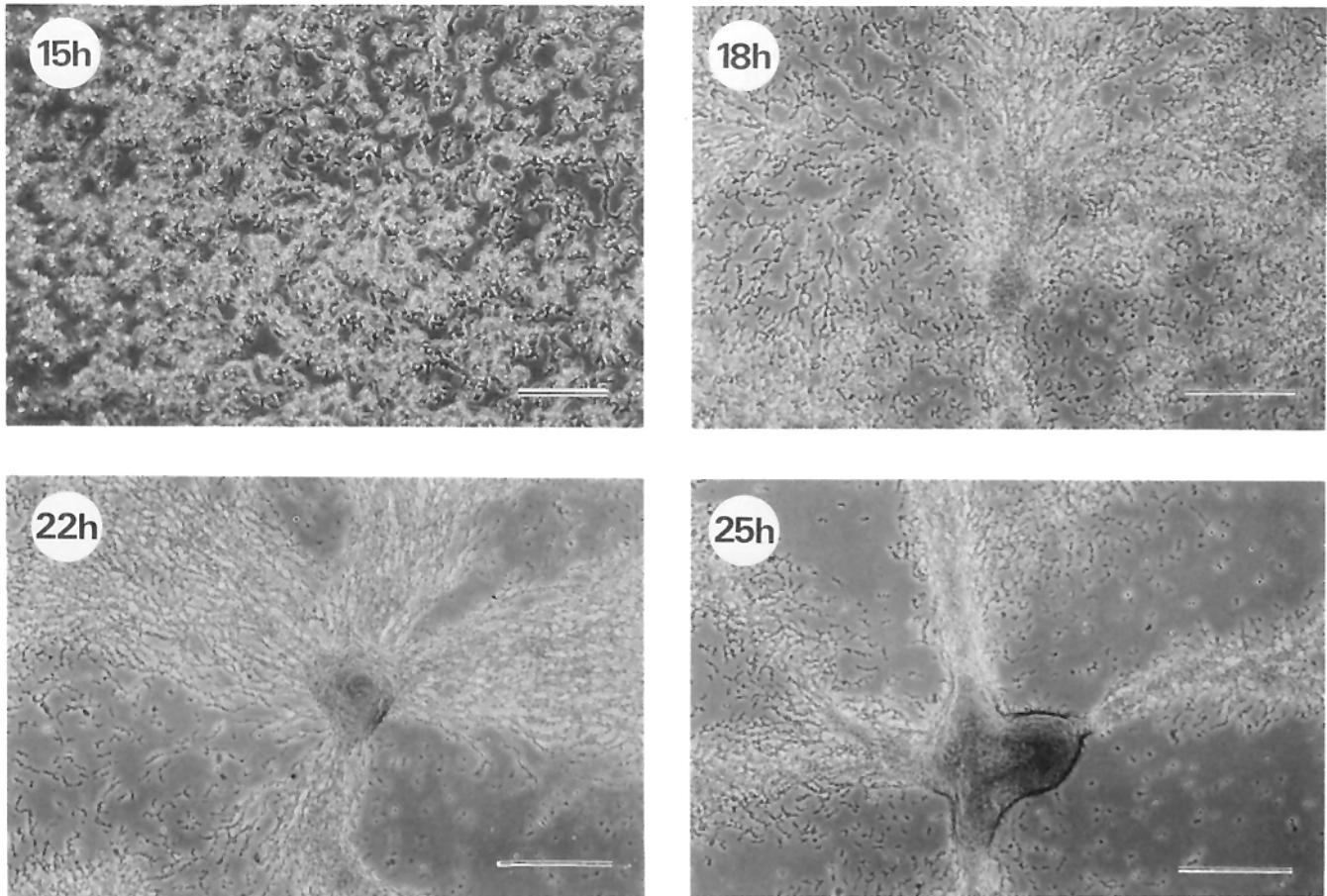
Figure 6. Phase-contrast micrographs of DMIHC⁻ cell line DMI⁻/6 streaming on a plastic surface. The cells were photographed at 15 (*top left*), 18 (*top right*), 22 (*bottom left*), and 25 h (*bottom right*) after being switched to starvation buffer. Bars: (*top left*) 50 μm; (*top right, bottom left* and *right*) 200 μm.

exist, the sequence of their ATP-binding domain, which is among the most highly conserved of myosin sequences, must be quite atypical.

