The EMBO Journal vol.8 no.3 pp.923-932, 1989

Gene replacement in *Dictyostelium*: generation of myosin null mutants

Dietmar J.Manstein, Margaret A.Titus, Arturo De Lozanne[1] and James A.Spudich

Department of Cell Biology, Stanford University School of Medicine, Stanford, CA 94305, USA
Present address: Department of Molecular Genetics, The University of Texas, Southwestern Medical Center at Dallas, TX 75235, USA

Communicated by G.Gerisch

The eukaryotic slime mold *Dictyostelium discoideum* has a single conventional myosin heavy chain gene (*mhc*A). The elimination of the *mhc*A gene was achieved by homologous recombination. Two gene replacement plasmids were constructed, each carrying the G418 resistance gene as a selective marker and flanked by either 0.7 kb of 5′ coding sequence and 0.9 kb of 3′ coding sequence or 1.5 kb of 5′ flanking sequence and 1.1 kb of 3′ flanking sequence. Myosin null mutants (*mhc*A$^-$ cells) were obtained after transformation with either of these plasmids. The *mhc*A$^-$ cells are genetically stable and are capable of a variety of motile processes. Our results provide genetic proof that in *Dictyostelium* the conventional myosin gene is required for growth in suspension, normal cell division and sporogenesis, and illustrate how gene targeting can be used as a tool in *Dictyostelium*.

Key words: Dictyostelium/gene replacement/homologous recombination/myosin

Introduction

One of the most important developments in eukaryotic molecular biology is the ability to introduce cloned sequence alterations at exact locations. The possible alterations include the introduction of point mutations, additional copies of a gene, or the complete removal of a certain gene from the genome. The application of these manipulations relies on the ability to introduce DNA into a cell, to integrate the DNA sequences by homologous recombination, and to use suitable selective markers. Gene targeting, the homologous recombination of DNA sequences residing in the chromosome with newly introduced DNA sequences, has been reported for mammalian cells (Smithies *et al.*, 1985; Thomas *et al.*, 1986; Song *et al.*, 1987) and several fungi, such as the ascomycete *Aspergillus nidulans* (Tilburn *et al.*, 1983; Yelton *et al.*, 1984), the basidiomycete *Coprinus cinereus* (Binninger *et al.*, 1987), and the acrasiomycete *Dictyostelium discoideum* (De Lozanne and Spudich, 1987; Witke *et al.*, 1987). The high frequency of homologous recombination in yeast has made gene targeting a very efficient genetic tool in this organism. Gene targeting has been used in *Saccharomyces cerevisiae* to make null mutations in each of the two α-tubulin genes (Schatz *et al.*, 1986), to obtain clathrin-deficient mutants (Lemmon and Jones, 1987; Payne *et al.*, 1987), and in the construction of temperature-sensitive actin mutants (Shortle *et al.*, 1984). The sometimes surprising similarities between yeast and higher eukaryotic cells makes yeast an important model system. The information acquired in these studies in yeast not only contributed considerably to the present perception of the function of α-tubulin, clathrin and actin in yeast but also in higher eukaryotic organisms. Limitations of yeast as a model system are that it does not show cell motility and carries out only the simplest morphogenetic changes. Studying the processes involved in cell migration and differentiation by molecular genetics requires another model system.

The eukaryotic microorganism *Dictyostelium* is an excellent system to study many cellular processes (for review see Spudich, 1987). For example, *Dictyostelium* displays many forms of cell motility. It extends filopodia and pseudopodia, undergoes chemotactic movements, and changes shape during cytokinesis as well as during the morphogenesis which is associated with its unique development cycle. Development initiated upon starvation first leads to the organization of cells into aggregates. These multicellular structures then undergo a series of morphological changes which culminate in the formation of a fruiting body. Three types of specialized cells can be distinguished within the fruiting body: stalk cells, spore cells at the tip of the stalk, and cells converted into a supporting disc at its base (Gerisch, 1987).

The recent advances in the application of molecular techniques in *Dictyostelium* include construction of transformation vectors (Nellen *et al.*, 1987), which made possible both the introduction of antisense constructs (Knecht and Loomis, 1987) as well as genes modified by site-directed mutagenesis (Datta and Firtel, 1987; Pears and Williams, 1987), and the construction of extrachromosomal transformation vectors (Firtel *et al.*, 1985). In addition, the recent demonstrations of high frequency homologous recombination in *Dictyostelium* (De Lozanne and Spudich, 1987; Witke *et al.*, 1987; Katz and Ratner, 1988) point to the possibility that one might be able to develop a system for specific gene deletion and replacement similar to that reported for yeast (Orr-Weaver *et al.*, 1981; Rothstein, 1983). It is therefore important to determine what elements are critical to obtain frequent homologous recombination in *Dictyostelium*. To elucidate these elements, we attempted to replace the *mhc*A gene by targeting different regions of the gene with plasmids containing different sizes of myosin gene segments.

To study the accessibility of the *Dictyostelium* genome to gene targeting, two plasmids were constructed using *mhc*A sequences. The *mhc*A gene codes for the only conventional myosin found in *Dictyostelium* and it has been cloned and sequenced (De Lozanne *et al.*, 1985; Warrick *et al.*, 1986), which considerably facilitated the genetic analysis of the transformants. The gene replacement plasmids each contained a selectable marker gene conferring G418

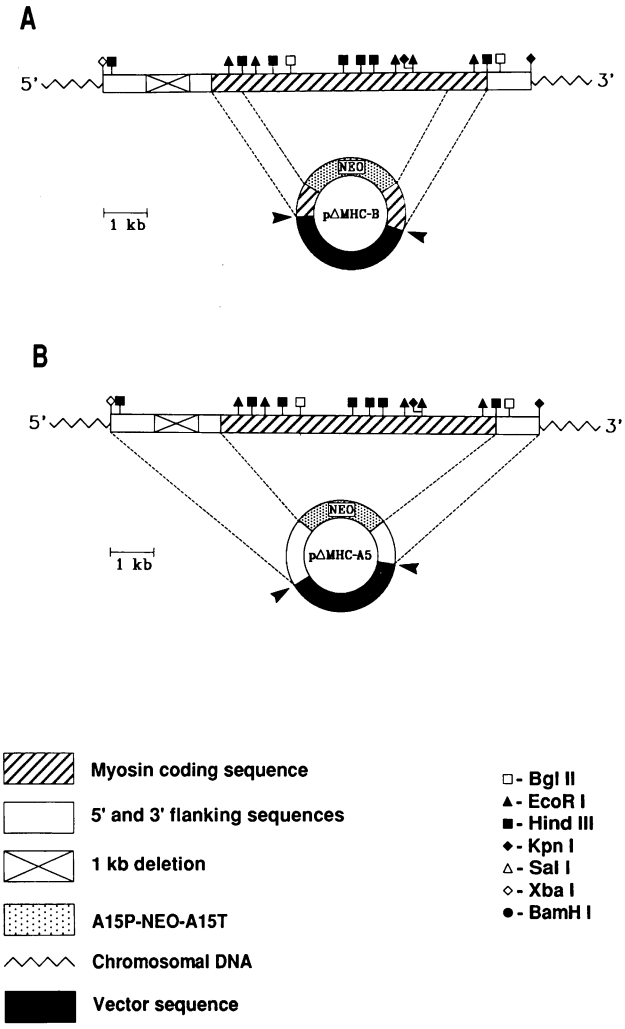


Fig. 1. Physical map of the *Dictyostelium mhc*A locus and the gene replacement plasmids pΔMHC-B and pΔMHC-A5. The transforming plasmids contain a neomycin phosphotransferase gene driven by the *Dictyostelium* actin-15 promoter and terminated by the actin-15 terminator. These sequences are flanked by cloned *mhc*A sequences which can recombine with the homologous sequences in the chromosome. The plasmid pΔMHC-B contains 0.7 kb of 5′ coding sequence and 0.9 kb of 3′ coding sequence (**A**). The plasmid pΔMHC-A5 contains 1.5 kb of 5′ flanking sequence and 1.1 kb of 3′ flanking sequence (**B**). The arrows indicate the sites where the plasmids were cut to liberate the gene replacement fragments from vector DNA and to create recombinogenic ends. The crossed box in the 5′ flanking region of the *mhc*A gene indicates a region of 1 kb that is absent from the cloned gene.

