

The *Saccharomyces cerevisiae* MYO2 Gene Encodes an Essential Myosin for Vectorial Transport of Vesicles

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Abstract. After the initiation of bud formation, cells of the yeast *Saccharomyces cerevisiae* direct new growth to the developing bud. We show here that this vectorial growth is facilitated by activity of the *MYO2* gene. The wild-type *MYO2* gene encodes an essential form of myosin composed of an NH₂-terminal domain typical of the globular, actin-binding domain of other myosins. This NH₂-terminal domain is linked by what appears to be a short α -helical domain to a novel COOH-terminal region. At the restrictive temperature the *myo2-66* mutation does not impair DNA, RNA, or

protein biosynthetic activity, but produces unbudded, enlarged cells. This phenotype suggests a defect in localization of cell growth. Measurements of cell size demonstrated that the continued development of initiated buds, as well as bud initiation itself, is inhibited. Bulk secretion continues in mutant cells, although secretory vesicles accumulate. The *MYO2* myosin thus may function as the molecular motor to transport secretory vesicles along actin cables to the site of bud development.

CELLS of the yeast *Saccharomyces cerevisiae* display an asymmetric pattern of growth: a progeny cell is produced as a bud on the surface of a mother cell. Initiation of bud formation to produce a daughter cell involves localized cell surface growth (Ballou, 1982; Sloat et al., 1981). Continued growth and maturation of the bud requires the directed delivery of new cellular constituents to the site of bud development, so that the bud becomes the major recipient of new cell mass (Hartwell and Unger, 1977; Johnston et al., 1977). Mother cells thus need not only to specify the site for bud formation, but must subsequently direct the allocation of new cellular constituents to the growing bud itself. This pattern of localized growth at the bud site is accomplished by vectorial transport of secretory vesicles to the cell surface (Thacz and Lampen, 1972, 1973; Novick and Schekman, 1979; Field and Schekman, 1981; Tschopp et al., 1984).

Recently components of many intracellular transport systems have been identified in a variety of organisms, including yeast (Drubin et al., 1988; Huffaker et al., 1988; Meluh et al., 1990; for reviews see Kelly, 1990; Kiehart, 1990; Vale and Goldstein, 1990). These various transport systems include force-generating proteins that are capable of propelling cellular cargoes along anchored structures such as microtubules or actin filaments (Vale and Goldstein, 1990). Investigation of actin-based transport systems has revealed a wide variety of myosin-related proteins that serve as intracellular molecular "motors" (Kiehart, 1990). These myosin-like proteins share a common "head" domain capable of interacting with actin (Korn and Hammer, 1988), but have a variety of COOH-terminal domains that specify interaction with the

transported component (reviewed in Kiehart, 1990). Myosin molecules of the myosin-II class, the conventional myosin found in muscle cells, have a COOH-terminal domain that exhibits a high degree of α -helix structure. This α -helical domain can adopt a coiled-coil configuration and cause myosin-II molecules to undergo self-assembly and filament formation. In these cases the transported component specified by COOH-terminal domain interactions is the myosin filament itself, and associated structures. Recent studies of nonmuscle myosin II in *Drosophila*, *Dictyostelium*, and *Saccharomyces cerevisiae* have demonstrated a role for this class of myosin in cytokinesis (Watts et al., 1987; De Lozanne and Spudich, 1987; Knecht and Loomis, 1987). For the yeast *S. cerevisiae*, disruption of the *MYO1* gene, which encodes a myosin II, does not affect cell viability but causes mutant cells to grow in chains of cells that show aberrant segregation of nuclei, indicating that myosin II in this yeast plays a role in both cytokinesis and nuclear migration (Watts et al., 1987).

Another, more heterogeneous, class of myosin proteins contains unconventional myosins. These molecules, collectively termed myosin I, also contain the characteristic myosin-head domain but possess unique and diverse COOH-terminal domains. The COOH-terminal domains of myosin I can interact with a wide variety of cellular targets, such as actin, organelles or plasma membrane, to facilitate intracellular movement (Adams and Pollard, 1986; Lynch et al., 1986). Studies with *Acanthamoeba* suggest that myosin I is responsible for directed movement of membrane vesicles (Adams and Pollard, 1989).

Here we report the characterization of the *S. cerevisiae*

MYO2 gene that encodes an essential myosin involved in localized cell surface growth. The NH₂-terminal region of the *MYO2* protein closely resembles a typical myosin-head domain that imparts force-generating activity, and is coupled to a unique COOH-terminal domain. Between these two domains is a region of potential coiled-coil structure suggesting that the *MYO2* protein may undergo some self-assembly, perhaps to form a dimer. We show here that the novel *MYO2* protein plays a determinant role in the spatial regulation of bud formation. The *MYO2* myosin may be the molecular motor that moves secretory vesicles along actin cables for the process of bud formation.

Materials and Methods

Strains and Culture Conditions

The wild-type strain GR2 (*Mata ural his6*) has been described (Johnston and Singer, 1978). Strains GR663-7 (*MAT α myo2-66 his6 ural*) and GR663-13 (*MAT α myo2-66 his6 ural*) were generated by repeated backcrosses of an initial *myo2* mutant strain (Prendergast et al., 1990) with strain GR2 or the isogenic strain GR6X-7A (*MAT α ade2 ural*). Diploid strains GRD7 and GRD66 were the results of matings between strains GR2 and GR6X-7A and strains GR663-7 and GR663-13, respectively. Strain JP7A (*MAT α myo2-66 adel his6 leu2-3,112 ura3-52*) is a transformable strain produced by successive crosses with strain 21R (Johnston and Hopper, 1982). Strains JY383 (*Mata sst1-3*) and XBH8-2C (*MAT α sst2-4*), kindly provided by J. P. McGrath (Massachusetts Institute of Technology, Cambridge, MA) were tester strains for pheromone secretion assays. Cells were grown in YMI complex medium (Hartwell, 1967) or YNB defined medium (Johnston et al., 1977) supplemented to satisfy auxotrophic requirements. Liquid cultures were maintained in gyrotory shaking water baths (New Brunswick Scientific, New Brunswick, NJ). *Escherichia coli* cells of strain RRI, obtained from J. E. Hopper (M. S. Hershey Medical Center, Hershey, PA) were grown in YT medium (Miller, 1972) and used for plasmid isolation and maintenance.

Assessment of Cellular Parameters

Cell concentrations and morphologies were determined as described (Hartwell, 1970). Before assessment, cells were fixed with formalin and sonicated briefly. DNA content was quantified by a modified diphenylamine procedure (Storms et al., 1984). To determine rates of RNA and protein synthesis, cells were transferred to medium containing radiolabeled precursors of RNA or protein (New England Nuclear, Boston, MA) and incubated for 10 min, after which acid-precipitable radioactivity was quantified (Hanic-Joyce et al., 1987). To visualize chitin, formalin-fixed cells were stained with calcofluor (fluorescent brightener 28; Sigma Chemical Co., St. Louis, MO; Hayashibe and Katohda, 1973; Johnston et al., 1979). Nuclei were visualized using the DNA stain 4'-diamidino-2-phenylindole (DAPI;¹ Sigma Chemical Co.). Determinations of individual cell volumes were performed as described (Murray et al., 1987).

