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18. Samples (usually 20 μl) were diluted to 85 μl with phosphate-buffered saline (PBS; 0.85% NaCl, 10 m*M* phosphate, *p*H 7.4) containing 1 μg of trypsin per milliliter. Five microliters of 1*M* tris, *p*H 8, were added to raise the *p*H of the low *p*H samples. After 10 min at room temperature to convert proaerolysin to aerolysin, 90 μl of PBS containing 0.1% bovine serum albumin were added, and the samples were serially diluted 1:2 with the same buffer in microtiter plates with V-shaped bottoms. Each well then received 90 μl of 0.8% (v/v) human erythrocytes, and the plates were incubated for 60 min at 37°C. After incubation, the plates were centrifuged and 135-μl samples of the supernatants were removed from wells in which partial hemolysis had occurred. They were diluted with 850 μl of PBS and their absorbances were read at 413 nm. The amount of aerolysin in each sample was determined from a standard curve prepared in the same microtiter plate with known amounts of purified aerolysin. Neither CCCP nor any of the buffers interfered with the assay system used to measure proaerolysin.
19. We thank S. P. Howard, S. MacIntyre, and F. Pattus for useful criticism and stimulating discussion and M. Cahill for help in preparation of the manuscript. Supported by a grant from the National Sciences and Engineering Research Council of Canada.

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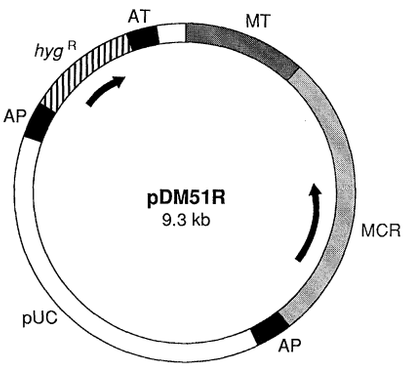

Fig. 1. Physical map of the myosin head fragment expression vector pDM51R. The expression vector is a derivative of the hygromycin resistance vector pDE102 (*6*) carrying 2.6 kb of 5′ coding region of myosin (MCR). The MHF expression cassette lies in the opposite orientation (shown by arrows) to the hygromycin phosphotransferase gene (hyg^R), and the two genes are separated in this construct by 1.1 kb of *mhc*A terminator sequence (MT) and 0.3 kb of *act*-15 terminator sequence (AT). Both the MCR and hyg^R sequences are controlled by the *act*-15 promoter (AP). The white segments represent pUC119 sequences (pUC).

Expression and Characterization of a Functional Myosin Head Fragment in *Dictyostelium discoideum*

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The isolated head fragment of myosin is a motor protein that is able to use energy liberated from the hydrolysis of adenosine triphosphate to cause sliding movement of actin filaments. Expression of a myosin fragment nearly equivalent to the amino-terminal globular head domain, generally referred to as subfragment 1, has been achieved by transforming the eukaryotic organism *Dictyostelium discoideum* with a plasmid that carries a 2.6-kilobase fragment of the cloned *Dictyostelium* myosin heavy chain gene under the control of the *Dictyostelium* actin-15 promoter. The recombinant fragment of the myosin heavy chain was purified 2400-fold from one of the resulting cell lines and was found to be functional by the following criteria: the myosin head fragment copurified with the essential and regulatory myosin light chains, decorated actin filaments, and displayed actin-activated adenosine triphosphatase activity. In addition, motility assays in vitro showed that the recombinant myosin fragment is capable of supporting sliding movement of actin filaments.

MYOSINS CONSTITUTE A FAMILY of diverse proteins that bind to actin and have Mg^{2+}-dependent adenosine triphosphatase (ATPase) activity that is stimulated by actin at low ionic strength. Interaction of myosins with actin in the presence of adenosine triphosphate (ATP) results in the conversion of chemical energy into mechanical force and displacement. Myosins occur in nearly every eukaryotic cell examined, where they participate in many fundamental cellular processes ranging from muscle contraction to cytokinesis. Two subgroups of myosins are known. The first group consists of the double-headed or conventional myosins, genereally referred to as myosin or sometimes as myosin II. The second group consists of the single-headed or unconventional myosins, referred to as myosin I. In this report we will only refer to conventional myosins, all of which show the same structural pattern. They consist of two heavy chains (~ 200 kD each) and two pairs of light chains (15 to 20 kD each). The NH_2-terminal half of the myosin heavy chain (MHC) forms the globular head, which contains the binding sites for the myosin light chains, and the COOH-terminal half forms the extended coiled-coil rod. The globular head fragment of the myosin molecule, also called subfragment-1 or S1, can be released as a soluble fragment by proteolytic cleavage of myosin and has the catalytic and actin-binding properties of the myosin molecule (*1, 2*). It has been shown that S1 alone is sufficient to cause sliding movement of actin filaments in vitro (*3*).