resistance ($G418^R$), flanked by sequences coming from the 5′ and 3′ ends of the *mhc*A gene. The first construct, pΔMHC-B, contained 0.7 kb of 5′ coding and 0.9 kb of 3′ coding sequence from the *mhc*A gene. Yeast replacement plasmids containing homologous elements of comparable size are known to induce high frequency gene replacement following transformation (Rothstein, 1983). The second construct, pΔMHC-A5, contained 1.5 kb of 5′ flanking and 1.1 kb of 3′ flanking sequences. Flanking sequences are known to be very AT-rich (>90%) in *Dictyostelium* (Firtel *et al.*, 1976). It was important to establish whether such regions could be used in gene targeting. Both plasmids were linearized by cutting at the border of vector and *Dictyostelium* DNA to create recombinogenic ends and enhance the efficiency of gene targeting events, in accordance with

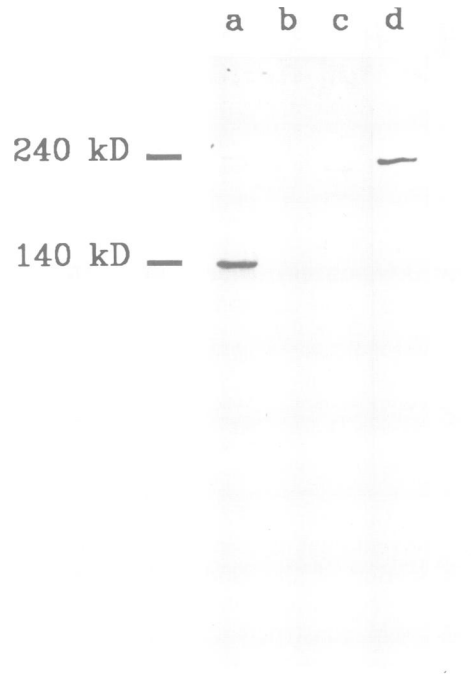


Fig. 2. Immunoblot analysis of the *mhc*A⁻ cells. Whole cell lysates from cells (AX4) producing only a truncated form of myosin (HMM-140) (**lane a**), the null mutant cell lines *mhc*A⁻/B (**lane b**) and *mhc*A⁻/A5 (**lane c**), and wild type cells (**lane d**) were subjected to electrophoresis on a 7.5% SDS–polyacrylamide gel, transferred to nitrocellulose paper, and probed with a polyclonal antibody directed against *Dictyostelium* myosin. Antibody binding was detected using horseradish peroxidase conjugated goat anti-rabbit IgG.

experiments in yeast and mammalian cells (Orr-Weaver *et al.*, 1981; Thomas *et al.*, 1986).

We report the isolation and characterization of transformants obtained with these two different gene replacement constructs. Transformation with both constructs led to the isolation of cells which were no longer able to express myosin, following the elimination of either the majority or all of the *mhc*A gene coding sequence. The establishment of cell lines that are missing the *mhc*A gene gives definite proof that it is neither an essential gene nor is it needed in many forms of cell motility.

Results

Experimental plan

To achieve replacement of the *Dictyostelium* myosin heavy chain gene (*mhc*A gene) we constructed the plasmids pΔMHC-B and pΔMHC-A5 (Figure 1) which contain the bacterial neomycin phosphotransferase gene (conferring resistance to the drug G418) as a selectable marker inserted between two cloned fragments of the *mhc*A gene. The neomycin phosphotransferase gene is under the control of the *Dictyostelium* actin-15 promoter and terminator sequences (Knecht *et al.*, 1986) in both these constructs. In the case of pΔMHC-B, the cloned *mhc*A gene sequences correspond to 0.7 kb of 5′ coding and 0.9 kb of 3′ coding sequence, and in the case of pΔMHC-A5, to 1.5 kb of 5′ flanking and 1.1 kb of 3′ flanking sequence. The procedure for the isolation of myosin null mutants (*mhc*A⁻cells) consisted of the following stages. (i) Introduction of the gene replacement plasmid into an axenic strain of *Dictyostelium* (AX4). Using gene replacement in yeast as a paradigm

(Rothstein, 1983), the plasmids were first linearized by restriction enzyme digestion on the border between the *Dictyostelium mhc*A sequences and the vector DNA (pTZ-18R). (ii) Selection with G418 of those cells which incorporated the plasmid fragment in their genomes. (iii) Screening of transformant colonies for the absence of the myosin heavy chain by immunoblot analysis. (iv) Southern blot analysis of transformants which were no longer able to express myosin.

Dictyostelium mhcA$^-$*/B cells obtained by transformation with pΔMHC-B*

Transformation of *Dictystelium* with the gene replacement fragment liberated from vector DNA of pΔMHC-B, by cutting with the restriction enzymes *Bam*HI and *Sal*I, led to the isolation of three G418^R cell lines. One of these cell lines failed to produce myosin and was named *mhc*A$^-$/B. The absence of myosin was determined by immunoblot analysis with a polyclonal antibody directed against myosin (Figure 2). The molecular organization of the *mhc*A locus of these cells was determined by gel transfer hybridization experiments on genomic DNA. The probe used was the *Xba*I−*Bcl*I fragment of the *mhc*A gene shown in Figure 3A. The patterns generated by hybridizing this probe to three different restriction digests of genomic DNA, isolated from the transformant cell line, are shown in Figure 3 (top). The hybridization pattern was compared to that of DNA isolated from the parent strain, AX4.

The replacement of the *mhc*A gene with the G418^R construct is expected to cause the disappearance of the 7.4 and 4.8 kb bands in the *Sal*I−*Bgl*II digest, the 6.0, 3.2, 1.5, 0.56 and 0.40 kb bands in the *Sal*I−*Eco*RI digest, and the 3.2, 2.7, 1.6, 0.66, 0.39 and 0.31 kb bands in the *Sal*I−*Hin*dIII digest (see Figure 3). Inspection of Figure 3 shows that, with the exception of the 0.40 kb band (the the presence of which is explained below) in the *Sal*I−*Eco*RI digest, this is the case for the transformant. However, pattern obtained can not be explained by a simple gene replacement event with a single copy of the introduced construct. Instead, the pattern is explained by the map shown in Figure 3B. This map shows the expected loss of the *mhc*A gene sequence and its replacement by two complete copies of the transforming plasmid pΔMHC-B in addition to two copies of the replacement fragment, arranged as shown in Figure 3B. The head to tail arrangement of the two gene replacement fragments may have arisen by blunt end-to-end ligation. This arrangement results in unique bands in the Southern blot shown in Figure 3: the 10.2 kb band in the *Sal*I−*Bgl*II digest, the 0.6 kb band in the *Sal*I−*Eco*RI digest, and the 3.7 kb band in the *Sal*I−*Hin*dIII digest. The 0.40 kb band in the *Sal*I−*Eco*RI digest results from cutting the *Eco*RI site in the 5′ coding fragment and the *Eco*RI site in the polylinker of the vector. A prediction of the map shown in Figure 3B is that an *Xba*I−*Bcl*I digest would create a unique 0.7 kb band. The presence of this band is confirmed by a gel transfer hybridization experiment of genomic DNA from the *mhc*A$^-$/B cell line, where the DNA digested with *Xba*I and *Bcl*I is probed with the same 7.8 kb *Xba*I−*Bcl*I fragment. Four more bands of 3.6, 3.4, 3.3, and 2.9 kb are expected in this digest. These fragments are indeed observed although the 3.4 and 3.3 kb bands are not resolved (Figure 3). A band migrating at approximately 6.5 kb indicates that the *Xba*I digest did not go to completion. A digest of DNA from untransformed AX4 cells shows a single 8.8 kb band (Figure 3).