Immunofluorescence Procedures

Cells were fixed for 2 h by addition of formalin directly to the culture medium, and then stained with fluorescein isothiocyanate (FITC)-conjugated antibodies to visualize actin and microtubules as described (Adams and Pringle, 1984; Haarer et al., 1990). Rabbit anti-yeast actin and rat anti-yeast tubulin antibodies were kindly provided by S. Lillie (University of Michigan, Ann Arbor, MI). FITC-conjugated goat anti-rabbit IgG and goat anti-rat IgG secondary antibodies were obtained from Sigma Chemical Co. Cells were examined using a Nikon microphot FX equipped with epifluorescence.

Electron Microscopy

Cells were fixed and processed for thin sectioning as described (Byers and Goetsch, 1975). Cells were postfixed in 1% osmium tetroxide for 3 h, rinsed in distilled water, and incubated in 1% uranyl acetate overnight at 4°C. Dehydration and infiltration were carried out with acetone and Taab embedding medium, respectively. Thin sections were viewed with a Philips 300 electron microscope.

1. Abbreviation used in this paper: DAPI, 4'-diamidino-2-phenylindole.

For freeze fracture analysis, cells were collected by centrifugation, transferred to gold stubs, and frozen directly in Freon 22. Fracturing was achieved using a Balzers 360 freeze fracture unit (Balzers AG, Liechtenstein) at 10⁻⁶ Torr. Fracture surfaces were shadowed at 45° with platinum-carbon and coated with carbon.

Secretion Assays

Cells were assayed for secretion of invertase as described (Bankaitis et al., 1989). Cells were grown to a density of 1–3 × 10⁷ cells per ml and then transferred to low-glucose medium, incubated at 23°C for 15 min and then incubated further at the restrictive temperature of 36°C to assess invertase secretion. External invertase levels were measured by the method of Goldstein and Lampen (1979). Secretion of the mating pheromones α -factor and a-factor was assessed by plate assays described by McGrath and Varshavsky (1989). Haploid wild-type and mutant cells of each mating type were harvested and washed by centrifugation to remove residual mating pheromones secreted during proliferation at 23°C, and were then transferred to fresh medium and incubated for 3 h at either 23 or 36°C. The cells were then removed by centrifugation and dilutions of the culture supernatants were applied to lawns of tester yeast cells. Manipulations involving transfer to 36°C were carried out in an environmental room.

Genetic Analysis

Standard yeast genetic techniques were used (Mortimer and Schild, 1981).

Cloning and Characterization of the *MYO2* Gene

The *MYO2* gene was cloned by complementation (Rothstein, 1986) of the *myo2-66* temperature sensitivity of strain JP7A with genomic DNA from a YCp50-based recombinant yeast DNA library, kindly provided by D. Botstein (Genentech, San Francisco, CA). One complementing plasmid, pJP10-2B, was used for further study. Restriction analysis was performed as described (Maniatis et al., 1982). Probes for Southern analysis were prepared by random-primer extension (Boehringer, Mannheim, FRG) of a *MYO2* restriction fragment. An integration plasmid was constructed by substituting the smaller EcoRI-SalI fragment of plasmid YIp5 with the EcoRI-SalI fragment from the genomic insert of plasmid pJP10-2B (Fig. 3 A). The resultant plasmid, pL4, contained the wild-type *URA3* gene but did not contain yeast sequences that allow autonomous replication, so that only the integrated plasmid could be transmitted to progeny cells. Integration of plasmid pL4 at the homologous chromosomal site was directed (Rothstein, 1986) by transformation with pL4 DNA that had been linearized within the genomic insert by digestion with HindIII (Figure 3 A). The diploid strain JPD7, heterozygous at the *MYO2* locus but homozygous for *ura3-52*, was transformed to uracil prototrophy by the linearized pL4 plasmid. Integration at the homologous chromosomal locus was confirmed by Southern analysis (data not shown). Subsequent genetic analysis showed that homologous integration of pL4 caused the integrated *URA3* vector gene to be tightly linked to the *myo2* locus (data not shown). Therefore the genomic insert in pJP10-2B harbors the *MYO2* gene. Integration of pL4 into JPD7 diploid cells also caused disruption of one copy of the *MYO2* gene with the result that, upon sporulation, only two spores in each tetrad gave rise to viable cells. Incubation of sporulated diploid cells for varying lengths of time (2–6 d) or germination of spores at different temperatures (14, 23, 36°C) did not improve spore viability.

For DNA sequencing, sets of 3' and 5' *MYO2* deletion plasmids were generated from plasmids containing subcloned regions from the genomic insert of pJP10-2B (Dale and Arrow, 1987). The deleted single-stranded DNA templates in M13 were sequenced by the dideoxy method (Sanger et al., 1977). In this way both strands of the pJP10-2B insert were sequenced. Nucleotide sequence analysis was performed using the University of Wisconsin Genetics Computer Group (UWGCG) software package (Devereux et al., 1984).

Results

Isolation of the *myo2* Mutant

A recently described centrifugation procedure (Prendergast et al., 1990) has been shown to enrich for abnormally large cells. Use of this procedure to enrich for enlarged cells at the restrictive temperature of 36°C enabled us to identify temperature-sensitive mutant cells defective in cell prolifera-

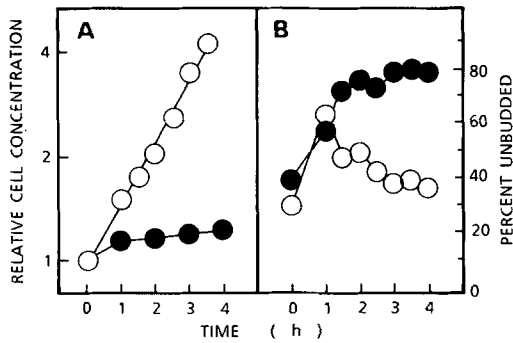


Figure 1. Uniform unbudded arrest of *myo2-66* mutant cells at 36°C. Proliferating wild-type (open circles) and *myo2-66* mutant cells (closed circles) were transferred to the nonpermissive temperature of 36°C and sampled at intervals. (A) Cell concentration; (B) percent unbudded.

tion but unimpaired in overall mass accumulation. All of the mutant cells identified in this way displayed significant residual biosynthetic activity at the restrictive temperature, but many were unable to perform aspects of the mitotic cell cycle.

