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The yeast *MYO1* gene encoding a myosin-like protein required for cell division

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A yeast gene *MYO1* that contains regions of substantial sequence homology with the nematode muscle myosin gene (*unc*54) has been isolated and sequenced. Although the disruption of *MYO1* is not lethal, it leads to aberrant nuclear migration and cytokinesis. The 200-kd myosin heavy chain-like protein, the product of *MYO1*, cross-reacts with anti-nematode myosin heavy chain IgG and is present in wild-type strains but not in strains carrying the disrupted gene. Instead, a truncated polypeptide with a molecular mass of 120 kd can be detected in some *myo1* mutants.

Key words: myosin gene/yeast/myosin-deficient mutants/nuclear segregation/cytokinesis

Introduction

Cytoskeletal proteins have been found in a wide range of eukaryotic cells and, in general, are highly conserved. Amongst such proteins, myosin, actin and tubulin have been discovered in yeast (Watts *et al.*, 1985; Koteliansky *et al.*, 1979; Baum *et al.*, 1978) and the genes encoding yeast actin and tubulin have been cloned and sequenced (Ng and Abelson, 1980; Gallwitz and Sures, 1980; Neff *et al.*, 1983; Botstein, personal communication).

The overall secondary structures of myosin heavy chain (MHC) proteins from both muscle and non-muscle cells are generally very similar and consist of a globular head region containing an ATP-binding site and active thiol region, and an α-helical rod. Sequence comparison of the nematode and rabbit proteins has underlined the extensive homology between the head regions. The rod portions of these proteins are much less homologous though both have been predicted to form α-helical structures.

We have previously reported that DNA sequences from the nematode MHC gene hybridize to yeast genomic DNA, suggesting sequence conservation in the two organisms (Watts *et al.*, 1985). Here, we report the cloning and partial sequence analysis of the myosin heavy chain-like gene *MYO1* from the yeast *Saccharomyces cerevisiae*. We also describe the possible physiological role of the yeast MHC protein.

DNA sequence analysis of the yeast myosin-like gene demonstrates that it contains regions which have been conserved throughout evolution. To study its *in vivo* role, the *MYO* gene was replaced with a mutated allele by disrupting the wild-type gene *in vitro* and transforming it into yeast cells. Although *myo* mutants are viable, their nuclei do not migrate normally. This results in incomplete cell division with the cells forming clusters. The mutants are osmotically sensitive and seem to overproduce actin-binding proteins. This overproduction may underlie the survival of cells in the absence of the *MYO* gene product.

Results

Southern blot analysis

We have previously shown that a DNA probe encoding the ATP-binding site of the nematode myosin heavy chain gene hybridizes to yeast genomic DNA (Watts *et al.*, 1985). This probe (probe 1) as well as two additional nematode MHC DNA probes 2 and 3 (Karn *et al.*, 1983) were hybridized to S288C (Table I) genomic DNA digested with *Bam*HI (B) and *Eco*RI (E) (Figure 1). Probe 1 hybridized to several fragments suggesting either that there is

Table I.

Strain	Genotype	Source
S. cerevisiae		
S288C	*SUC2, mal, gal2, CUP1*	D.Mortimer
DBY747	*Matα, his3, leu2, ura3, trp1*	D.Botstein
83	*Mata, spo11, ura3, can1, cyh2, ade2, his7, hom3, tyr1*	M.Esposito
85	*Mata, spo11, ura3, ade6, arg4, aro7, asp5, met14, lys2, pet17, trp1*	M.Esposito
87	*Mata, spo11, ura3, his2, leu1, lys1, met4, pet8*	M.Esposito
89	*Mata, spo11, ura3, ade1, his1, leu2, lys7, met3, trp5*	M.Esposito
471	*Mata, cdc12, ade1, ade2, ura1, his7, lys2, tyr1, gal1*	L.Hartwell
471L	*cdc12, leu2*	Progeny of cross carried out in this laboratory by crossing 471 × DBY747
S150-2B	*Matα, leu2, his3, trp1, ura3*	Cold Spring Harbor Laboratory
MC16	*Mata, ade2, his4, leu2, SUF2*	J.D.Beggs
MCS150	*Mata/α, leu2/leu2, HIS3/his3, TRP1/trp1, URA3/ura3, ADE2/ade2, HIS4/his4, SUF2*	Diploid produced in this laboratory by crossing MC16 × S150-2B
L1442	*Mata, leu2, pet2, arg4, ade8, trp4, aro1C, rna3*	Cold Spring Harbor Laboratory
842	*Mata/α, ade2-1/ade2-1, trp1-1/trp1-1, leu2-3/leu2-112, his3-11/his3-15, ura3/ura3, can^{R}1-100*	K.Nasmith
842-1	*ade2-1, trp1, LEU2/leu2, his3, ura3, myo1*	*Haploid produced in* this laboratory
E. coli		
JM83	*ara, Δ(lac-pro), rpsL, thi1, supE44, φ80dacI, ZΔM15*	J. Messing
JM101	*Δ(lac-pro), thi1, supE44,* F′ *traD36, proAB, ZΔM15*	J.Messing