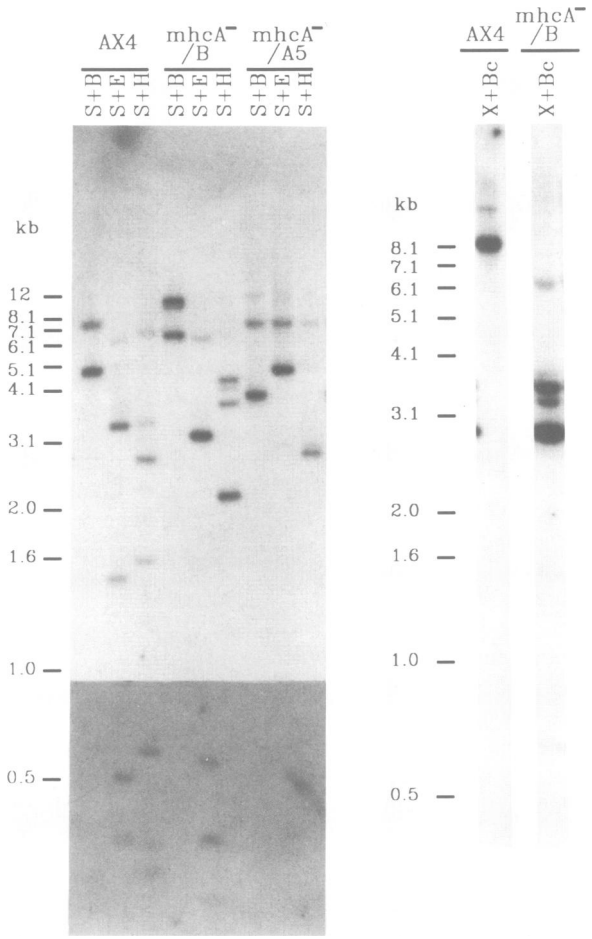
Comparison of the maps and Southern blots in Figure 3 shows that for both wild type and mutant cells the bands derived from the 5′ flanking sequence are weaker than those originating from coding regions. This finding may be due to the AT-richness of the *Dictyostelium* non-coding sequences. It is possible that this composition leads to an increased loss of probe during the washing procedure, due to the lower melting temperature of the probe in this AT-rich region.

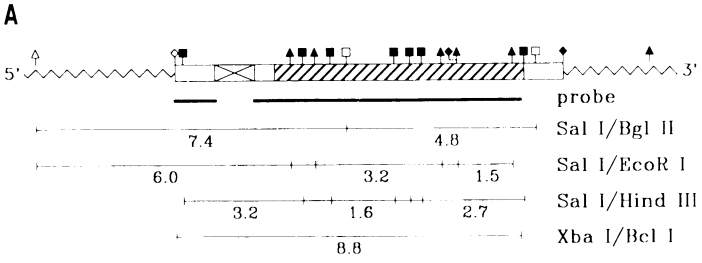
Bands derived from the 5′ flanking region of the *mhc*A gene are 1 kb larger than the size precicted from a restriction map of the cloned gene (De Lozanne *et al.*, 1988). These results can be explained by the occurrence of a 1 kb deletion within the 5′ flanking sequence (indicated by the crossed box in all schematic diagrams) during the cloning of the *mhc*A gene. This finding explains previous results (De Lozanne and Spudich, 1987), where the cloned 5′ flanking sequence of the *mhc*A gene is not functional as a promoter. Presumably the 1 kb deletion contains the *mhc*A gene promoter or an essential part of it.

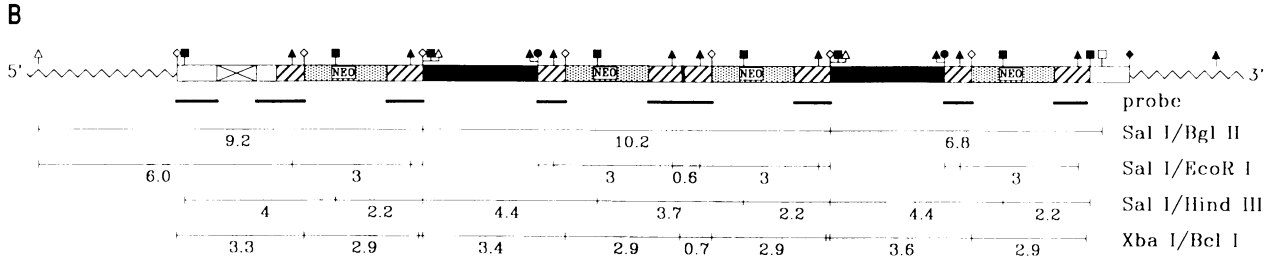
Dictyostelium mhcA$^-$*/A5 cells obtained by transformation with pΔMHC-A5*

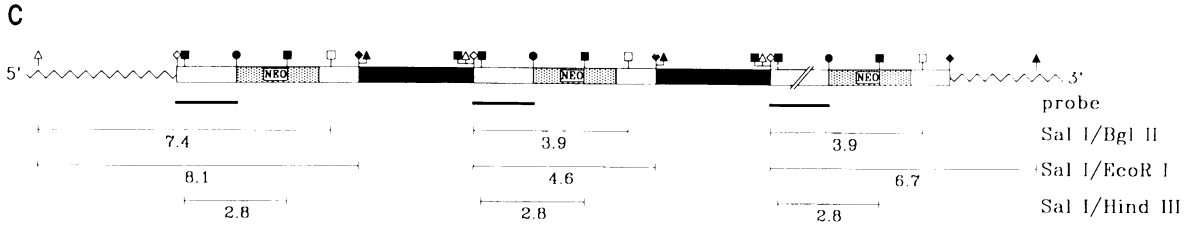
The plasmid pΔMHC-A5 differs from pΔMHC-BV in that it contains two fragments of homologous non-coding region flanking the resistance marker instead of fragments coming from the *mhc*A gene coding regions. Because of the deletion which occurred in the cloning of the *mhc*A gene, it also lacks a fragment of ~1 kb in the homologous 5′ non-coding region (crossed box in Figure 4A).

The gene replacement fragment can be liberated from vector sequences by digestion of the plasmid pΔMHC-A5 with *Kpn*I and *Xba*I. After transformation with the unpurified *Kpn*I−*Xba*I fragment, seven colonies of G418^R transformants were obtained. Western analysis showed that one of these transformants no longer produced myosin (Figure 2). Southern transfer patterns of these cells, which we called *mhc*A$^-$/A5, and of wild type AX4 cells were generated using two different probes. The first probe, the *Sal*I−*Xba*I fragment shown in Figure 4A, is homologous to a region 2.5 kb upstream of the *mhc*A gene. In wild type cells this probe hybridized to a 7.4 kb band in the *Sal*I−*Bgl*II digest, a 6.0 kb band in the *Sal*I−*Eco*RI digest and a 10.2 kb band in the *Sal*I−*Kpn*I digest (Figure 4, top). Probably, due to the large size of the expected fragment (>16 kb), no hybridization was detected to the DNA digested with *Sal*I. The *Sal*I−*Xba*I fragment hybridized to genomic DNA isolated from *mhc*A$^-$/A5 cells to give a 7.4 kb band in the *Sal*I−*Bgl*II digest, a 8.1 kb band in both the *Sal*I−*Eco*RI and *Sal*I−*Kpn*I digests, and to a 11.0 kb band in the *Sal*I digest. This last fragment is derived from the integration of a complete copy of the transforming plasmid pΔMHC-A5 next to the region of homology of the probe (see Figure 4B). The sizes of these fragments indicate that the transformants acquired the 5′ flanking sequence of the incoming replacement fragment. Thus, in addition to the deletion of the entire coding region, these transformants also lack 1 kb of 5′ flanking sequence that presumably contains the *mhc*A promoter. The presence of trace amounts of vector DNA in the probe was useful in showing the integration of the