One temperature-sensitive mutation, now termed *myo2-66* but originally designated *cdc66-1* (Prendergast et al., 1990), impaired bud formation. Transfer of proliferating *myo2-66* mutant cells to the restrictive temperature of 36°C led to a concerted arrest of proliferation, with arrested cells displaying a uniform unbudded cell morphology (Fig. 1). Despite the cessation of proliferation, *myo2-66* mutant cells at 36°C remained biosynthetically active and mass accumulation continued, as evidenced by continued synthesis of protein and RNA (Fig. 2 B), and the continued increase in size of arrested cells. At the restrictive temperature *myo2-66* mutant cells also continued to replicate DNA and accumulated DNA to a level twice that for proliferating cells (data not shown). Nuclear staining revealed that a significant proportion of arrested mutant cells (168 out of 360, or 47%) displayed more than one nucleus (Fig. 2 A), whereas each wild-type cell treated in the same way displayed only one nucleus (data not shown). These observations showed that the *myo2-66* mutation does not block the nuclear cycle, but makes mutant cells defective in proper morphological development.

The MYO2 Gene

The *MYO2* gene was isolated by complementation of the *myo2-66* temperature-sensitive defect. Approximately 30,000 transformants were selected by uracil prototrophy and assessed for growth at the restrictive temperature of 36°C. The plasmids from three temperature-resistant transformants that showed concomitant loss of both uracil prototrophy and temperature resistance under nonselective conditions were found to contain overlapping genomic inserts (data not shown). The integration by homologous recombination (Rothstein, 1986) of a plasmid harboring the EcoRI-SalI fragment from plasmid pJP10-2B (Fig. 3 A) into the *myo2-66* chromosomal site showed that pJP10-2B contained the wild-type *MYO2* gene.

The genomic insert in plasmid pJP10-2B was shown to contain the wild-type *MYO2* gene by integration of a plasmid

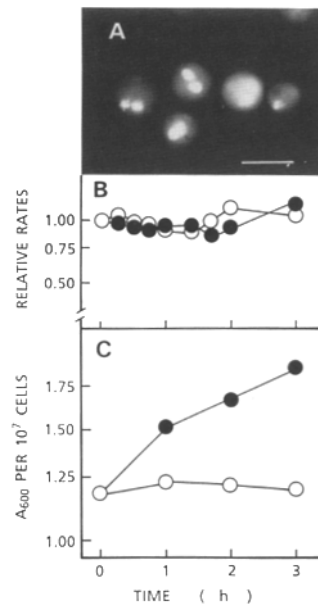


Figure 2. Biosynthetic activity of *myo2-66* mutant cells. Proliferating wild-type and *myo2-66* mutant cells were transferred to 36°C, and at intervals were assessed for biosynthetic activity. (A) Mutant cells stained with DAPI to visualize nuclei after 3 h at 36°C. (B) 1-ml samples were transferred to 36°C medium containing radiolabeled precursors and incubation was continued for a further 10 min. The rates of synthesis per mutant cell of RNA (open circles) and protein (closed circles) are expressed as a fraction of the per cell incorporation of wild-type cells at the same incubation time. (C) optical density (A_{600}) per cell for wild-type (open circles) and mutant (closed circles) populations. Bar, 10 μ m.

harboring part of that insert into the *myo2-66* chromosomal site by homologous recombination (Rothstein, 1986).

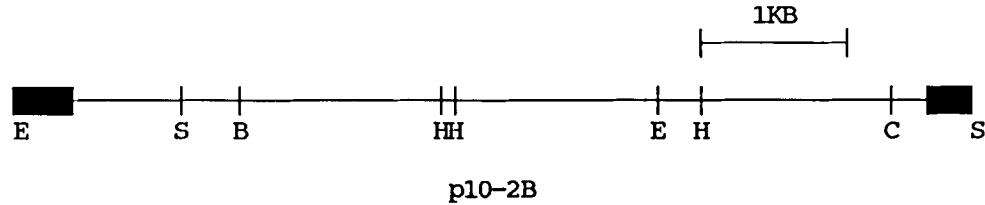
The MYO2 Gene Product Is Essential

Sequence analysis (below) showed that the EcoRI-SalI fragment is completely internal to the *MYO2* open reading frame, so that integration of this fragment at the *MYO2* locus disrupts that chromosomal *MYO2* allele. Therefore integration of pL4 into JPD7 diploid cells heterozygous for the *myo2-66* allele resulted in disruption of either the wild-type *MYO2* allele or the mutant *myo2-66* allele. Upon sporulation of one integrant diploid strain, only 2 spores in each of 43 tetrads were viable, in marked contrast to the high spore viability for the untransformed diploid. Each viable spore product from that transformed diploid was a temperature-resistant uracil auxotroph, indicating that viable spores contained neither the integrated plasmid nor the *myo2-66* allele. The nonviable spore products must therefore have contained the plasmid integrated into the *myo2-66* allele, disrupting the *myo2-66* locus. Microscopic examination revealed that all of these nonviable spores germinated and formed a bud, but most (72%) remained as a single budded cell. This finding demonstrates that *MYO2* is an essential gene.

myo2-66 Supplies Only Partial Function

The transformed diploid described above was temperature-resistant and grew well with only a single intact copy of the wild-type *MYO2* gene. Thus the *MYO2* gene product is normally present in excess. However, other Ura⁺ transformants of the same diploid strain remained temperature-sensitive, and therefore must have been disrupted for the wild-type *MYO2* allele. These diploid integrants with only the *myo2-66* mutant gene still intact were severely impaired in growth even at 23°C, and were unable to sporulate (data not shown). This growth defect demonstrates that the mutant *myo2-66* gene product is only partially functional even at the permis-