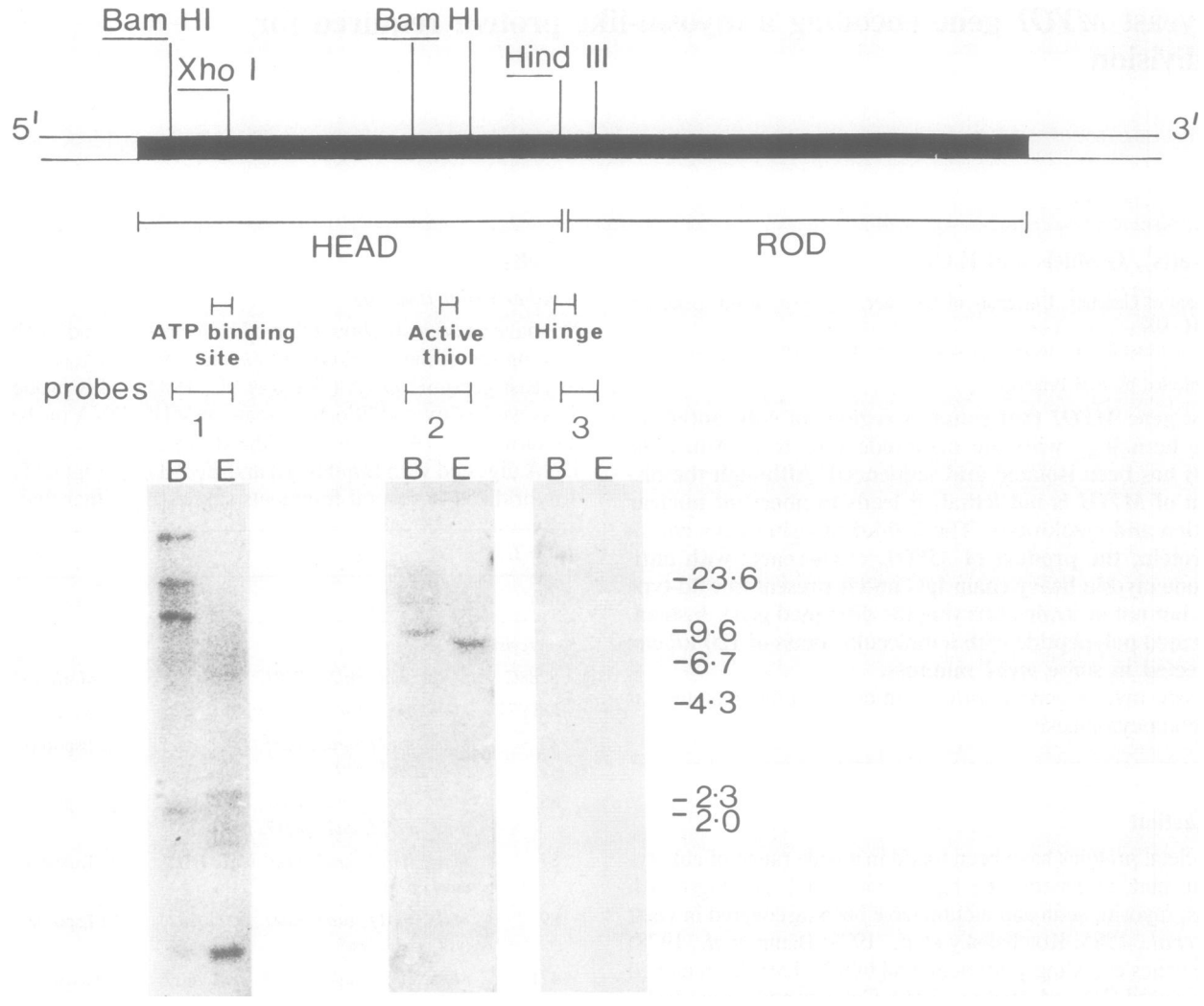

Fig. 1. Southern blots of nematode MHC probes hybridized to yeast genomic DNA. Yeast genomic DNA from strain S288C was digested with either *Bam*HI (B) or *Eco*RI (R), subjected to electrophoresis on a 0.6% agarose gel and blotted to nitrocellulose. The filter was hybridized in 0.3 M NaCl/0.03 M sodium citrate at 60°C, with one of three nematode myosin heavy chain gene (*unc*54) DNA probes. The solid box indicates the nematode myosin heavy chain gene (introns not shown). The probes were (**1**) a *Bam*HI−*Xho*I fragment encoding the ATP-binding site, (**2**) a *Bam*HI fragment encoding the active thiol region and (**3**) a *Hin*dIII fragment encoding the hinge region of the MHC protein. *Hin*dIII-digested λ DNA was used as size markers.

more than one MHC gene in yeast or that this probe is not specific for the yeast myosin gene. The DNA used as probe 2 contains part of the *unc*54 gene encoding the active thiol region of the MHC protein. It hybridized to a single *Eco*RI fragment (8 kb) and to a single *Bam*HI fragment (~10 kb) suggesting that this probe may be more specific for the yeast myosin gene. The third DNA probe (probe 3) encoding the hinge region of the protein did not hybridize to yeast DNA under the same conditions.

Construction and screening of a yeast genomic library

A yeast genomic library was constructed in λL47.1 and duplicate filters of the 10^5 recombinant clones were screened with either probe 1 or probe 2 (see Figure 1). As anticipated from Southern blot analysis, probe 1 produced four times as many positive signals (>100) as probe 2 (30). Six positive clones identified with probe 2 were retained for further study. Mapping by restriction endonuclease digestion demonstrated that these clones contained overlapping DNA fragments (see restriction map in Figure 2).