AX4
mhcA⁻ /B
mhcA⁻ /A5
S+B
S+E
S+H
kb
12
8.1
7.1
6.1
5.1
4.1
3.1
2.0
1.6
1.0
0.5
AX4
mhcA⁻ /B
X+Bc
X+Bc
kb
8.1
7.1
6.1
5.1
4.1
3.1
2.0
1.6
1.0
0.5


A
5'
3'
probe
7.4
4.8
Sal I/Bgl II
6.0
3.2
1.5
Sal I/EcoR I
3.2
1.6
2.7
Sal I/Hind III
8.8
Xba I/Bcl I


B
5'
NEO
3'
probe
9.2
10.2
6.8
Sal I/Bgl II
6.0
3
3
0.6
3
3
Sal I/EcoR I
4
2.2
4.4
3.7
2.2
4.4
2.2
Sal I/Hind III
3.3
2.9
3.4
2.9
0.7
2.9
3.6
2.9
Xba I/Bcl I


C
5'
NEO
3'
probe
7.4
3.9
3.9
Sal I/Bgl II
8.1
4.6
6.7
Sal I/EcoR I
2.8
2.8
2.8
Sal I/Hind III

transforming plasmid pΔMHC-A5. This integration resulted in the appearance of a 3.6 kb band in the *SalI−BglII* digest, a 2.9 kb band in both the *SalI−EcoRI* and the *SalI−KpnI* digests, and a 7.5 kb band in the *SalI* digest of the *mhc*A$^-$/A5 cells. This 7.5 kb band shows that at least two copies of the transforming plasmid were tandemly integrated (see Figure 4B).

The second probe is the same *XbaI−BclI* fragment used to analyse the *mhc*A$^-$/B cells (Figure 3). The pattern obtained with this probe, shown in Figure 3, confirms the results obtained with the first probe. The replacement of the *mhc*A gene by the transforming plasmid pΔMHC-A5 causes the disappearance of the 4.8 kb band in the *SalI−BglII* digest, the 6.0, 3.2, 1.5, 0.56 and 0.40 kb bands in the *SalI−EcoRI* digest, and the 3.2, 1.6, 0.66, 0.39 and 0.31 kb bands in the *SalI−HindIII* digest. The absence of the 7.4 kb band in the *SalI−BglII* digest and the 2.7 kb band in the *SalI−HindIII* digest (Figure 3) cannot be confirmed since the replacement of the *mhc*A gene with pΔMHC-A5 leads to the appearance of a 7.4 kb band in the *SalI−BglII* digest and a 2.8 kb band in the*SalI−HindIII* digest. The presence of a 3.9 kb *SalI−BglII* band and a 4.6 kb *SalI−EcoRI* band indicates a multicopy tandem integration of the transforming plasmid pΔMHC-A5. The relative intensity of these two bands also indicates the integration of several copies of the transforming plasmid. Multiple insertions of the gene replacement plasmid are indicated by the break in Figure 3C. Although the genomic DNA was digested extensively with the respective restriction endonucleases, the presence of a 7.5 kb band in the *SalI−HindIII* digest and of bands >12 kb in the *SalI−BglII* and *SalI−EcoRI* digests indicates that the enzymic reactions were incomplete. For this reason, an unambiguous confirmation of the expected 7.4 kb *SalI−BglII* band and the 8.1 kb *SalI−EcoRI* band is not possible, since the incomplete digestion results in a co-migrating 7.5 kb fragment corresponding to a complete copy of pΔMHC-A5. This is especially true since these fragments, as well as the 6.7 kb *SalI−EcoRI* fragment, are expected to occur only once per cell while all other fragments should be present in greater number. Upon longer exposure of the autoradiograph, it is possible to detect the 6.7 kb *SalI−EcoRI* fragment (not shown).

Generation of Dictyostelium mhcA$^-$ cells by the integration of a single gene replacement fragment

Two conclusions were suggested by the results described above. First, under the selection conditions used there may be a requirement for the integration of multiple copies of the G418^R gene. Second, the DNA fragments may be religated after introduction into the cell. According to the yeast paradigm recircularization of the replacement plasmids should lead to the appearance of *mhc*A disruption mutants, resulting from a single cross over event, instead of gene replacement mutants, which require the occurrence of two cross overs. Indeed, in subsequent transformations we often found that essentially all *mhc*A$^-$ mutants were not a result of gene replacement events but were gene disruption mutants.

As a consequence of these observations, several modifications were introduced into the transformation protocol. The most important modifications were that the concentration of G418 during selection was reduced to 6 μg/ml of HL5 medium and the digested fragments of the gene replacement plasmids were treated with T4 DNA polymerase in the presence of a single nucleotide. The treatment with T4 DNA polymerase results in a limited degradation of the fragments due to the 3′−5′ exonuclease activity of the enzyme and should decrease the efficiency with which the fragments can be religated in *Dictyostelium*. The modified transformation protocol (see Materials and methods, protocol 2) led to an increase in the transformation frequency to 10^{-5}, which is similar to that observed using circular plasmids. About 80 G418 resistant cell lines were obtained per experiment in three transformations using linearized pΔMHC-A5. Seven out of 45 transformants, which were isolated and screened for alterations in the *mhc*A gene, failed to produce myosin. Southern analysis showed that the replacement of the *mhc*A gene with a single gene replacement fragment was the cause for the failure to produce myosin in all seven cell lines. Two of these transformants, which derived from independent transformations, are shown in Figure 5 (*mhc*A$^-$/A5-2 and -3).

Phenotype of the Dictyostelium mhcA$^-$ cells

Both the transformants obtained with pΔMHC-B and pΔMHC-A5 show the same phenotype characteristics described for cells which only express a truncated form of myosin (HMM-140) or have greatly reduced levels of myosin (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987). The *Dictyostelium mhc*A$^-$ cells show many forms of cell movement. They are able to extend and retract filopodia and lamellopodia, are capable of chemotaxis and of forming streams that lead to aggregates. These cells can also ingest bacteria by phagocytosis. Cytoplasmic organelle movement is apparently unaffected by the lack of myosin. When *mhc*A$^-$ cells are placed in starvation conditions they start their developmental cycle. However, their development proceeds more slowly than that of wild type cells and is blocked after the formation of aggregated mounds.

Fig. 3. Southern transfer demonstration of gene replacement induced by the transforming plasmids pΔMHC-B and pΔMHC-A5. DNA was purified from untransformed *Dictyostelium* cells (AX4) and cell lines that failed to produce myosin after transformation with pΔMHC-B (*mhc*A$^-$/B) and pΔMHC-A5 (*mhc*A$^-$/A5). Following digestion with restriction endonucleases, DNA (40 μg) was loaded onto a 0.8% agarose gel, electrophoresed, transferred to a nylon membrane, and hybridized to the ^{32}P-labeled DNA probe. The probe was the cloned 7.8 kb *XbaI−BclI* fragment, corresponding to the cloned *mhc*A gene, which lacks 1 kb of 5′ flanking sequence. The hybridization pattern obtained with this probe is shown in the top part of the figure. The length of the fragments are given in kb and were determined by the co-electrophoresis of plasmid fragments of known length. The bottom part of the figure shows a schematic representation of the Southern analysis data. (**A**) Known map of wild type *mhc*A locus. The lighter lines with numbers underneath indicate the size of the restriction fragments (in kb) hybridizing with the probe when genomic DNA is digested with the enzymes shown on the right. The position of the *BclI* site, which is not illustrated by a symbol, is 92 bp upstream from the 3′ end of the coding region. The sizes of the smaller fragments not indicated in the figure are (from left to right): 0.56 and 0.40 kb in the *SalI−EcoRI* digest; 0.66, 0.39 and 0.31 kb in the *SalI−HindIII* digest. The occurrence of a 1 kb deletion within the 5′ flanking region of the cloned fragment, that was used as probe, is illustrated by the crossed box in the map and the gap in the dark line. The dark line beneath the maps indicates the region of homology between the probe and *mhc*A sequences. (**B**) Map of the *mhc*A locus of the transformant cell line *mhc*A$^-$/B as deduced from the hybridization pattern shown above. The sizes of the smaller fragments in the *SalI−EcoRI* digest are (from left to right): 0.25, 0.40, 0.25 and 0.4 kb. (**C**) Map of the *mhc*A locus of the transformant cell line *mhc*A$^-$/A5 as derived from the Southern blots shown in Figures 3 and 4. The following abbreviations are used: B, *BglII*; Bc, *BclI*; E, *EcoRI*; H, *HindIII*; S, *SalI*; X, *XbaI*. The symbols used are explained in Figure 1.