Understanding the mechanism by which myosin catalyzes the transduction of energy stored in chemical bonds into mechanical work will require knowledge of the high-resolution structure of S1 and the ability to manipulate the protein in specific manners at the molecular level. The S1 crystals obtained by Rayment and Winkelmann (*4*) should help in determining the high-resolution structure of the molecule. However, a source of S1 without the inherent heterogeneity of preparations made by proteolytic digestion and that allows the introduction of specific alterations into the molecule by molecular genetic approaches would be desirable. We have addressed this problem by exploring the use of the cellular slime mold *Dictyostelium discoideum* as an expression system for myosin fragments.

The plasmid pDM51R (Fig. 1), which was constructed to achieve expression of a myosin head fragment (MHF) nearly equivalent to S1, carries a translational fusion of the eighth codon of the *Dictyostelium act*-15 gene to the second codon of the *Dictyostelium mhc*A gene. The polypeptide encoded by this fusion has 871 amino acids and extends 46 amino acids beyond the proline residue that marks the region of proteolytic cleavage in muscle myosin. The vector also contains a bacterial hygromycin resistance gene for selection in *Dictyostelium* (*5*) as well as pUC119 sequences (*6*) for selection and autonomous replication in *Escherichia coli*. Transformations of *Dictyostelium* axenic strain AX2 by the calcium phosphate precipitate technique were carried out according to the modified protocol described by Egelhoff *et al.* (*5*). Transformation efficiencies of $\sim 10^{-6}$ were observed. As pDM51R cannot replicate autonomously in *Dictyostelium*, all stable transformants must result from an integration event. The relative amounts of MHF produced by the transformants were estimated from immunoblots of whole-cell

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lysates probed with antibodies directed against the myosin head, in conjunction with Coomassie blue–stained SDS-polyacrylamide gels (7). For the immunoblotting, we used both a polyclonal antibody (P-5020) to a peptide corresponding to amino acids 600 to 616 in the *Dictyostelium* myosin sequence, a region that corresponds to the junction of the 20- and 50-kD myosin head domains (*8*), and a monoclonal antibody (M II.42) to the head region of *Acanthamoeba* myosin II. Expression of a polypeptide of the appropriate size was only detected in cells transformed with the expression vector. Cell line HS2210 produced the largest amounts of MHF and was used for the purification and characterization of the recombinant protein. These cells show normal morphology and growth rate and upon starvation undergo a complete developmental cycle. No changes in the rate of MHF expression have been observed in 8 months of maintaining this cell line.

MHF was purified from HS2210 cells that had been starved for 4 hours to induce development (*9*). Development was found to enhance the expression of MHF and to decrease the activity of proteases in crude cell extracts. The purification procedure resulted in an approximately 2400-fold enrichment of the recombinant MHF. An average of 0.05% of the total cell protein was recovered as purified MHF, which corresponds to a yield of 2.5 mg of MHF from 100 g of cells.

The expression of a functional MHF in *Dictyostelium* relies in part on the fact that this organism can supply the expressed fragment of the MHC with its endogenous essential (ELC) and regulatory (RLC) myosin light chains. Analysis of the purified MHF by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) revealed the presence of all three polypeptides. The relative mobility of the expressed fragment corresponded to that of a 95-kD polypeptide. The two bands corresponding in size to the *Dictyostelium* light chains had molecular masses of 18 kD (RLC) and 16 kD (ELC) (Fig. 2A). Furthermore, the presence of the RLC was confirmed by immunoblotting with a monoclonal antibody, My 8 (*10*), to the *Dictyostelium* RLC (*11*).