Sequence analysis

A 6.8-kb *Pst*I−*Bam*HI fragment (Figure 2) hybridizing to probe

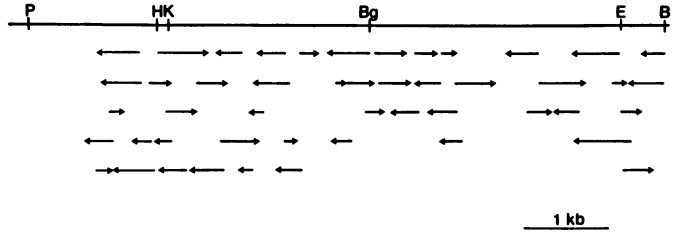

Fig. 2. Restriction map of the yeast myosin gene, indicating regions sequenced using the dideoxy chain termination method. Bar indicates 1 kb. H = *Hin*dIII, P = *Pst*I, K = *Kpn*I, Bg = *Bgl*II, E = *Eco*RI, B = *Bam*HI.

2 was digested with either *Alu*I, *Sau*3A or *Taq*1 and subcloned into M13 mp18. This fragment encodes a protein with a molecular mass of ~200-kd in an *in vitro* transcription translation system prepared from *Escherichia coli* (Orr, submitted). In addition, it encodes a 200 kd protein in yeast that is recognized by immunoglobulins raised against nematode myosin heavy chain (see below). The dideoxy chain termination method (see Figure

Fig. 3. Sequence analysis of the yeast myosin heavy chain gene. The first ATG of the longest open reading frame is taken as position +1.

2) was employed to determine the DNA sequence of the 6.8-kb *Pst*I− *Bam*HI fragment. The DNA sequence and the predicted amino acid sequence of the 5′ end region of the putative gene is shown in Figure 3. There are two in-frame ATG codons near the 5′ end of the identified open reading frame (ORF) (at +1 and +30) both with the required A at position −3 from the ATG. The sequence CAACAA is present nine nucleotides upstream from the first ATG. This sequence is often found at the 5′-untranslated region of yeast genes (Kingsman *et al.*, 1983), suggesting that initiation occurs at the first ATG. Possible TATA boxes for the yeast gene are TAATT at −175 and TAATTT at −158. Dot matrix analysis comparing the predicted amino acid sequence in the N-terminal region of the yeast protein with that of the nematode MHC protein is shown in Figure 4. It reveals a substantial homology throughout the head sequence (40% overall).