The observation that DNA probes encoding portions of the DMIHC tail domain do not recognize other myosin I heavy chain genes in Southern blots of *Dictyostelium* genomic DNA (Jung et al., 1989a), together with the observation that a polyclonal antibody generated specifically against the DMIHC tail domain does not recognize other small myosin heavy chain polypeptides in Western blots (Fig. 2), suggest that the tail domain sequences of these additional small myosin isoforms diverge considerably from that of DMIHC. Therefore, while the DMIHC gene is not single copy in the sense of being the only small myosin heavy chain isoform in *Dictyostelium*, it may be the only isoform whose tail domain amino acid sequence is very similar to that of the *Acanthamoeba* myosins I. This conclusion is borne out, at least in part, by the fact that the sequence of the tail domain of abm a (Titus et al., 1989) is similar to that of DMIHC only in the amino-terminal approximate one-half (∼200 residues) and this similarity is only ∼40% (exact matches plus conservative substitutions). Furthermore, the tail domain of abm a is devoid of the glycine/proline/alanine-rich regions found in DMIHC, *Acanthamoeba* myosin IB, and *Acanthamoeba* myosin IC.

One apparent contradiction in our results is that while probe A identified these additional small myosin heavy chain genes in Southern blots (Fig. 8), it did not identify their transcripts in Northern blots of vegetative DMIHC⁻ cell RNA (Fig. 5), even though transcripts for these additional genes can be seen in both vegetative DMIHC⁻ cells (Fig. 9) and vegetative Ax3 cells (data not shown) using homologous probes. This discrepancy is probably due to a combination of two factors: (*a*) the signal intensities obtained with probe A that correspond to the additional genes are one-tenth or less than that of the DMIHC gene (this can be seen in the Southern blots (Fig. 8), although shorter exposures indicate more clearly the large difference in the signal intensities); and (*b*) the signal strength of the control Northern blot (Fig. 5, lane *1*) is already quite low.

Phenotype

The initial examination of these DMIHC⁻ cells indicates that while the DMIHC isoform is not required for phagocytosis and chemotaxis, it does appear to play a role in supporting these motile processes. One seeming discrepancy in the results, however, is that while streaming on plastic is delayed by 8–10 h, the rate of formation of fruits on filter supports is essentially normal. One possible explanation for this dis-
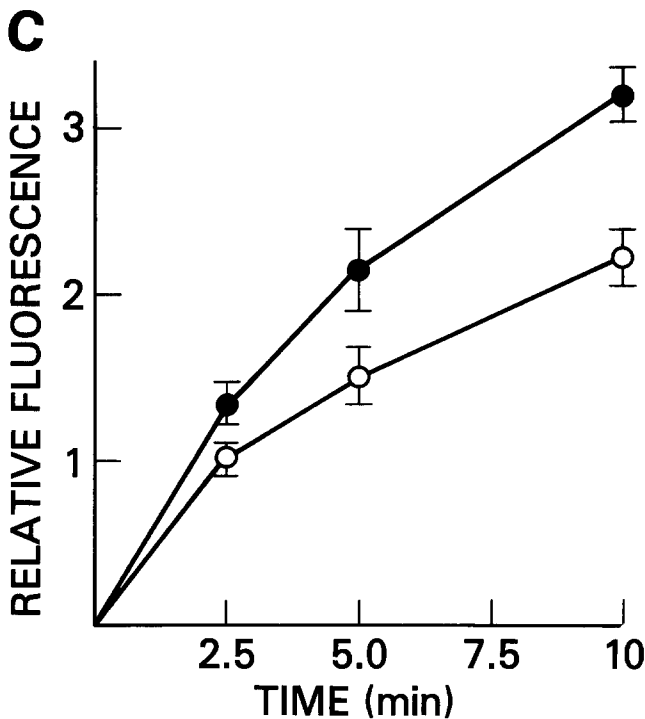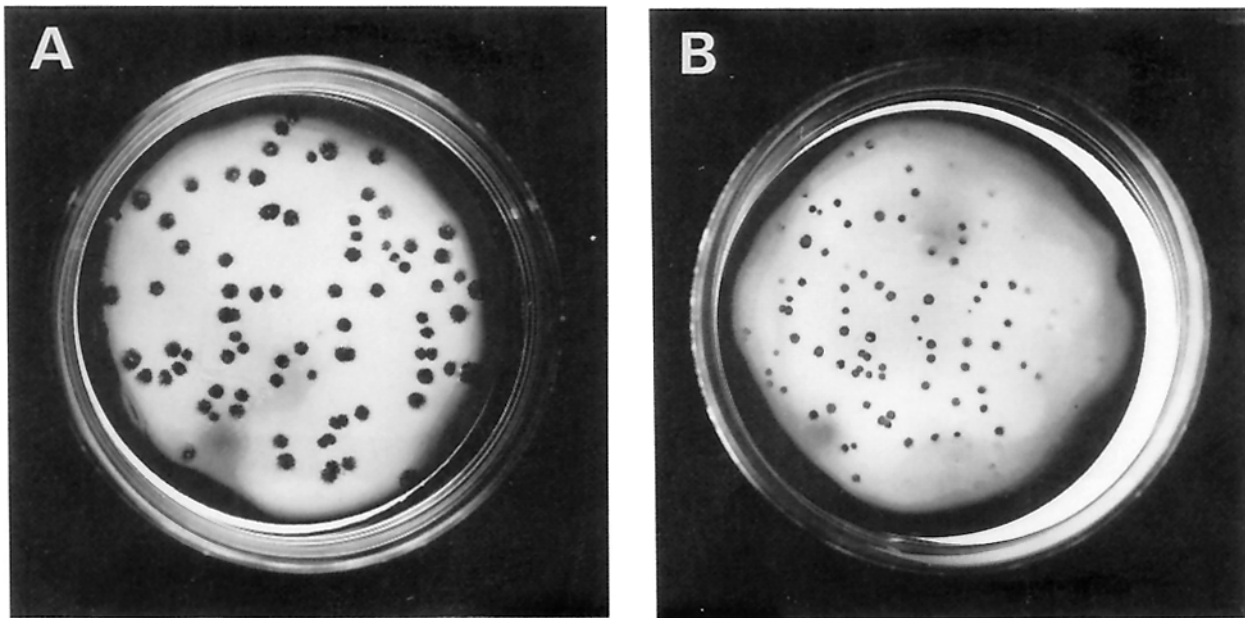
Figure 7. Growth on lawns of heat-killed *Klebsiella aerogenes*, as revealed by formation of clear plaques (*A*, Ax3 cells at 6 d; *B*, DMI⁻/6 cells at 6 d), and the initial rate of uptake of FITC-labeled *E. coli* B/r by Ax3 cells (●) and cell line DMI⁻/6 (○) (*C*; mean ± standard deviation; $n = 4$).