One of the most distinctive properties of a functional myosin head is the ATP-dependence of its interaction with actin. In the absence of ATP, the head portion of the myosin molecule forms a rigor complex with actin filaments, giving rise to a typical arrowhead structure that can be detected by electron microscopy (*12*). The expressed MHF formed an arrowhead structure with F-actin (Fig. 2B). Similar to the situation found for muscle myosin and endogenous *Dictyostelium* myosin, the expressed MHF had ATPase activity at low ionic strength in the presence of Mg^{2+} that was greatly stimulated by actin (*13*). The maximal actin-activated ATPase activity measured with rabbit skeletal muscle actin was 480 nmol of inorganic phosphate (P_i) per minute per milligram of MHF. This corresponds to a turnover rate of one ATP molecule per second, which matches the turnover rate of 0.9 ATP molecules per second per head observed for *Dictyostelium* myosin (*14*). In the absence of actin and at high ionic strength, the expressed MHF had a high ATPase activity in the presence of Ca^{2+} (950 nmol of P_i per minute per milligram of MHF) and a considerably lower ATPase activity in the presence of K^+ and EDTA (65 nmol of P_i per minute per milligram of MHF), as is the case for native *Dictyostelium* myosin. Further evidence for the expression of a functional MHF was obtained when we tested the ability of the purified protein to move actin filaments labeled with tetramethylrhodamine-phalloidin in an in vitro assay system (*3*). Fragmentation and sliding movement of filaments occurred after infusion of the magnesium salt of ATP into the flow cell. The MHF supported actin movement at a mean rate of 130 nm/s (Fig. 2C). Similar to the situation observed with muscle myosin (*3*), the rate of movement of the head fragment is slower than the rate of 1 μm/s observed with intact *Dictyostelium* myosin (*15*).

The expression and purification of an MHF capable of creating motive force demonstrates that it should now be possible to dissect the motor function of myosin by molecular genetic approaches. Indeed, we have successfully expressed a myosin head fragment that includes approximately 50 nm of coiled-coil rod. This myosin fragment, equivalent in size to the proteolytically defined muscle heavy meromyosin heavy chain, binds both light chains, decorates actin filaments to produce arrowheads, and has a maximal actin-activated ATPase activity of 880 nmol of P_i per minute per milligram of protein (*16*).

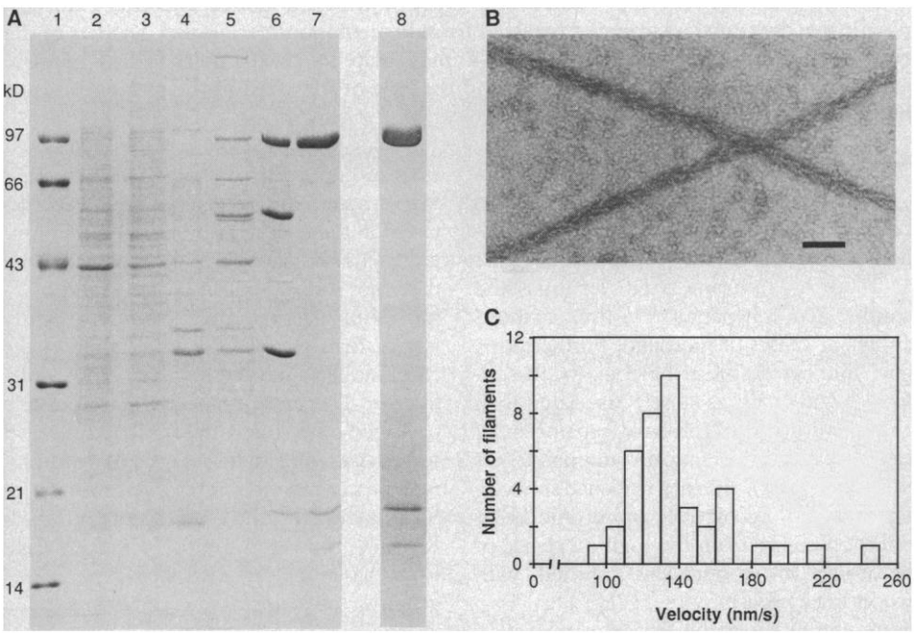

Fig. 2. (**A**) SDS-polyacrylamide electrophoresis of *Dictyostelium* MHF at various stages of purification. Samples subjected to SDS-PAGE (12.5% separating gel) were molecular mass markers (lane 1), crude cell lysate (lane 2), supernatant after actomyosin precipitation (lane 3), actomyosin pellet after Mg-ATP extraction (lane 4), Mg-ATP–extracted protein (lane 5), flow-through after first DEAE column (lane 6), pool of MHF-containing fractions after second DEAE column (lane 7), pool of MHF-containing fractions after gel filtration column (lane 8). In each case, 3 μg of protein was loaded, except for lane 8, where 5 μg was loaded. The gel was stained with Coomassie brilliant blue R. (**B**) Electron micrograph of filamentous actin from rabbit muscle decorated with the expressed MHF. The arrowhead pattern is like that of filamentous actin decorated with S1 obtained by proteolytic cleavage of muscle myosin. Because of the decoration, the 9-nm-wide actin filament appears much wider (*17*). Scale bar, 100 nm. (**C**) Histogram showing the sliding speed at 25°C of 40 actin filaments moving on a nitrocellulose surface covered with *Dictyostelium* MHF. MHF was applied to the flow cell at a concentration of 100 μg/ml.