In a detailed study of the head region, the amino acid sequence of the ATP-binding site and the active thiol regions from different organisms were compared (Figure 5). In the ATP-binding site region there is a substantial homology around a glycine-rich sequence. Similar glycine-rich sequences are present in nucleotide binding sites of many different enzymes (McLachlan, 1984). The active thiol region in both the nematode and the rabbit MHC proteins contains two cysteine residues (McLachlan, 1984). In

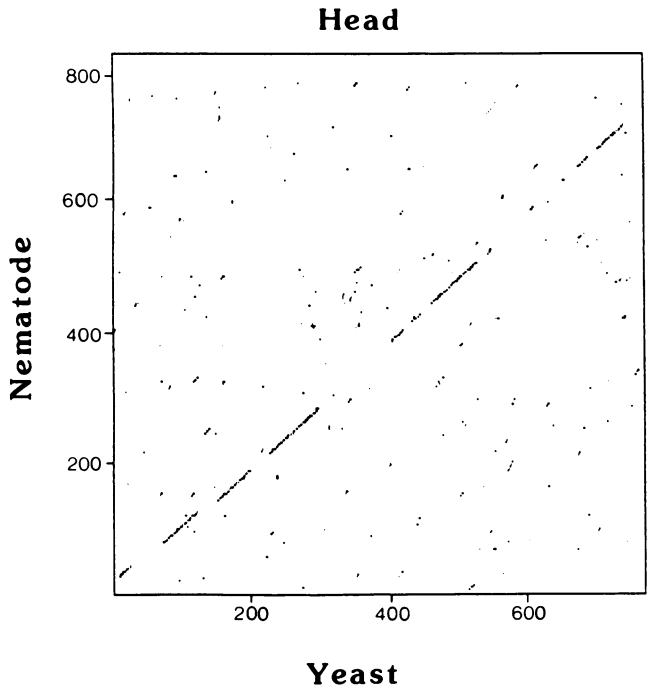

Fig. 4. Dot matrix comparison of yeast and nematode myosin heavy chain protein sequences. Dot matrix analysis of the head regions of the yeast and nematode MHC protein sequences was performed using the Staden program, with a match figure of 8/11.

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                    ATP-binding site

YEAST       PPHIFAIAEEAYENLLSEGKDQSILVTGESGAGKTENTK

NEMATODE    ---L--VSD---R-M-QDHEN--M-I-------------

RABBIT      -----S-SDN--QFM-TDREN----I---------V---

            Active thiol region

YEAST                   VIQLRCNGVLEGIRLARGYPS
DICTYOSTELIUM           L------------IT-KGFP
NEMATODE                ---T---------IC-KGFP
RABBIT                  HF-----------IC-KGFP

                          |           |
                         SH2         SH1
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Fig. 5. Comparison of the ATP-binding sites and active thiol regions from different organisms. A nucleotide match is indicated as −. SH1 and SH2 refer to the active thiol groups (McLachlan, 1984).

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   defgabc defgabc defgabc defgabc

   DVTLEKN SNIAISR LQSLVTE NSDLRSK
   NENFKKE KAALNNQ LKNKESE LLKMKEK
   IDNHKKE LATFSKQ RDDAVSE HGKITAE
   SKETRIQ LTEYNLI YQKIKEE YSNFQTR
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Fig. 6. Part of the yeast myosin rod sequence. The sequence is arranged in four sets of seven amino acids. The letters in italics refer to equivalent positions in the proposed coil.

the yeast sequence however this region is more similar to that of the *Dictyostelium* MHC protein, having only one cysteine, SH2 (Warrick *et al.*, 1986). The significance of the absence of the SH1 cysteine in these proteins is as yet unclear.