crepancy is that in the filter development assay the cells are seeded at extremely high density ($\sim 1 \times 10^7/\text{cm}^2$) and, therefore, have very short distances to travel (<1 mm) before they are in aggregates. In contrast, the cell density is ~ 25 times lower in the streaming assay, so that the cells have much longer distances to travel to form aggregates (in some cases, probably >10 mm). Any abnormality in the ability of DMIHC⁻ cells to locomote and chemotax should be greatly amplified in the streaming assay.

There are at least two possible explanations for the delay in streaming, both of which may contribute to causing the delay. First, Northern blot analyses have indicated that the steady-state level of DMIHC transcript rises about sevenfold over the time during which cells are actively forming aggregates (Jung et al., 1989a). Consequently, when the process of streaming should begin the DMIHC⁻ cells may be waiting for the DMIHC gene to be upregulated and for increased amounts of the protein to appear. This obviously cannot occur. The reason why this defect results in a delay rather than an absolute block in chemotaxis could be that other small myosin isoforms eventually accumulate to levels where they can take the place of the DMIHC isoform. A second reason for the delay could be that the chemotaxis of DMIHC⁻ cells, when it does occur, is somehow less efficient than in wild-type cells, perhaps because of subtle differences in the dynamics of the leading edge (for example,
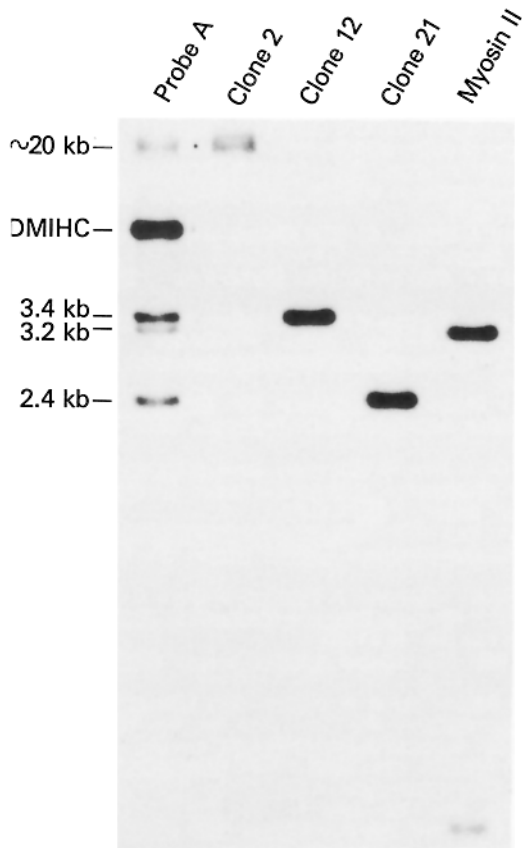
Figure 8. Southern blot analyses of the three additional putative *Dictyostelium* small myosin heavy chain genes. *Dictyostelium* genomic DNA was restricted with Eco RI and hybridized under conditions of moderate stringency with probe A, and under conditions of high stringency with the inserts of clones 2, 12, and 21 and a *Dictyostelium* myosin II heavy chain clone. The results are from a single agarose gel transferred to a single sheet of nitrocellulose. After transfer, the lanes were cut into strips for use with the different probes. The autoradiograms were then aligned using the well positions.