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9. Cell line HS2210 was grown in 6-liter Erlenmeyer flasks, each containing 2.5 liters of HL5 medium. Cells were shaken on a gyratory shaker at 200 rpm at 22°C. MHF was typically purified starting with 40 liters of culture medium, which yielded about 250 g of cells. Cells were harvested at a density of 5×10^6 cells per milliliter by centrifugation for 7 min at 2500 rpm in an IEC PR-6000 centrifuge. To initiate development, cells were suspended at a density of 2×10^7 cells per milliliter in a solution containing 20 m*M* 2-(*N*-morpholino)ethanesulfonic acid (*p*H 6.8), 0.2 m*M* $CaCl_2$, and 2 m*M* $MgSO_4$ and shaken at 140 rpm for 4 hours. All remaining manipulations were performed at 4°C. The developed cells were washed once with a solution containing 10 m*M* tris (*p*H 8.0) and 1 m*M* EDTA and suspended in 1 ml of lysis buffer [50 m*M* Hepes (*p*H 7.5), 20 m*M* sodium pyrophosphate, 2 m*M* EDTA, 1 m*M* dithiothreitol (DTT), 30% (w/v) sucrose, 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF), 5 m*M* benzamidine, *N*-tosyl-L-phenylalanine chloromethyl ketone (20 μg/ml), *Nα-p* tosyl-L-lysine chloromethyl ketone (20 μg/ml), and leupeptin (10 μg/ml)] per gram of cells. Cells were lysed by sonication with a Heat-System Ultrasonic model W 220-F sonicator equipped with a 0.75-inch flat tip. Unbroken cells and most of the cell debris were removed by centrifugation for 15 min at 40,000*g*. The supernatant was adjusted to 0.1*M* KCl using a 3*M* stock solution and then centrifuged at 100,000*g* for 3 hours. A key step in the purification scheme, resulting in a 140-fold enrichment in MHF, was the formation of an actomyosin pellet by removal of the sucrose from the resulting supernatant [M. Clarke and J. A. Spudich, *J. Mol. Biol.* **86**, 209 (1974)] and the specific release of MHF from this pellet by addition of the magnesium salt of ATP (Mg-ATP). The supernatant was dialyzed for 48 hours against 10 m*M* 1,4-piperazinediethanesulfonic acid (*p*H 6.9), 50 m*M* KCl, 2 m*M* EDTA, 0.5 m*M* DTT, 0.2% NaN_3, 1 m*M* benzamidine, and 0.5 m*M* PMSF. The actomyosin precipitate formed during dialysis was collected by centrifugation at 27,000*g* for 15 min and washed with HKE buffer [10 m*M* Hepes (*p*H 7.5), 50 m*M* KCl, 1 m*M* EDTA, 0.5 m*M* DTT, and 1 m*M* benzamidine]. The MHF wa solubilized by extracting the actomyosin pellet with HKE buffer containing 10 m*M* Mg-ATP in a Dounce-type homogenizer. The suspension was centrifuged at 48,000*g* for 15 min. To induce the formation of actin paracrystals, we increased the magnesium concentration of the supernatant to 12 m*M*. After incubation on ice for 30 min, 1 m*M* ATP was added to the now turbid solution, and the actin paracrystals were removed by centrifugation at 100,000*g* for 1 hour. A further fourfold enrichment in MHF was achieved by passing the resulting clear supernatant through a column (1.5 by 20 cm) containing a weak anion-exchanger matrix (Toyopearl DEAE-650 S) equilibrated with HKE buffer. The flow-through, containing the MHF, was collected and dialyzed overnight against HE buffer [20 m*M* Hepes (*p*H 7.5), 0.5 m*M* EDTA, 0.5 m*M* DTT, and 1 m*M* benzamidine]. The dialysate was then loaded onto a column (1.5 by 20 cm) containing a strong cation-exchanger matrix (Toyopearl SP-650 S) connected to a DEAE-650 S column (1.0 by 18 cm). Washing with HE buffer was continued as long as the flow-through showed absorbance at 280 nm. The SP-650 S column was then removed, and MHF was eluted from the DEAE-650 S column with a 75-ml gradient from 0 to 500 m*M* KCl in HE buffer. This combined cation-anion exchanger step led to a further fourfold enrichment for MHF. After this step the MHF could be used successfully both for the decoration of actin filaments and in an in vitro movement assay. Traces of impurities could be removed by passing the MHF over a Superose 12 gel filtration column.
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13. Actin-activated ATPase activity was measured by adding 10 μg of MHF to 0.5 ml of assay solution containing 17 m*M* KCl, 2.7 m*M* $MgCl_2$, 0.7 m*M* EGTA, 0.7 m*M* DTT, 17 m*M* Hepes (*p*H 7.4), and actin. The final concentrations of actin in the assay solution were 0, 1, 2.5, 5, 10, and 20 μ*M*. All assays were performed at 30°C. Samples (100 μl) were removed after 5, 10, 15, and 20 min, added to 50 μl of stop-buffer containing 13.3% (w/v) SDS and 0.12*M* EDTA, and mixed vigorously. The release of phosphate was then determined by addition of 350 μl of freshly prepared developer solution [0.5*M* H_2SO_4, 0.5*M* ammonium molybdate, and 0.5% (w/v) $FeSO_4$]. The absorbance of 550 nm was measured [H. D. White, *Methods Enzymol.* **85**, 698 (1982)]. ATPase activity in the presence of Ca^{2+} was measured with 10 μg of MHF in a 0.5-ml assay volume. The assay solution contained 0.6*M* KCl, 5 m*M* $CaCl_2$, 4 m*M* ATP, and 10 m*M* Hepes (*p*H 7.4). ATPase activity in the presence of EDTA was measured in a similar way except that 25 μg of MHF was used and the assay solution contained 0.6*M* KCl, 5 m*M* EDTA, 4 m*M* ATP, and 10 m*M* Hepes (*p*H 7.4).
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A Liver-Specific Enhancer in the Core Promoter Region of Human Hepatitis B Virus