The predicted N-terminal sequence of the yeast gene product

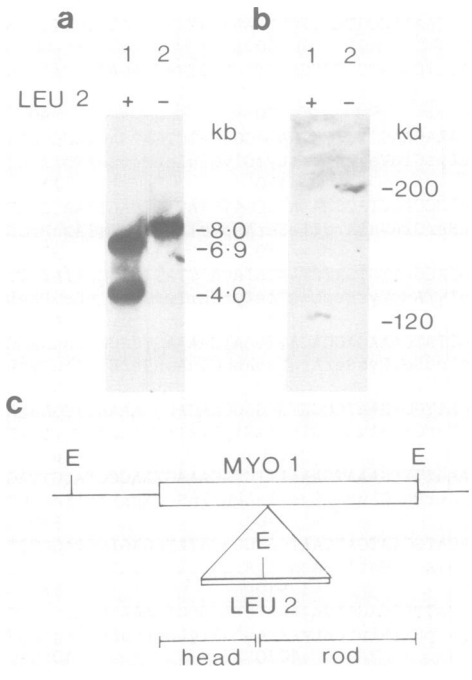

Fig. 7. Disruption and replacement of the yeast myosin heavy chain gene. Strain MCS150 was transformed with a disrupted yeast *myo*1 gene and allowed to sporulate. Spores from dissected tetrads were tested for growth on leucine-deficient medium; +, growth in the absence of leucine; −, no growth in the absence of leucine. (**a**) Genomic DNA from the two spores was digested with *Eco*RI (E) and subjected to electrophoresis through a 0.6% gel, blotted to nitrocellulose. The filter was hybridized at 65°C in 0.3 M NaCl/0.03 M sodium citrate with a ^{32}P-labelled *Pst*I−*Eco*RI yeast *myo1* DNA probe. (**b**) Protein from crude extracts of the same two spores was subjected to the procedure for the preparation of actomyosin complexes (Pollard and Korn, 1973; Watts *et al.*, 1985), run on a 5% SDS−polyacrylamide gel and blotted to nitrocellulose using the method of Bowen *et al.* (1980). The filter was then incubated with polyclonal anti-nematode MHC IgG (MacLeod *et al.*, 1981) followed by goat anti-rabbit IgG peroxidase. (**c**) Diagram showing the construction of the disrupted myosin gene. The *LEU2* gene on a *Bgl*II fragment was cloned into the *Bgl*II site in the rod portion of the yeast MHC gene.

again shows similarity to the *Dictyostelium* MHC protein rather than to the nematode or rabbit proteins. In particular, it does not contain the loop sequences (lysine-rich stretches) which in rabbit and nematode proteins separate the 25-, 50- and 20-kd regions of the head (Warrick *et al.*, 1986).

The yeast DNA sequence which presumably encodes the rod portion of the myosin-like protein, the C-terminal region, does not show any homology with the nematode MHC protein as determined by dot matrix analysis. A similar observation was reported for the rod portion of the *Dictyostelium* protein (De Lozanne *et al.*, 1985; Warrick *et al.*, 1986). The rod regions of the MHC proteins so far studied consist of repeating units made up of four sets of seven amino acids with a distinctive arrangement of hydrophobic and charged amino acids (McLachlan and Karn, 1982). This arrangement permits the formation of a coiled coil structure comprising two myosin peptides. Figure 6 shows a short protein sequence predicted from the yeast gene for the C-terminal region. The sequence appears to consist of a 28-amino acid repeat unit. Amino acids b, c and f are highly charged, whilst a high proportion of the amino acids a and d are the hydrophobic amino acids Ala, Val and Leu. The predicted C-terminal sequence of the yeast protein contains no proline residues and very few glycine residues, both of which are strong α-helix breakers (Chou and

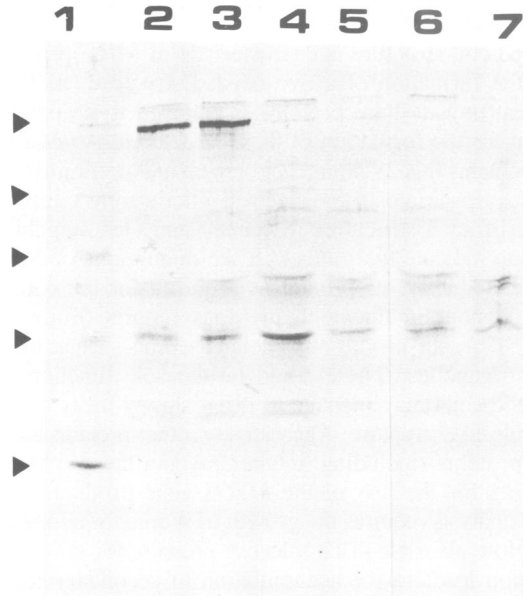

Fig. 8. Immunoprecipitation of myosin. Myosin-like proteins were precipitated from cells labelled with [^{35}S]methionine, run on 10% SDS acrylamide gels and autoradiographed. (**1**) Mol. wt markers, from top: myosin heavy chain (200 kd), phosphorylase b (92.5 kd), bovine serum albumin (69 kd), ovalbumin (946 kd), carbonic anhydrase (30 kd). (**2**) Wild-type strain (842). (**3**) 842-1 (*myo*) transformed with *MYO*. (**4**) 842-1 (*myo*) incubated with anti-myosin and anti-tubulin IgG. (**5**) 842-1 (*myo*). (**6**) Wild-type (842) treated with pre-immune serum. (**7**) 842-1 (*myo*) incubated with pre-immune serum.