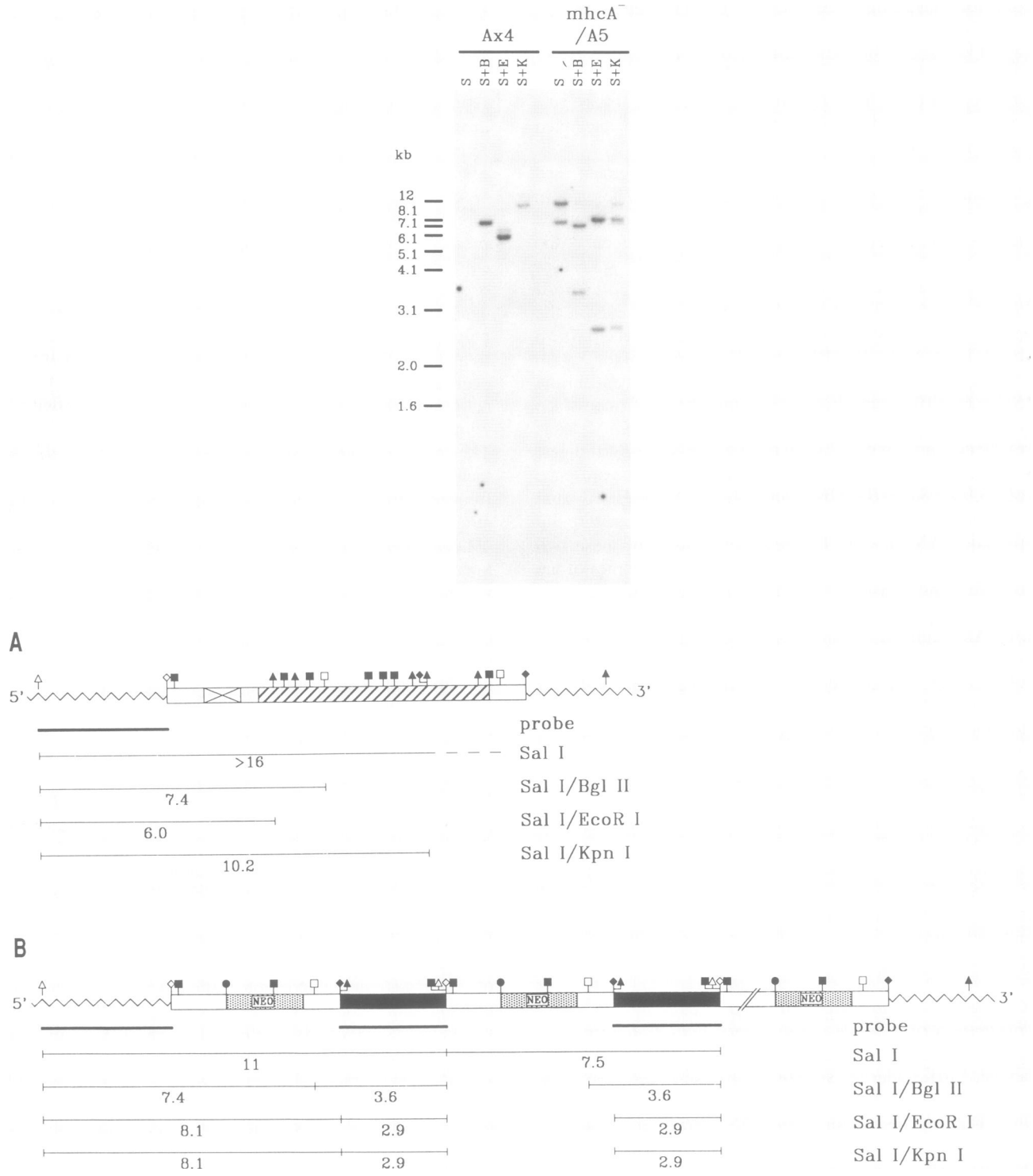


Fig. 4. Southern transfer demonstration of gene replacement induced by the transforming plasmid pΔMHC-A5. A cloned 3.1 kb *SalI*−*XbaI* fragment containing sequences located 2.5 kb upstream of the *mhc*A gene was used as the probe. DNA was purified from untransformed *Dictyostelium* cells (AX4) and cells transformed with the plasmid pΔMHC-A5 that failed to produce detectable levels of myosin (*mhc*A⁻/A5). The length of the fragments are given in kb and were determined by the co-electrophoresis of plasmid fragments of known length. A schematic representation of the organization of the *mhc*A locus in wild type (**A**) and *mhc*A⁻/A5 cells (**B**) is shown in the bottom part of the figure. The dark line beneath the maps indicates the region of homology between the probe and *mhc*A sequences. The following abbreviations for restriction enzymes are used: B, *Bgl*II; E, *Eco*RI; K, *Kpn*I; S, *Sal*I. All other symbols used are explained in Figure 1.

Revertants showing a normal development cycle were not observed when either cell line was grown without selection for several months.

A second phenotypic effect of the null mutation is a defect in cytokinesis which leads to the frequent appearance of large multinucleated cells. Nevertheless, the *mhc*A⁻ cells can grow on a Petri plate with a doubling time as short as 12 h, only slightly slower than the doubling time of 10−11 h