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An 88–base pair fragment in the core promoter of the human hepatitis B virus (HBV) contains a functional promoter and a strong liver-specific enhancer. This enhancer functions in human hepatoma cells, where it is much more active than the previously described HBV enhancer in stimulating expression of the linked bacterial chloramphenicol acetyltransferase gene expressed from heterologous promoters. Studies of the role of this enhancer-promoter in HBV may help to clarify mechanisms of gene expression in cells infected with HBV and the role of the virus in the pathogenesis of hepatitis and hepatocellular carcinoma.

HEPATITIS B VIRUS (HBV) IS A human hepatotropic virus that has infected approximately 200 million people worldwide. HBV causes either an acute or a chronic viral hepatitis, and chronic active hepatitis and liver cirrhosis are major causes of mortality. Furthermore, epidemiological studies have shown that infection with HBV is closely associated with an increased risk of primary hepatocellular carcinoma, one of the most common cancers in the world (*1*). Further evidence supporting the tumorigenic role of chronic HBV infection comes from the high incidence of hepatoma in woodchucks infected with woodchuck hepatitis virus (WHV) (*2*). Two years after initial viral infection, almost all of the animals developed hepatoma. However, since the precise role of HBV in hepatocellular tumorigenesis remains unclear, it is particularly important to elucidate the mechanisms that control the HBV life cycle and regulate its gene expression.

The principal site of clinical pathology for HBV is the liver, and HBV actively replicates in human hepatocytes (*3*). Two major HBV-specific mRNA species have been detected in the liver of HBV-infected chimpanzees (*4*); a subgenomic 2.1-kb RNA encoding the major surface antigen (HBsAg) and a 3.5-kb terminally redundant RNA, slightly longer than genome length, encoding the core antigen and possibly the DNA polymerase. The latter transcript is ultimately packaged into the viral nucleocapsid and serves as a template for viral DNA synthesis. A sequence resembling the late promoter of SV40 may direct the synthesis of the 2.1-kb RNA (*4*), but the promoter for the 3.5-kb RNA has not been identified. The 3.5-kb genomic transcript has never been detected in nonhepatic cells transfected with HBV DNA, but it has been found in well-differentiated human hepatoma cell lines (Hep G2, HuH6, and HuH7) transfected by the cloned HBV genome (*5*), suggesting that liver-specific factors are needed for correct transcription from the core promoter. In contrast, the 2.1-kb subgenomic HBV mRNA has been observed in a wide variety of transfected cell lines of diverse tissue and species origin (*6*), suggesting a relative lack of tissue specificity for the promoter of the

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