A



B

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-579 ATCAATAAATAAATAGGCTCGAAGACGCCTCAGAACTCCGGTCACTGGTGTGCTTGTGGTATACGATGTCCAAGCGCCGTTCTCGATGCTTATCTGGTTAGTTACGCTGTTAAA
-459 ACCAAAAACCAACAGATTTTCGACCCTAACGTATGTAGGCTAAAATAGATATTGAGTAGGTACAATAATTATTGGCAATTGCACCTAGTGACACATTTACGAAAACGTAGGCAAAA
-339 AACTATTACCCGACCCAGGGCTATTTGTGATTTTTCTTTTTTTGTTTATGATCGCGCTTCTCGAAAAGCCAAATATCAGAAATCCCAAAACACGCCCTTCATTGATACGATTCGTAG
-219 CCTGCGTTTCAGAGATCTATCAACTTTCGAAGGCCAATCAGAGAACAAAAAGTCTCGCAAAGTCACTTTCTCGCTTGAATATTCTGTCGATTTCTGGCTGCTTGCCTGTTTT
- 99 TTTGTTTTCTAAGGTACTATTGCACACCATTCCATTGGACAGCGATCTTATACCATTGTACATATAGGACATAAAAAACAGCAGATATTACAGCGTATAATGCTTTTGAAGTGGGTACA
                                     M S F E V G T 7
22  CGATGCTGGTATCCCCATAAAGAATTGGGCTGGATTGGGGCGGAAGTAATCAAAAATGAGTTCACACGACGGCAAGTACCACCTGGAGTTACAATTGGAAGACGATGAAATCGTGTCCGTTG
   R C W Y P H K E L G W I G A E V I K N E F N D G K Y H L E L Q L E D D E I V S V 47
142 GACACAAAAGACTTGAATAACGATAAGGACCAATCTCTACCGCTTCTTAGAAACCCCTCCATTTTGGAAAGCAACGGAAGATTTGACCTCTTTATCTTACTTGAATGAGCCAGCTGTTTTA
   D T K D L N N D K D Q S L P L L R N P P I L E A T E D L T S L S Y L N E P A V L 87
262 CATGCCATCAAACAGCGCTATTCTCAATTGAATATCTACACATACTCGGATTTGTTCTGATGCTACAAAACCCCTTTGATCGTGTGACACAGCTTTATACACAAGACATGATCCAAGCA
   H A I K Q R Y S Q L N I Y T Y S G I V L I A T N P F D R V D Q L Y T Q D M I Q A 127
382 TATGGGGAAAGCGCAGAGGTGAAGTGAACCTCACTTGTTCATGTCGGAAGAAGCGTATAGGTGTGATAAATGACAAAACAAAATCAAACCATTGTGGTAAAGTGGTGAATCTGGT
   Y A G K R R G E L E P H L F A I A E E A Y R L M K N D K Q N Q T I V V S G E S G 167
502 GCTGGA AAAACGGTTTCTGCCAAGTATATTATCGCTTATTTGCTTCTGTAGAAGAGGAAAATCCCGTACTGTACAACATCAAGTGGAAAATGTCGGAACAGAACAAAAGATTCTAGCT
   A G K T V S A K Y I M R Y F A S V E E E N S A T V Q H Q V E M S E T E Q K I L A 207
622 ACAAAACCCATCATGGAAGCATTGTTGTAATGCTAAGACTACCAGAAATGACAATTCTCCAGATTTGGTAAATCTAGAAAATTTATTTCGATAAGGACACATCTATTATTGGAGCAAGG
   T N P I M E A F G N A K T T R N D N S S R F G K Y L E I L F D K D T S I G A R 247
742 ATCCGCACATACTTGTGGAAACGGTCCAGATTAGTTTACCAGCGCCAAATTGAGAGAACTACCACATATTTTCAATTAATGGCTGGATTACCAGCTCAAACCAAGGAGGAATTGCAT
   I R T Y L L E R S R L V Y Q P P I E R N Y H I F Y Q L M A G L P A Q T K E E L H 287
862 CTTACCGATGCCTCAGATTACTTCTACATGAACCAAGCGGTGACACCAAGTCAACGGTATTGATGATGCCAAGAATACAAAATACAGTAGATGCAATTAGTTCGGAATCACC
   L T D A S D Y F Y M N Q G G D T K I N G I D D A K E Y K I T V D A L T L V G I T 327
982 AAGGAACTCAACACCAAAATATTAAGATCTTGGCCGCACTTCTGCATATCGGTAACATAGAAAATAAAAAACTAGAAAATGATGCATCACTATCAGCTGATGAGCCAAAACCTGAACTG
   K E T Q H Q I F K I L A A L L H I G N I E I K K T R N D A S L S A D E P N L K L 367
1102 GCGTGGCAATGCTGGGAATTGATGCCTACAACCTTTGCCAAAATGGGTCAACAAAAGCAGATCATTACAAGGTGAGAGAAAATGTTTCGAATCTAAAATATAGTCAAGCTCTGGTTGCC
   A C E L L G I D A Y N F A K W V T K K Q I I T R S E K I V S N L N Y S Q A L V A 407
1222 AAAGATCCCGTGCTAAGTTATTTATTCGCCCTTTTCGATTGGCTTGTGGAAAATATCAACACCGTGTATGCAACCCGCTGTGAACGACCAAAATAGCTCATTATTGGTGTCTG
   K D S V A K K F I Y S A L F D W L V E N I N T V L C N P A V N D Q I S S F I G V L 447
1342 GATATTTATGGTTGAACTTTGAAAAAATTCATTTGAACAAATTTGATTAACATATGCCAAACGAAAACATAACAAGAGTTCAACCAACATGTTTTCAAATAGAGCAAGAAGAA
   D I Y G F E H F E K N S F E Q F C I N Y A N E K L Q Q E F N Q H V F K L E Q E E 487
1462 TACGTTAAAGAAGAAATGAATGGTCTTTTATAGAGTTAATGATAATCAACCTTGTATTGATCTGATTGAAAACAAGTGGGTATTTTACTGCTTACGCAAGAAAGTAGGTTACCT
   Y V K E E I E W S F I E F N D N Q P C I D L I E N K L G I L S L L D E E S R L P 527
1582 GCTGGTCCGACGAATCTTGACCCAAAACCTTTATCAAACCTTTGATAAATCTCCTACGAAACAAAGTATTTCTAAACCAAGATTGGGCAAACTAAATTTATCGTGAGCCATTATGCT
   A G S D E S W T Q K L Y Q T L D K S P T N K V F S K P R F G Q T K F I V S H Y A 567
1702 CTAGATGTCGCTTATGATGTGGAAGGATTTATTGAAAAAATAGAGACACCGTATCTGACGGACATTTGGAAGTGTGGAAGCTTCTACCAACGAGACACTAATAATATCTTAGAGGGA
   L D V A Y D V E G F I E K N R D T V S D G H L E V L K A S T N E T L I N I L E G 607
1822 TTAGAAAAGCTGCCAAAACCTGGAAGAAGCGAAAAGCTTGAATTAGAGCAGGCTGGCAGTAAAAAGCCAGGTCGATAAAGAACGGTTAACAGGAAACCCACTTTAGGTTCCATGTT
   L E K A A K K L E E A K K L E L E Q A G S K K P G P I R T V N R K P T L G S M F 647
1942 AAGCAATCTTTGATTGAACATAATGAATACCACTCAACTCAACTAATGTTTCATTAATCGTTGTATAAAGCCTAATCGAGATAAAGAAGCTTGGCAATTTGATAATTTGATGGTGTGCT
   K Q S L I E L M N I N S T N V H Y I R C I K P N A D K E A W Q F D N L M V L S 687
2062 CAACTCAGAGCCTGTGGTGTGTTTGGAAAATATTAGAATATCTGTGCTGGGTTCCCTTAGGTTGGACTTTTGAAGAATTTGATTAAGATATTACATCTTGATACCACATGAGCAGTGG
   Q L R A C G V L E T I R I S C A G F P S R W T F E E F V L R Y Y I L I P H E Q W 727

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Figure 3. The *MYO2* gene. (A) Restriction map of plasmid pJ10-2B. The thin line represents the genomic insert containing the *MYO2* gene, and the thick lines flanking the insert represent vector sequences. E, EcoRI; S, SalI; B, BamHI; H, HindIII; C, ClaI. (B) The nucleotide sequence of *MYO2* and deduced amino acid sequence. The nucleotide and amino acid sequences are numbered on the left and right, respectively. The A nucleotide of the presumed ATG initiator codon is designated +1. The potential p34^{cdc2} phosphorylation site is boxed. The ATP-binding and active thiol regions are underlined with a single or double line, respectively. The potential splice donor and acceptor sites are underlined with a dashed line and the splice signal recognition sequence is overlined. (C) A schematic representation of proposed regions within the *MYO2* protein. These sequence data are available from EMBL/GenBank/DBJ under accession number M35532.