Fasman, 1974). This further suggests that the C-terminal part of this protein can form an α-helical structure.

Various yeast DNA probes carrying either the ATP-binding site or the active thiol region were hybridized to yeast genomic DNA. The detection of a single band, under different conditions of stringency, strongly suggests that this gene is present as a single copy in yeast (Figure 7).

Northern blot analysis using a DNA fragment carrying the active thiol region as a probe yielded a transcript of ~5.7 kb. This size of RNA is consistent with the predicted amino acid sequence of the yeast gene and with the size of the yeast myosin heavy chain protein (~200 kd) previously described (Watts *et al.*, 1985).

Gene disruption

Gene disruption experiments have been carried out on several yeast genes to determine whether their products are essential for cellular growth. In a similar experiment, the yeast *LEU2* gene (on a *Bgl*II fragment) was cloned into the *Bgl*II site of the yeast myosin-like gene (Figure 7c). To avoid possible lethality resulting from the disruption of the gene, we transformed the diploid strain MCS150 (Table I) with a 9.3 kb *Pst*I–*Bam*HI fragment containing the disrupted gene. Three LEU2$^+$ transformants were plated onto sporulation medium and 12 tetrads from each transformant were disected. In 33/36 tetrads all the four spores were viable and the LEU2$^+$ segregated 2$^+$:2$^-$. Southern blot analysis of spores from dissected tetrad demonstrates that in the two *LEU2*$^+$ spores the wild-type myosin-like gene has been replaced by the disrupted sequence (Figure 7a). This result suggests that the disruption of the yeast myosin-like gene in the putative rod region does not confer lethality. Gene disruption experiments with the myosin-like gene containing a deletion at the 5′ end (the 2.3-kb *Kpn*I–*Bgl*II fragment, see Figure 2) also yielded viable spores. When germinated, the cells appeared morphologically similar to the tail-disrupted mutants (see below). They did not contain, however, any polypeptide which could be recognized by the anti-nematode MHC IgG (see below).

Expression of MYO in yeast

Actomyosin complexes from spores carrying the disrupted myosin-like gene were prepared to determine whether they contained the myosin heavy chain-like protein, previously identified in yeast (Watts *et al.*, 1985). Actomyosin proteins were analysed by acrylamide gel electrophoresis and immunoassays of Western blots using anti-nematode myosin heavy chain IgG. Two actin-binding proteins of ~200 kd were detected in the wild-type strains (Shiels and Orr, unpublished). The slightly smaller protein has been shown to react with anti-nematode MHC IgG in wild-type strains (Watts *et al.*, 1985; Figure 7b, lane 2) but was absent in all strains carrying the disrupted gene. A truncated protein of ~120 kd which cross-reacted with the anti-nematode MHC IgG was nevertheless detected in the tail-disrupted mutants (Figure 7b, lane 1).

Immunoprecipitation of myosin from total cell proteins failed to detect any myosin-like polypeptide in the *myo* mutants carrying a deletion in the 5′ end of the gene (Figure 8, lanes 4 and 5). One of these *myo* mutants was transformed with the entire *MYO* gene and fast growing colonies were selected at 37°C. A transformant that was shown by DNA hybridization to regain the wild-type allele also produced the 200-kd protein which could be precipitated by the anti-myosin immunoglobulins (Figure 8, lane 3). The presence of the wild-type allele further restored the wild-type phenotype of the *myo* mutants.

Cell division

Strains carrying the disrupted myosin gene grow much slower than the wild-type strains and form chains or broad sheets because of an incomplete cell division (Figure 9a). Most cells appear larger than either haploid or diploid wild-type strains and are distorted and sensitive to hypotonic solutions. Multiple buds often emerge from various regions of the cell. DAPI staining reveals that many cells do not contain nuclei while others have several, often clustered together (Figure 9b), suggesting that nuclear migration is affected in these strains. This interpretation was supported by indirect immunofluorescence with anti-tubulin IgG which reveals a random orientation of the microtubules (to be published elsewhere).