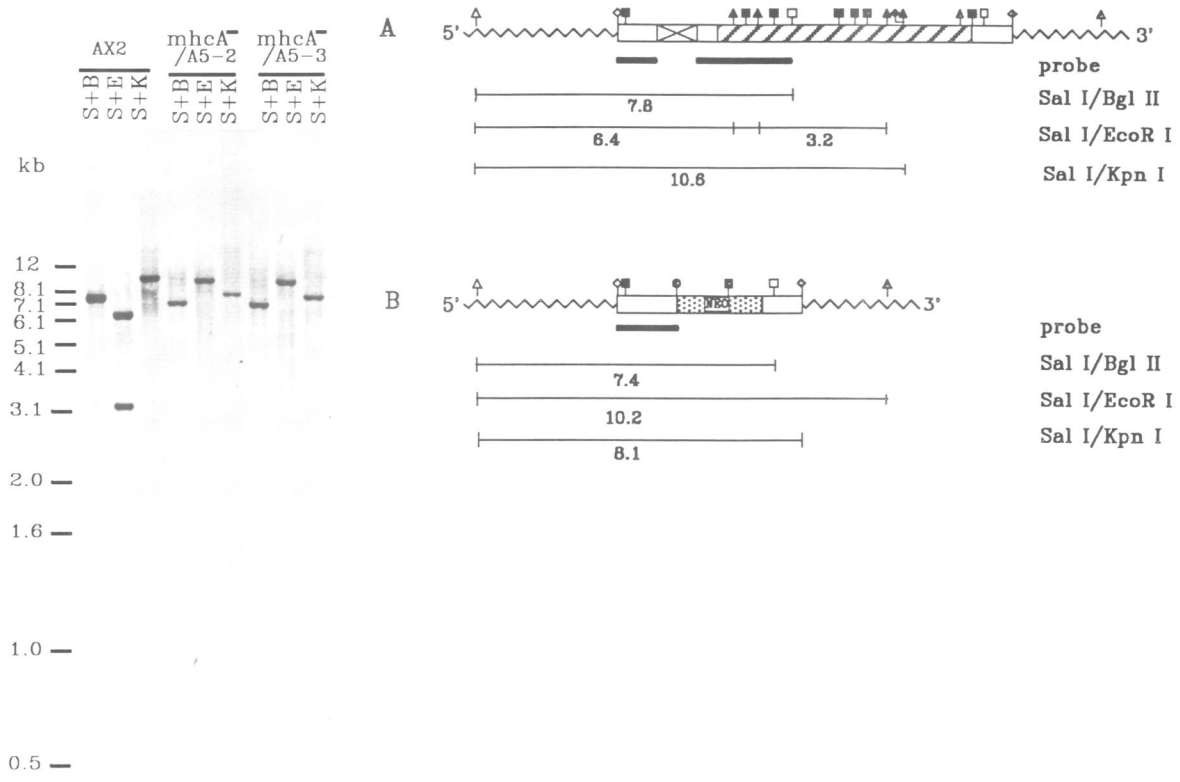


Fig. 5. Generation of *mhc*A⁻ cells by the integration of a single gene replacement fragment. A cloned 3.2 kb *Xba*I–*Bgl*II fragment containing 1.5 kb of 5′ flanking sequence and 1.7 kb of coding sequence was used as the probe. The probe was labeled with digoxigenin-dUTP by extension of random primers. Hybrids were detected using alkaline phosphatase conjugated antibodies. DNA was purified from untransformed *Dictyostelium* cells (AX2) and from cells transformed with pΔMHC-A5 using protocol 2. Two cell lines *mhc*A⁻/A5-2 and *mhc*A⁻/A5-3 derived from independent transformations with pΔMHC-A5 are shown. Both these transformants show the hybridization pattern expected for the replacement of the *mhc*A gene caused by the insertion of a single gene replacement fragment. A map of the wild type *mhc*A locus (**A**) and a schematic representation of the organization of the *mhc*A locus in cells which have their *mhc*A gene replaced by the insertion of a single copy of the gene replacement fragment from pΔMHC-A5 (**B**) is shown. The following abbreviations for restriction enzymes were used: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I, S, *Sal*I. All other symbols used are explained in Figure 1.

observed for untransformed cells. The defect in cytokinesis becomes particularly obvious when the *mhc*A− cells are transferred from Petri plates to suspension culture. Cells grow uniformly bigger and massive lysis starts to occur after 4–5 days. Therefore, we were unable to maintain these cells in continuous suspension culture in contrast to the parent strain. However, when returned to a surface, the multinucleated cells from a 3-day suspension culture can fragment giving rise to smaller cells and an increase in cell number (Figure 6).

Discussion

We report here the successful isolation of *mhc*A⁻ cell lines from *Dictyostelium* strains AX2 and AX4. The two gene replacement constructs that gave rise to these cell lines are different in their content of homologous sequences. The gene replacement fragment cloned into pΔMHC-B contains only *mhc*A coding sequences, while the fragment cloned into pΔMHC-A5 contains only flanking sequences, which are rich in AT-content. The smallest homologous region used in this study was 0.7 kb and none of the homologous regions exceeded 1.5 kb. The finding that transformation with both of these constructs yielded *mhc*A⁻ cells shows that there is no special region in the *mhc*A gene responsible for high frequency homologous recombination. The results reported here also indicate that there is no requirement for more extensive homology between the homing sequence and the target sequence in *Dictyostelium* than observed for yeast (Orr-Weaver *et al.*, 1983). Gene targeting, therefore, may be a general phenomenon in *Dictyostelium* and it should be possible to extend the principles we have used here to any other gene in the *Dictyostelium* genome. Indeed, homologous recombination has recently been reported in the *Dictyostelium* α-actinin gene (Witke *et al.*, 1987) and between unlinked non-replicating plasmid molecules (Katz and Ratner, 1988).

It was reported previously that a circular plasmid can integrate by homologous recombination in *Dictyostelium* with a frequency of at least 15–30% (De Lozanne and Spudich, 1987). Using the same conditions (protocol 1), transformation with linear DNA led to the isolation of *mhc*A⁻

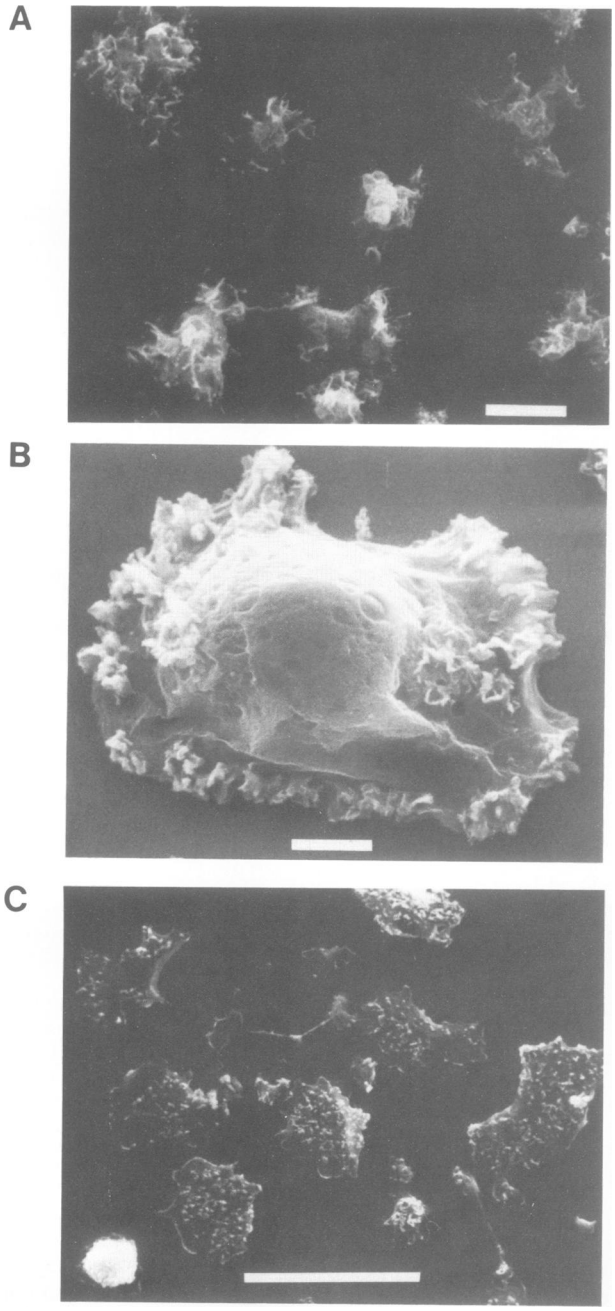


Fig. 6. Scanning electron micrographs of untransformed *Dictyostelium* cells and *mhc*A$^-$ cells. A defect in cytokinesis becomes particularly obvious when *mhc*A$^-$ cells (*mhc*A$^-$/B) are transferred from Petri plates to suspension culture. Cells grow uniformly bigger and will eventually lyse. When returned to a surface the multinucleated cells can fragment again giving rise to smaller cells and an increase in cell number. (**A**) Untransformed *Dictyostelium* cells grown in suspension were allowed to attach for 5 min to a cover slip before fixation. (**B**) Typical *mhc*A$^-$ cell as obtained after 3 days of growth in suspension. Cells were allowed to attach for 5 min to the cover slip before fixation. (**C**) Fragmentation of the large *mhc*A$^-$ cells could be observed when cells from 3-day suspension cultures were returned to a surface 4 h before fixation. Bar = 10 μm (A and B), 100 μm (C).

cells, but the frequency of tranformation was low (10^{-7}). Furthermore, Southern analysis showed that religation of cut plasmid had occurred leading to chromosomal insertion of multiple copies of the complete gene replacement plasmid. These results led us to search for conditions that would result in gene replacement by insertion of a single copy of the gene replacement plasmid, and with high frequency. Repeated attempts to transform *Dictyostelium* with gel-purified gene replacement fragments were not successful. All of these results suggested that more than one G418^R gene was required to confer drug resistance to the cell using protocol 1. We therefore reduced the G418 concentration in the event that protocol 1 was selecting for multiple insertions. Furthermore, we treated the linearized gene replacement plasmids with T4 DNA polymerase. This treatment might be expected to suppress the religation and recircularization of the transforming DNA, especially in the case of the *Kpn*I digested pΔMHC-A5 which would have a 3′ GTAC extension. Reduction of the G418 concentration in combination with the T4 DNA polymerase treatment (protocol 2) consistently resulted in frequencies of transformation comparable to those observed with circular DNA.