2182 GACCTAATCTTCAAAAAAGGAACTACAGAAGAAGATATCATATCAGTGGTAAAATGATCCTAGTAGCTACTGTAAGGACAAAATCCAAGTACCAGATTGGTAATACAAAAATTTTC
D L I F K K K E T T E E D I I S V V K M I L D A T V K D K S K Y Q I G N T K I F 767

2302 TTCAAAGCAGGTATGCTTGCATATCTGGAAAACTTAGAAGCAATAAGATGCATAATCAATTGTTATGATCCAGAAGAAAATAGAGCTAAATATACCGTAAGCAGTATTTGCAATA
F K A G M L A Y L E K L R S N K M H N S I V M I Q K K I R A K Y R K Q Y L Q I 807

2422 TCTCAGGCCATCAAGTATTGCAGAACACATCAAAGTTTCATCATTGCTCAACGGCTTAATGATGAAATGAAAGTTAACTGTGCAACTTTATTACAGCGCGCTACAGGGGTCATTCC
S Q A I K Y L Q N N I K G F I I R Q R V N D E M K V N C A T L L Q A A Y R G H S 847

2542 ATCCGTGCCAATGTGTTGAGCAATTAACAATTTGCAAAAAGAAAATAGAAAGGAACTAAAAACAAGACAACCTGAAAACAAGAACATGAATATAATGCTGCGGTAACATT
I R A N V F S V L R T I T N L Q K K I R K E L K Q R Q L K Q E H E Y N A A V T I 887

2662 CAAAGTAAAGTATGAGACCTTTGAGCCGAGATCGAGATTTTACGCACATAAAAAGACACTGTTGTTGTCCAATCTTTGATCAGAAGAAGAGCTGCTCAAAGGAAATGAAACAATTGAAG
Q S K V R T F E P R S R F L R T K K D T V V V Q S L I R R R A A Q R K L K Q L K 927

2782 GCAGCGCTAAATCAGTAAATCATCTGAAAGAAGTGAGCTATAAATAGAGAATAAAGTGATTGAACTGACGCAGAACTAGCATCCAAGGTCAAAGAAAATAAAGAAATGACAGAAAAGA
A D A K S V N H L K E V S Y K L E N K V I E L T Q N L A S K V K E N K E M T E R 967

2902 ATTAAGAAGTACAGGTTCAAGTGGAAAGAAAGTCCCAAGTTACAAGAGACATTAGAAAAATGAAAAAAGAGCACTTAATAGATATTGATAATCAGAAAATCAAGGATATGGAATTACAA
I K E L Q V Q V E E S A K L Q E T L E N M K K E H L I D I D N Q K S K D M E L Q I 1007

3022 AAACTATTGAGAACAATTTGCAATCCACTGAACAACTCTAAAGGACGCTCAATTAGAGTTGAGGACATGGTTAAACAACATGATGAATTGAAAGAAGAACTAAAAAGCAACTTGAA
K T I E N N L Q S T E Q T L K D A Q L E L E D M V K Q H D E L K E E S K K Q L E 1047

3142 GAATTAGAGCAAAACAAGAAACATTGGTTGAATACCAGACATTAACCGGAGACTTGAACCAAGAAAGTAAATCTTTAAAGGAAGAAATGCTAGGTTACAACTGCCATGTCGCTGGGC
E L E Q T K K T L V E Y Q T L N G D L Q N E V K S L K E E I A R L Q T A M S L G 1087

3162 ACCGTTACTACTAGTACTACTACCTCAAACACCATTAAGGATGTAATGGGAGCGGTGCTTCAAATTTCAACAATATGATGCTTGAGAATTCGACTTATCTCTAATGATTTGAATCTA
T V T T S V L P Q **T P L K** D V M G G G A S N F N M M L E N S D L S P N D L N L 1127

3282 AAGTCTAGTCTACTCCATCGTCCGAAACAACCACATTGATTGATTGAGTGTGATCGCGAAAATGGTGTCAATGCTACACAATCAATGAAGATTATACAGGTTATGGAGGACACT
K S R S T P S S G N N H I D S L S V D R E N G V N A T Q I N E E L Y R L L E D T 1167

3402 GAAATTTTGAATCAAGAAATCAGCGAAGGCTGTTAAAGGGATTGCAAGTACCGGATGCTGGTGTAGCTATCAACTAAGTAAAAAGAGCGTTGTTATCCGGCTAGAATACTGATTATA
E I L N Q E I T E G L L K G F E V P D A G V A I Q L S K R D V V Y P A R I L I I 1207

3522 GTTTAAGTGAATGGAGTTGGGCTGACCAAGCAAAGTGAAGCTTTCTGCCAAGTATTGACTACAAATGAAAAAGTGTCACTCAATTGAAGGTAACGATTAATTCACAA
V L S E M W R F G L T K Q S E F L A Q V L T T I Q K V V T Q L K G N D L I P S 1247

3642 GGTGATTCTGGTTAGCAACGTTAGAGLTTATACTCATTGTTGGTGTTCCTCTAACTCTATTTTAAACGAAGAAACGTTCAAACCGGATGACCGATGAGGAGTAAAGGAGTAT
G V F W L A N V R E L Y S F V V F A L N S I L T E E T F K N G M T D E E Y K E Y 1287

3762 GTTTCATTGGTACAGAACTAAAGGATGATTTCGAAGCTCAAGTATAATATATAACATTTGGCTGAAGAAATGCAGAAGCAATGCAAAAAAGGCCATCAATGCTGTGGTCATC
V S L V T E L K D D F E A L S Y N I Y N I W L K K L Q K Q L Q K K A I N A V V I 1327

3882 TCCGAATCATTACAGGTTTCCAGCGGGAGAAACAGCGGGTTTTTGAACAAAATTTTGTCTAACTGAAGAAATACAATGGACGACATTTTACCTTTTCAACAGCATATACTGG
S E S L P G F S A G E T S G F L N K I F A N T E E Y T M D D I L T F F N S I Y W 1367