reduction in the size of pseudopods formed, or in their rate of formation). More detailed analyses of this mutant are underway in order to assess both of these possibilities.

Conclusions

The fact that there are multiple small myosin isoforms in *Dictyostelium*, together with the lack of a severe phenotype when the DMIHC gene is rendered nonfunctional, suggests that there may be considerable overlap of function between these different myosin I isoforms. Therefore, in order to generate a more striking myosin I⁻ phenotype it may be necessary to block the expression of two or more small myosin heavy chain genes within a single cell. Currently, it is possible to introduce two additional mutations into G418ᴿ cells, using hygromycin resistance (Egelhoff et al., 1989) and a *ura* selection system (Dingermann, T., personal communication). Furthermore, if some of these myosin I heavy chain genes are not linked, it may be possible to create multiple mutations in a single cell using parasexual genetics (Loomis, 1987).

While considerable redundancy of function may exist among the various *Dictyostelium* small myosin isoforms, it
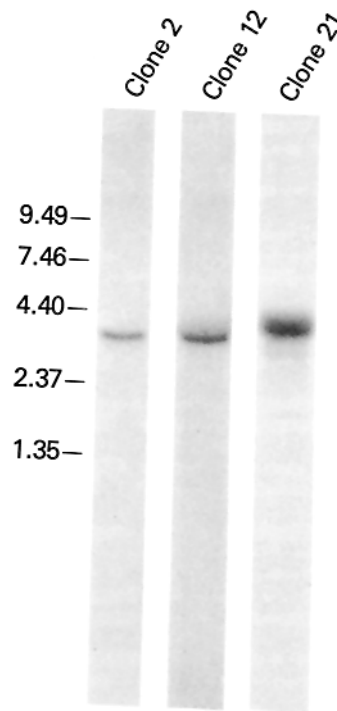

Figure 9. Northern blot analysis of total RNA (10 μg/lane) from vegetative DMIHC⁻ cells (DMI⁻/6), probed with the inserts of clones 2, 12, and 21.

is also possible that individual isoforms or subgroups of isoforms may be solely responsible for particular functions. For example, one subgroup may be responsible for intracellular vesicle transport (Adams and Pollard, 1986), while another subgroup may be responsible for generating force at the leading edge (Fukui et al., 1989). Heavy chain sequence information, and in particular tail domain sequence information, may provide an initial basis for dividing up the small myosin isoforms into different subgroups with potentially distinct functions. For example, whereas the DMIHC tail domain appears to have both the putative membrane binding site (Adams and Pollard, 1989) and the second actin binding site (Korn and Hammer, 1990), abm a appears to have only the membrane binding site (Titus et al., 1989), suggesting that it falls into a different functional group. Information like this should facilitate the prioritizing of gene targeting experiments.

Finally, it will be very valuable to localize each of these myosin I isoforms within the cell by immunofluorescence, using isoform-specific antibodies. For example, using the antibody generated against the TRPE/DMIHC fusion protein, Ax3 cells show intense leading edge staining while DMIHC⁻ cells are essentially devoid of leading edge staining (Fukui, Y., G. Jung, and J. A. Hammer III, unpublished results). This result suggests that the DMIHC isoform is concentrated at the leading edge of motile cells, although other small myosin isoforms not seen by this antibody may be located there as well.

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