Many studies have indicated the importance of myosin function in cytokinesis (for a review see Warrick and Spudich, 1987). For example, immunofluorescence studies using *Dictyostelium* (Yumura *et al.*, 1984) and sea urchin blastomeres (Schroeder, 1987) demonstrate that myosin concentrates and disperses in the contractile furrow in coincidence with the beginning and end of the furrowing that leads to cell division. In other experiments, microinjection of antibodies directed against myosin was shown to disrupt cytokinesis (Mabuchi and Okuno, 1977; Kiehart *et al.*, 1982). The observations reported earlier for *Dictyostelium* (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987) and for yeast (Watts *et al.*, 1987), as well as those reported here, offer genetic proof that myosin is indeed required for normal cytokinesis.

The results reported here constitute conclusive genetic proof that the conventional myosin heavy chain gene is not an essential gene in *Dictyostelium*. This conclusion had been suggested previously by the finding that *Dictyostelium* cells expressing only a truncated form of myosin or very low amounts of myosin are viable (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987). The unexpected finding that those cells could perform many forms of cell movement contradicted the prevailing view of the function of myosin in non-muscle cells. However, it could not be excluded that the motility of those cells was due to the presence of the truncated myosin heavy chain or to the low amounts of myosin still present in those cells. Our current results rule out these possibilities.

An important feature of the *mhc*A$^-$ cells is that they are genetically stable. The complete absence of myosin in the *mhc*A$^-$ cells provides a system to study the structure and function of myosin. Transformation of the *mhc*A$^-$ cells with plasmids carrying altered forms of the *mhc*A gene provides a way to elucidate the critical features of the protein *in vivo*, by screening for restoration of the wild type phenotype, and *in vitro*, by purifying the recombinant forms of myosin and characterizing them with biochemical means. The *mhc*A$^-$ cells may also be used to identify the motors responsible for the various forms of cell movement that now can no longer be attributed to the conventional myosin. A candidate protein resembling myosin I from *Acanthamoeba* has already been purified from *Dictyostelium* (Côté *et al.*, 1985). The purification and identification of other actin dependent cellular motors should be greatly facilitated using *mhc*A$^-$ cells and will help to establish the role of these proteins in cell motility.

Materials and methods

General methods

The axenic AX2 and AX4 strain of *D.discoideum* were grown in suspension in HL5, as previously described (Spudich, 1982). DNA was prepared in the following manner: 5×10^8 cells were collected by centrifugation, washed twice in cold, sterile TE (10 mM Tris, pH 8.0, 1 mM EDTA) and resuspended in 6 ml of TE. Sarkosyl (BDH Chemicals Ltd., Poole, England) was added to 5% (v/v) and the tube was gently mixed a few times by hand. The DNA was then sequentially extracted with phenol, phenol:chloroform and chloroform, treated with 300 μg of RNase (Sigma Chemicals, St. Louis, MO, USA) for 1 h at 37°C, and then incubated with 150 μg of proteinase K (BMB, Indianapolis, IN, USA) for an additional 1 h at 37°C. The solution was extracted twice with phenol:chloroform and then ethanol precipitated.

Basic DNA manipulations were performed as described in Maniatis *et al.* (1982) and genomic blotting using the method of Southern (1975). The *Xba*I−*Bcl*I and *Xba*I−*Bgl*II fragments used for probing Southerns were obtained by digestion of pMyD (De Lozanne and Spudich, 1987). The *Xba*I−*Bcl*I fragment included 6.3 kb of the myosin coding sequence and the *Xba*I−*Bgl*II fragment included 1.4 kb of the myosin coding sequence. Both fragments also contained 1.5 kb of 5′ non-coding sequence (see Figures 3A and 5A). The 3.5 kb *Sal*I−*Xba*I fragment used for probing Southerns was obtained by the digestion of pΔMHC-3.5, a vector containing this fragment from λDdMHC-13 (De Lozanne *et al.*, 1988) cloned into pTZ-18R, and it included only 5′ non-coding sequence (see Figure 4A). The fragments of interest, in both cases, were gel-purified and labeled with [α-^{32}P]dATP (Amersham, Arlington Heights, IL, USA) by extension of random primers (BMB, Indianapolis, IN, USA). Alternatively, blots were probed using DNA randomly labeled with digoxigenin-dUTP and detection of hybrids was by enzyme immunoassay (BMB, Indianapolis, IN, USA).

Dictyostelium whole cell lysates were prepared as described by De Lozanne and Spudich (1987) and subjected to electrophoresis on a 7.5% separating and a 4% stacking SDS−polyacrylamide gel (Laemmli, 1970). Immunoblotting was performed according to Towbin *et al.* (1979), and the blots were probed with a rabbit polyclonal antibody directed against *Dictyostelium* myosin (Berlot *et al.*, 1985). Antibody binding was detected using a horseradish peroxidase conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, Richmond, CA, USA).

Scanning electron micrographs of critical point dried cells were obtained using a Philips SEM 505.

Plasmid constructs

The transformation vector pΔMHC-A5 was generated by first constructing pTZ-1.5 which contained a 1.5 kb *Xba*I−*Bam*HI fragment from the 5′ flanking region of pTZ-HMMB cloned into pTZ-18R (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ, USA). pTZ-HMMB is identical to pHMM-140 (De Lozanne and Spudich, 1987) except for introduction of a *Bam*HI site at the myosin start codon by oligonucleotide-directed mutagenesis [pTZ-HMMB and pTZ-S1 (see below) were constructed by E.M.McNally]. A 1 kb *Hin*dIII−*Kpn*I fragment from the 3′ flanking region was excised from pDE1 (De Lozanne, 1988). The *Hin*dIII site was blunted with Klenow enzyme and the fragment ligated into the *Sma*I−*Kpn*I sites of pTZ-1.5. The resultant plasmid, pTZ-D1, contains a single *Bam*HI site between the 5′ and 3′ flanking sequences of the *mhc*A gene. A 2.1 kb *Bam*HI−*Bgl*II fragment from pNEO MLS$^-$ was then ligated into the *Bam*HI site of pTZ-D1, producing the final construct, pΔMHC-A5. pNEO MLS$^-$ was derived from the vector pA15TX (Cohen *et al.*, 1986) which was digested with *Xba*I and *Bam*HI, blunted with Klenow enzyme and blunt-end ligated, recreating the BamHI site and removing the polylinker. The 2.1 kb *Bam*HI−*Bgl*II fragment from this vector contains the gene for neomycin resistance fused to the *Dictyostelium* actin-15 promoter.

The first steps in the generation of pΔMHC-B were the digestion of pTZ-18R with *Xba*I, filling in the overhang with Klenow enzyme and then digesting with *Bam*HI. A 665 bp fragment from pTZ-S1 was generated by first digesting with *Hin*dIII, blunting with Klenow enzyme, and subsequently digesting with *Bam*HI. This small fragment was then ligated into the digested pTZ-18R, creating pTZ-I which retained the *Xba*I site. pTZ-S1 contained a 2.6 kb fragment of the myosin head coding region from the *mhc*A gene created by the digestion of pTZ-HMMB with *Bam*HI and *Rsa*I and then ligating the fragment into the *Bam*HI and *Sma*I sites of pTZ-19R (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ, USA). The second plasmid, pTZ-II, was generated by first digesting pBgl-4.5 (De Lozanne *et al.*, 1988) with *Hin*dIII, blunting with Klenow enzyme, then digesting the *Kpn*I. This resulted in a 1.76 kb fragment which included the terminal 3′ coding region of the *mhc*A gene. pTZ-18R was then digested with *Xba*I, blunted with Klenow enzyme and subsequently digested with *Kpn*I. This 1.76 kb fragment from pBgl-4.5 was then ligated into the cut pTZ-18R, resulting in pTZ-II (which still had an intact *Xba*I site). The final construct, pΔMHC-B, was generated by a three-piece ligation of the *Sal*I−*Xba*I-cut pTZ-I with the 2.1 kb *Xba*I−*Bam*HI fragment of pNEO MLS$^-$ (containing the neomycin resistance) and an 888 bp *Sal*I−*Xho*II fragment from pTZ-II.