2 μm-mediated chromosome loss mapping

Preliminary immunofluorescence experiments (Watts *et al.*, 1985 and our unpublished data) have indicated that yeast myosin may be associated with the 10 nm ring structure present between mother cell and bud. These rings are absent in some genetically characterized cell cycle (*cdc*) mutants (Byers and Goetsch, 1976). Chromosomal mapping of the yeast myosin gene was used to determine whether this gene mapped to the same locus as any of these *cdc* mutations. The *Hin*dIII–*Bgl*II fragment of the yeast myosin gene was subcloned into Yep13 (pFW21). This plasmid enabled us to use the 2 μm-mediated chromosome loss procedure (Falco and Botstein, 1983). The plasmid was linearized by digestion with *Kpn*I and used to transform DBY747.

Stable LEU2$^+$ transformants were crossed with four strains containing *spo11* and several auxotrophic markers (Table I). 16/48 diploids obtained in crosses with strain 85 (Table I) were unable to grow in the absence of arginine, indicating the loss of chromosome VIII.

To map the location of the gene on chromosome VIII more

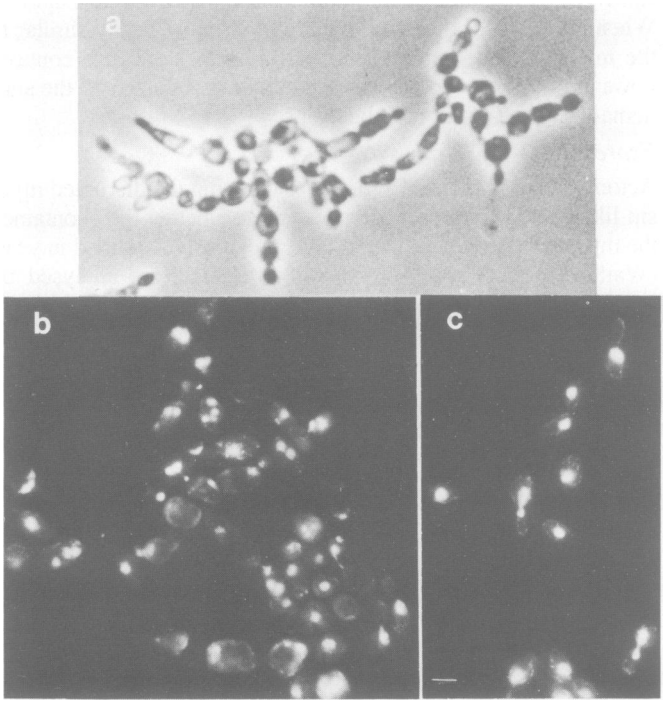

Fig. 9. Cell division and nuclei staining. Haploid strain carrying the head disrupted *myo1* gene (Δ *Kpn*I−*Bgl*II 2.3-kb fragment) was stained with DAPI and photographed under u.v. light. (**a**) Non-stained and (**b**) DAPI stained *myo1* mutant cells; (**c**) DAPI stained wild-type diploid strain (842). The bar represents 5 μM.

precisely, a haploid strain containing the disrupted myosin gene was crossed with strains L1442 and 471L (Table I). Tetrad analysis located the gene ~ 80 centimorgans from *cdc12* and 30 from *arg4*. This region does not contain any of the known *cdc* mutations associated with the 10 nm filaments present at the neck of budding cells.

Discussion

A myosin heavy chain (MHC)-like gene has been isolated from a yeast genomic library using DNA sequences encoding nematode MHC as a probe. The protein sequence predicted from the DNA sequence of this gene shows substantial homology with the head region of other MHC proteins. The product of this gene is recognized by immunoglobulins raised against nematode myosin heavy chain. For these reasons we have named the yeast gene *MYO1*.

Comparison of the yeast and the nematode MHC protein sequences demonstrates that variations in the head sequence occur at the C termini of the 25-, the 50- and the 20-kd segments which comprise the nematode MHC head. In cross-linking and photochemical labelling experiments, various functions have been assigned to each segment (Mornet *et al.*, 1981; Szilagyi *et al.*, 1979). The 25-kd segment contains the active site for ATP hydrolysis, while the 50- and the 20-kd segments are thought to bind actin and myosin light chains respectively. Comparing sequences of the yeast *MYO1* and the nematode 50-kd segment reveals a highly conserved region (73%) extending from amino acid 458 (Tyr) to amino acid 527 (Glu) in yeast, suggesting a common involvement in the binding of actin which is highly conserved in all eukaryote cells. Comparing the yeast *MYO1* gene and the nematode 20-kd segment did not reveal a recognizable light chain binding site. However, as myosin light chains show considerable diversity their binding sites also may vary and hence would be difficult to recognize.