4002 TGCAATGAAATCTTTTATATTGAGAATGAAGTGTCCATGCTGTAGTACAACTTATTGAATATTGTTGGATGCAATTTGTTTTAACGAATTAATCATGAAACGTAATTTCTTGTGCTGG
C H K S F H I E N E V F H A A V V T T L L N Y V D A I C F N E L I M K R N F L S W 1407

4122 AAAAGGGTCTTCAATTGACTACAACGTTACTAGATTAGAGGAATGGTCAAGACGCTGCTGACAGTGGTACTGAGTACTGCTTACAACATTTGATTGACAGCGCTAAGCTGCAAA
K R G L Q L N Y T R L E E W C K T H G L T D G T E C L Q H L I Q T A K L L Q 1447

4242 GTCGTAAGTATACTATCGAAGACATTGATATCTTAAGAGGAATTTGTTATTGCTGTAACCTGCACAATGCAAAAAATGATTTACAATACCAGGTGGCAGACTATGAGTCTCAAT
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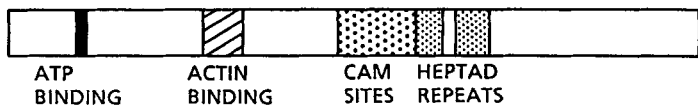
4362 CCACAGGAAATCTTAAGATACGTTGCTGATATAGTTAAGAAAGAAAGCTGCGTTATCTTATCAGGTAATGATTCTAAGGGTCAGGACATAGCAGCAGTATATTTACTCCAGAAACA
P Q E I L R Y V A D I V K K E A A L S S S G M D S K G H E H S S S I F I T P E T 1527

4482 GGTCCATTTACTGACCCATTGATTAAGACAAGAAAATTTGACCAAGTAGAAGCCTATATACCAGCGTGGTTATCCTTGCCTCAACTAAGAGAATAGTTGACCTGTTGCCCAA
G P F T D P F S L I K T R K F D Q V E A Y I P A W L S L P S T K R I V D L V A Q 1567

4602 CAAGTCGTTCAAGACGGCCATAAATGATGGCGGAGAAAACAAAATTTGATCATGAATGCTAAAAAAGAAATGACAAAAAAGAGAAAAAATGAAACTACATAGTTAATTAAT
Q V V Q D G H 1574

4722 AATAGAAGTATTTGCAATAGTATGATAATGAAATCGATATATGGAAGATATTAACCGCGCGCTATTAGTGTACACTATATAACTACATTTTCTTACTGAATTTATAAAT
4842 ATGATTATATFATTACTATTACTACTGATATATATTTTAGAATTAGATCGGGAACCGATGAGCGTTAGCTGAAATGGACGACGATAAGGAACGATAATTACCAGTAAAAATA
4962 ATAACAACCTAAGAATAAACACATTTCTCATTTTTA

C



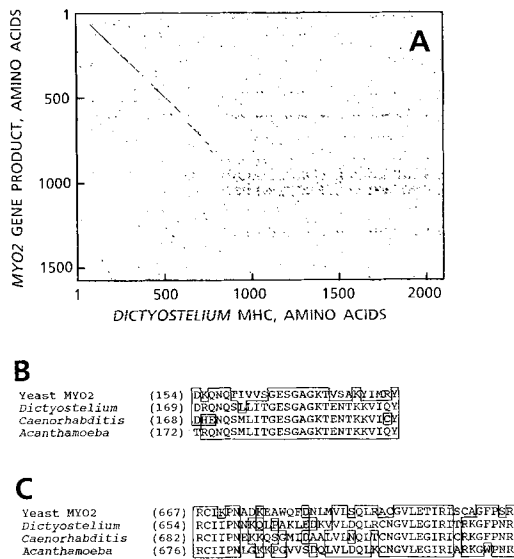


Figure 4. Comparison of MYO2 protein sequences. (A) Dot-matrix comparison of the MYO2 protein with myosin II from *Dictyostelium discoideum*. A dot was made when 9 or more residues matched within a window of 11 residues. (B and C) Comparisons of the ATP-binding (B) and active-thiol (C) regions. The amino acid position is shown in parentheses. Residues identical in at least two of the displayed sequences are boxed.

sive temperature. Furthermore, the impaired growth of a diploid cell with a single *myo2-66* gene at a permissive temperature is in marked contrast to the good growth of a haploid *myo2-66* cell. Such effects of gene dosage may indicate that the stoichiometry of interaction between the partially functional *myo2-66* gene product and other cellular components is critical for function. Alternatively, the truncated protein that results from disruption of the *MYO2* gene might interact with the mutant *myo2-66* gene product to further enfeeble the mutant protein. Differences in gene expression between haploid and diploid cells may also result in a suboptimal environment for mutant gene-product function.

MYO2 Encodes a Myosin I Protein

The nucleotide sequence of a 5,675-bp complementing insert from pJP10-2B revealed an extensive open reading frame that could encode a protein of 1,574 amino acids, with a predicted molecular weight of 180,567 (Fig. 3 B). We assume that the ATG codon at position +1 serves as the translation initiation codon: the sequences flanking this putative initiator codon are identical to the proposed consensus sequence for translation initiation in yeast (Cigan and Donahue, 1987).

The NH₂-terminal half of the deduced amino acid sequence of the *MYO2* gene shows striking similarity to the globular head domain of myosin heavy chain genes. Fig. 4 A shows a representative dot-matrix comparison between the MYO2 protein and a myosin heavy chain (a myosin II) from *Dictyostelium* (Warrick et al., 1986). Like other myosins, the MYO2 protein contains within the NH₂-terminal domain a putative ATP-binding pocket, centered at residue 167, with the characteristic glycine-rich GXXGXGKT motif (Walker

et al., 1982). This potential ATP-binding region (Fig. 4 B) displays significant sequence similarity to analogous regions in the myosins from *Dictyostelium* (Warrick et al., 1986), nematode (Karn et al., 1983), and *Acanthamoeba* (Hammer et al., 1987). In addition, the putative ATP-binding region of the *MYO2* gene shows 62% amino acid identity with a region of the yeast *MYO1* gene product (Watts et al., 1987; data not shown). A further region of sequence similarity is evident between residues 443 (phenylalanine) and 523 (glutamic acid). This 81-amino acid stretch has 65% identity to the proposed actin-binding region in nematode myosin and 62% identity to the analogous region in the yeast *MYO1* gene product (data not shown).