Dictyostelium transformation

Transformation of *Dictyostelium* was either performed as described by De Lozanne and Spudich (1987) (protocol 1) or by following a modified protocol (protocol 2). The following modifications were used in protocol 2: following digestion with the appropriate restriction enzymes to liberate the replacement fragment the DNA used for transformation was incubated for 5−8 min with T4 DNA polymerase (1 unit/20 μg of DNA) in the presence of a single deoxynucleotidetriphosphate (50 μM dTTP). Cultures of AX2 were grown to a density of $2-4 \times 10^6$ cells/ml. Approximately 3 h before transformation, 2×10^7 cells were transferred to 10 cm plastic Petri dishes and allowed to attach for 10 min. The medium was removed and replaced with Bis-Tris-HL5 (pH 7.1). The calcium phosphate co-precipitation procedure and the length of the glycerol shock remained unchanged but the concentration of glycerol was reduced to 15%. Cells were allowed to recover for 18−24 h, and then the media was replaced with HL5 (pH 6.5) containing G418 at 6 μg/ml every 24 h for the next 3 days. From day 5 on, the G418 concentration in the media was increased to 10 μg/ml and the media changed only every third day. Following the appearance of colonies, around the sixth day, cells from these colonies were removed with a pipet and directly transferred to 24-well microtiter plates. These cells were then screened for the absence of myosin by immunoblotting, and colonies which were derived from a single cell were selected by dilution into 96-well microtiter plates.

Acknowledgements

The authors would like to thank Beth McNally (Department of Microbiology and Immunology, Albert Einstein College of Medicine) for constructing the plasmids pTZ-HMMB and pTZ-S1. We thank Ken Niebling for help with the preparation of the figures and Dr R.M.Marshall for help with the preparation of cells for scanning electron microscopy. This research was supported by grant GM40509 (J.A.S.) and post-doctoral fellowship GM12000 (M.A.T.) from the National Institutes of Health. D.J.M. was a recipient of a fellowship from the Deutsche Forschungsgemeinschaft (Ma 1081/2-1). This paper is dedicated to Professor Arthur Kornberg on the occasion of his 70th birthday.

References

Berlot,C.H., Spudich,J.A. and Devreotes,P.N. (1985) *Cell*, **43**, 307−314.
Binninger,D.M., Skrzynia,C., Pukkila,P.J. and Casselton,L.A. (1987) *EMBO J.*, **6**, 835−840.
Cohen,S.M., Knecht,D., Lodish,H.F. and Loomis,W.F. (1986) *EMBO J.*, **5**, 3361−3366.
Côté,G.P., Albanesi,J.P., Takashi,U., Hammer,J.A. and Korn,E.D. (1985) *J. Biol. Chem.*, **260**, 4543−4546.
Datta,S. and Firtel,R.A. (1987) *Mol. Cell. Biol.*, **7**, 149−159.
De Lozanne,A. (1988) Ph.D. Thesis, Stanford University.
De Lozanne,A., Lewis,M., Spudich,J.A. and Leinwand,L.A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6807−6810.
De Lozanne,A. and Spudich,J.A. (1987) *Science*, **236**, 1086−1091.
De Lozanne,A., Warrick,H.M., Chasan,R., Leinwand,L.A. and Spudich,J.A. (1988) In Satir,P., Condeelis, J.S. and Lazarides,E.A. (eds) *Proc. UCLA Symp. Signal Transduction in Cytoplasmic Organization and Cell Motility*. Alan R.Liss Inc., New York, pp. 279−286.
Firtel,R.A., Cockburn,A., Frankel,G. and Hershfield,V. (1976) *J. Mol. Biol.*, **102**, 831−852.
Firtel,R.A., Silan,C., Ward,T.E., Howard,P., Metz,B.A., Nellen,W. and Jacobson,A. (1985) *Mol. Cell. Biol*, **5**, 3241−3250.
Gerisch,G. (1987) *Annu. Rev. Biochem.*, **56**, 853−879.
Katz,K.S. and Ratner,D.I. (1988) *Mol. Cell. Biol.*, **8**, 2779−2786.
Kiehart,D.P., Mabuchi,I. and Inoue,S. (1982) *J. Cell Biol.*, **94**, 165−178.
Knecht,D.A., Cohen,S.M., Loomis,W.F. and Lodish,H.F. (1986) *Mol. Cell. Biol.*, **6**, 3973−3983.
Knecht,D.A. and Loomis,W.F. (1987) *Science*, **236**, 1081−1086.
Laemmli,U.K. (1970) *Nature*, **227**, 80−81.
Lemmon,S.K. and Jones,E.W. (1987) *Science*, **238**, 504−509.
Mabuchi,I. and Okuno,M. (1977) *J. Cell Biol.*, **74**, 251−263.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Nellen,W., Datta,S., Reymond,C., Siversten,A., Mann,S., Crowley,T. and

Firtel,R.A. (1987) In J.A.Spudich (ed.), *Methods in Cell Biology, Volume 8. Dictyostelium discoideum*. Academic Press, New York, pp. 67–100.
Orr-Weaver,T.L., Szostak,J.W. and Rothstein,R.J. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6354–6358.
Orr-Weaver,T.L., Szostak,J.W. and Rothstein,R.J. (1983) *Methods Enzymol.*, **101**, 228–245.
Payne,G.S., Hasson,T.B., Hasson,M.S. and Schekman,R. (1987) *Mol. Cell Biol.*, **7**, 3888–3898.
Pears,C.J. and Williams,J.G. (1987) *EMBO J.*, **6**, 195–200.
Rothstein,R.J. (1983) *Methods Enzymol.*, **101**, 202–211.
Schatz,P.J., Solomon,F. and Botstein,D. (1984) *Mol. Cell Biol.*, **6**, 3722–3733.
Schroeder,T.E. (1987) *Dev. Biol.*, **124**, 9–22.
Shortle,D., Novick,P. and Botstein,D. (1986) *Proc. Natl. Acad. Sci. USA*, **81**, 4889–4893.
Smithies,O., Gregg,R.G., Boggs,S.S., Koralewski,M.A. and Kucherlapati,R.S. (1985) *Nature*, **317**, 230–234.
Song,K.-Y., Schwartz,F., Maeda,N., Smithies,O. and Kucherlapati,R. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6820–6824.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.
Spudich,J.A. (1982) *Methods Cell Biol.*, **25** (part B), 359–364.
Spudich,J.A. (ed.) (1987) *Methods in Cell Biology Volume 8, Dictyostelium discoideum*. Academic Press, New York.
Thomas,K.R., Folger,K.R. and Capecchi,M.R. (1986) *Cell*, **44**, 419–428.
Tilburn,J., Scazzocchio,C., Taylor,G.G., Zabiky-Zissman,J.H., Lockington,R.A. and Davies,R.W. (1983) *Gene*, **26**, 205–221.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
Warrick,H.M., De Lozanne,A., Leinwand,L.A. and Spudich,J.A. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9433–9437.
Warrick,H.M. and Spudich,J.A. (1987) *Annu. Rev. Cell Biol.*, **3**, 379–421.
Watts,F.Z., Shiels,G. and Orr,E. (1987) *EMBO J.*, **6**, 3499–3505.
Witke,W., Nellen,W. and Noegel,A. (1987) *EMBO J.*, **6**, 4143–4148.
Yelton,M.M., Hames,J.E. and Timberlake,W.E. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1470–1474.
Yumura,S., Mori,A. and Fukui,Y. (1984) *J. Cell Biol.*, **99**, 894–899.