A coiled coil structure is characteristic of MHC proteins and permits the formation of a two headed structure. A 28-amino acid repeat unit in these proteins is believed to be particularly important for the formation of these structures. A yeast rod sequence contains this 28-amino acid repeat and preliminary analysis of the yeast myosin by electron microscopy confirms the existence of such structures (Sweeney and Orr, unpublished).

Our data indicate that, although the disruptions of *MYO1* do not confer lethality, its product is required for the completion of cell division, in line with previous reports (Fujiwara and Pollard, 1976), but also for other cellular events such as the movement of organelles. These could be the sole functions of the protein and cells may manage to grow slowly in its absence in a syncytium-like structure. Alternatively, other proteins e.g. actin-binding proteins (including another myosin heavy chain) may compensate for the loss of the *MYO1* gene product.

Tetrad analysis requires the growth of spores for a long period. It is therefore plausible that a selective pressure for growth during germination leads to the accumulation of secondary mutations or to the increase in the expression of particular genes, compensating for the loss of the *MYO1* gene product so enabling these mutants to survive. The overproduction of several actin-binding proteins in the *myo1* strains (Shiels and Orr, unpublished) supports this possibility. One of these proteins is a cytoskeleton protein, albeit with a different cellular localization from that of myosin (Shiels and Orr, unpublished).

Finally, the abnormal segregation of nuclei and the formation of multiple buds in *myo* mutants are consistent with previous indications that yeast nuclei are physically attached to structures like the 10 nm filaments, localized between mother and bud (Byers, 1981). Such interaction could be crucial for normal mitosis and cell division.

Materials and methods

DNA and strains

*Bam*HI-digested λ*L*47.1 and *S. cerevisiae* S288C DNA were a gift from A. Blanchetot and P.Meacock respectively. Nematode *unc*54 DNA probes were a gift from J.Karn (Karn *et al.*, 1983). Anti-tublin antibodies were kindly donated to us by J.Kilmartin. Yeast and *E. coli* strains are shown in Table I.

Construction and screening of a yeast genomic library

The yeast genomic library contained partially *Sau3*A-digested genomic DNA from the yeast strain S228C inserted into *Bam*HI-digested bacteriophage λ*L*47.1 DNA. It was screened according to Benton and Davis (1977), using nematode MHC gene DNA probes. The DNA was labelled with [^{32}P]dCTP using the oligo-labelling method of Feinberg and Vogelstein (1984) (see text). Hybridization was carried out at 60°C in 0.3 M NaCl/0.03 M sodium citrate.

DNA sequence determination

The DNA sequence was determined by the dideoxy chain termination method (Sanger *et al.*, 1977; Messing *et al.*, 1980), using [α-^{35}S]dATP.

Transformation and microbiological procedures

Yeast transformtion was carried out as described by Lee *et al.* (1984). Yeast tetrad analysis was by the method of Mortimer and Hawthorne (1969), and gene disruption experiments were performed as described by Rothstein (1984). 2 μm-mediated chromosome loss mapping was carried out as described by Falco and Botstein (1983) using tester strains 83, 85, 87 and 89 (Table I).

Immunoprecipitation

Cells were labelled with [^{35}S]methionine and lysed by breaking them with glass beads. 5 μl of antiserum was added to 50 μl of cell extract and left on ice for 90 min. Immuno-complexes were precipitated with *Staphylococci* A cells and washed with 50 mM Tris pH 7.5 containing 150 mM NaCl, 0.1% bovine serum albumin, 0.1% SDS, 1% deoxycholate and 1% NP-40. The pellets were analysed by 10% SDS−PAGE followed by autoradiography.

Nuclei staining

Exponentially grown yeast were fixed at room temperature with 3% (final) for-

maldehyde for 2–4 h. Cells were washed with 10 mM Tris pH 7.4 containing 150 mM NaCl. Samples of cells were stained with 50–150 ng/ml DAPI (4,6-diamidino-2-phenylindole) and visualized with u.v. light.

Computer analysis

Sequence alignment and dot matrix analysis was carried out using the Staden computer programs. Open reading frames were analysed using programs from M.Stark (University of Leicester).

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