Reactive cysteine residues are considered important for the actin-activated ATPase activity of myosins (Harrington and Rodgers, 1984). Unlike the situation for the yeast *MYO1* gene product, in which the active-thiol region contains only a single cysteine (Watts et al., 1987), the MYO2 protein contains the typical two cysteine residues at positions 692 and 702 (Fig. 4 C). Over a 24-residue stretch encompassing these two cysteine residues, the MYO2 protein displays significant amino acid identity to the analogous regions from *Dictyostelium*, nematode and *Acanthamoeba* (Fig. 4 C).

Analysis of the remaining COOH-terminal domain of the MYO2 protein failed to indicate any significant sequence similarity to previously reported myosin proteins (see Fig. 4 C). These structural considerations point to a provisional assignment of the MYO2 protein to the myosin I class of myosins.

Chitin Deposition Delocalized But Septation Normal

At the time of bud emergence, a localized deposition of chitin occurs to form a ring structure, through which the cell surface evaginates to form the bud. This localized deposition of chitin marks the site of bud emergence and remains on the surface of the mother cell after cell separation as a characteristic bud scar (Ballou, 1982; Fig. 5 A). Chitin staining of *myo2-66* mutant cells under restrictive conditions revealed that chitin deposition was no longer localized but had become distributed over the entire cell surface (Fig. 5 B). This behavior is similar to the delocalization of chitin distribution seen in actin mutants (Novick and Botstein, 1985) and certain *cdc* mutant cells (Sloat et al., 1981; Roberts et al., 1983; Adams et al., 1990). After 6 h at the restrictive temperature, mutant cells were stained uniformly for chitin. By this time many cells also had become deformed and wrinkled in appearance, consistent with the loss of viability observed after several hours at the restrictive temperature (data not shown).

Chitin deposition also is required to form the final septum during cell separation (Silverman et al., 1988; Cabib et al., 1989). Success of this chitin deposition is indicated indirectly by the phenotype of *myo2-66* mutant cells: as shown in Fig. 1, these cells arrest proliferation promptly upon transfer to the restrictive temperature and accumulate as unbudded cells, suggesting that buds that were present at the time of transfer to the restrictive temperature are released normally. Thus, the *MYO2* gene, unlike the *MYO1* gene (Watts et al., 1987), is not involved in nuclear migration or cytokinesis. Furthermore, prompt completion of the budding process even at the restrictive temperature indicates that the *myo2-66* mutation does not compromise the chitin deposition involved in septation.

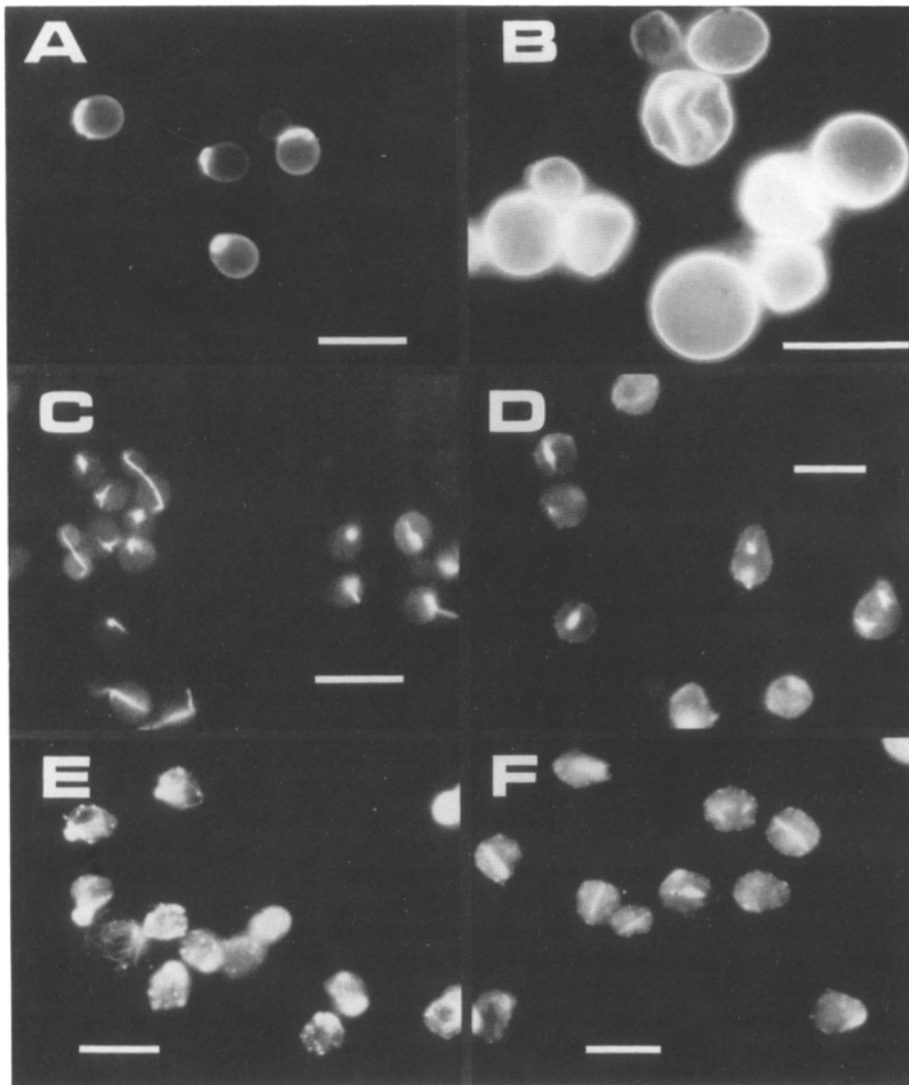


Figure 5. The *myo2-66* mutation disrupts actin cables. Proliferating homozygous *myo2-66* (*B*, *D*, *F*) or wild-type (*A*, *C*, *E*) diploid cells were incubated at 36°C for 3 h (*C-F*) or 6 h (*A* and *B*) and then stained for chitin (*A* and *B*), or subjected to immunofluorescence staining to reveal microtubules (*C* and *D*) or actin (*E* and *F*). Bars, 10 μ m.

***MYO2*-encoded Myosin Directs New Growth to the Bud**

The budding process produces a daughter cell that is nearly the same size as its mother cell. This asymmetric pattern of cell enlargement reflects the transport of vesicular material to a localized area of the cell surface (Thacz and Lampen, 1972, 1973; Novick and Schekman, 1979; Field and Schekman, 1980; Tschopp et al., 1984). To determine the role of the MYO2 protein in the vectorial transport of vesicles necessary for this asymmetric pattern of growth, we measured the sizes of the new daughter cells produced by *myo2-66* mutant cells after the imposition of restrictive conditions. This analysis was facilitated because at the restrictive temperature *myo2-66* mutant cells complete ongoing cycles of budding, cytokinesis and cell separation before arrest of proliferation as unbudded cells (Fig. 1). A defect in bud growth at the restrictive temperature should result in new daughter cells that are smaller than normal.

Mutant and wild-type cells were transferred to the restrictive temperature, and incubated for 3 h, at which time cell samples were fixed and cell volumes were determined. As shown in Fig. 6 *A*, the population of arrested *myo2-66* mu-

tant cells generated a disperse cell volume distribution compared to that of the wild-type population. Although most mutant cells were significantly larger than the largest wild-type cell, the population of arrested mutant cells also contained a significant fraction of extremely small cells. To determine the volume of individual wild-type daughter cells, fixed cells were first stained for chitin, and for wild-type cells only newly produced cells, easily identified by the absence of a chitin-rich bud scar, were measured (Fig. 6 *B*). For cells in the arrested mutant population the classification of cells according to bud scars was compromised by the generalized delocalization of chitin (see above), and for this reason cell volumes of all mutant cells were determined without regard for bud-scar status. As shown in Fig. 6 *B*, the arrested mutant population contained cells that were significantly smaller than the smallest daughter cells in the wild-type population. Although the arrested cells continued to increase in mass (see Fig. 2), this growth was no longer restricted to the developing bud. This finding indicates that the MYO2 myosin protein is required to direct new growth into the bud, as well as to localize secretion for bud initiation.

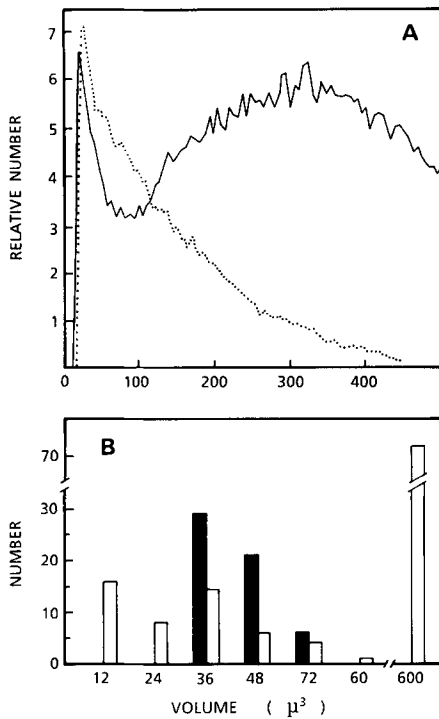


Figure 6. Cell volumes of wild-type and *myo2-66* mutant cells. (A) Cell volumes for wild-type and mutant cells after 3 h at 36°C were determined using a Coulter Channelyzer (Coulter Electronics, Hialeah, FL). (Dashed line) Wild-type cells; (solid line) *myo2-66* mutant cells. (B) A portion of each sample was also stained for chitin, and volumes of individual cells were determined using an ocular graticule. For wild-type cells, only new daughter cells were measured; these were identified because chitin staining was confined to the site of budding, so that new daughter cells were easily recognized as unbudded cells without a bud scar. For *myo2-66* mutant cells, the volumes of all unbudded cells were determined without consideration to bud scar status, because chitin distribution became delocalized. Cells were grouped into classes differing by 12 μm³ in volume. (Solid bars) Wild-type daughter cells; (open bars) unbudded mutant cells.

Intact Microtubules in Arrested Mutant Cells

Examination of *myo2-66* mutant cells using a yeast anti-tubulin antibody revealed normal spindle structures that persisted after transfer of cells to the restrictive temperature and the eventual arrest of proliferation. As shown in Fig. 5 D, arrested mutant cells maintained well-defined cytoplasmic microtubule structures. These findings are consistent with results showing that the array of extranuclear microtubules in yeast cells is not involved in continued bud growth (Hufaker et al., 1988; Jacobs et al., 1988), although this array may affect bud site selection (Byers, 1981; but see Jacobs et al., 1988).

An Aberrant Actin Cytoskeleton

Another structural component involved in localized surface growth is actin (Adams and Pringle, 1984; Novick and Bostein, 1985; Drubin et al., 1988; Haarer et al., 1990). Actin is found in cortical patches that concentrate on the surface of the bud (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Drubin et al., 1988), and in an intracellular network

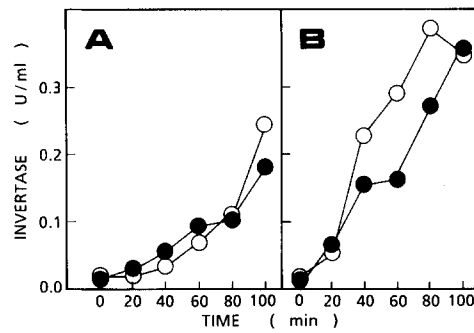


Figure 7. Invertase secretion by *myo2-66* mutant cells. Wild-type (open circles) and mutant (closed circles) cells were transferred to medium containing 0.1% glucose and incubated at either 23°C (A) or 36°C (B). At intervals, levels of external invertase were assessed.

that forms the actin cytoskeleton within the cytoplasm (Adams and Pringle, 1984; Drubin et al., 1988). As assessed by anti-actin antibody staining, proliferating wild-type and mutant cells displayed normal actin cytoskeletons, with pronounced actin cables coursing through the interior of each cell (Fig. 5 E and data not shown). Cortical actin patches were concentrated as expected in the growing bud. At the restrictive temperature the arrested mutant cells remained biosynthetically active (Fig. 2 B) and became enlarged (Fig. 2 C), but now all cells in the population displayed delocalized cortical actin patches (Fig. 5 F), suggesting that cell-surface growth was no longer restricted to specific sites. Moreover, the actin cables underwent a striking alteration in mutant cells to produce thick bars of actin within the cell (Fig. 5 F). The actin bars were randomly oriented with respect to the long axis of the arrested, unbudded mutant cells. After 6 h at the restrictive temperature, ~66% of the mutant cells (132 out of 200) contained these actin bars. In contrast, under permissive conditions only 23 out of 200 mutant cells (11%) contained actin bars, and at no time did we observe these structures in wild-type cells regardless of growth temperature (data not shown).

The aberrant actin cytoskeleton in mutant cells suggests that the *myo2-66*-encoded myosin interacts with the network of actin filaments. Furthermore, the aberrant actin cytoskeletons in some cells even at the permissive temperature reinforces the conclusion derived above from studies of gene dosage that the *myo2-66* gene product is partially dysfunctional under permissive conditions.

Impaired Vesicle Movement But Continued Secretion

A defect in bud development could indicate an impairment in general secretory processes, or in the localization of secretion. Many genes have been shown to be involved in the secretion activity of cells (Novick et al., 1980), and mutations that affect steps late in the secretory pathway block secretion and result in the accumulation of vesicles within the cytoplasm (Novick et al., 1981). Therefore in *myo2-66* mutant cells, the status of the secretory pathway was assessed. Mutant cells were examined by thin section EM and by freeze fracturing to determine if vesicle transport was affected, and we also measured the export of two well-studied polypeptides, the periplasmic enzyme invertase (Carl-

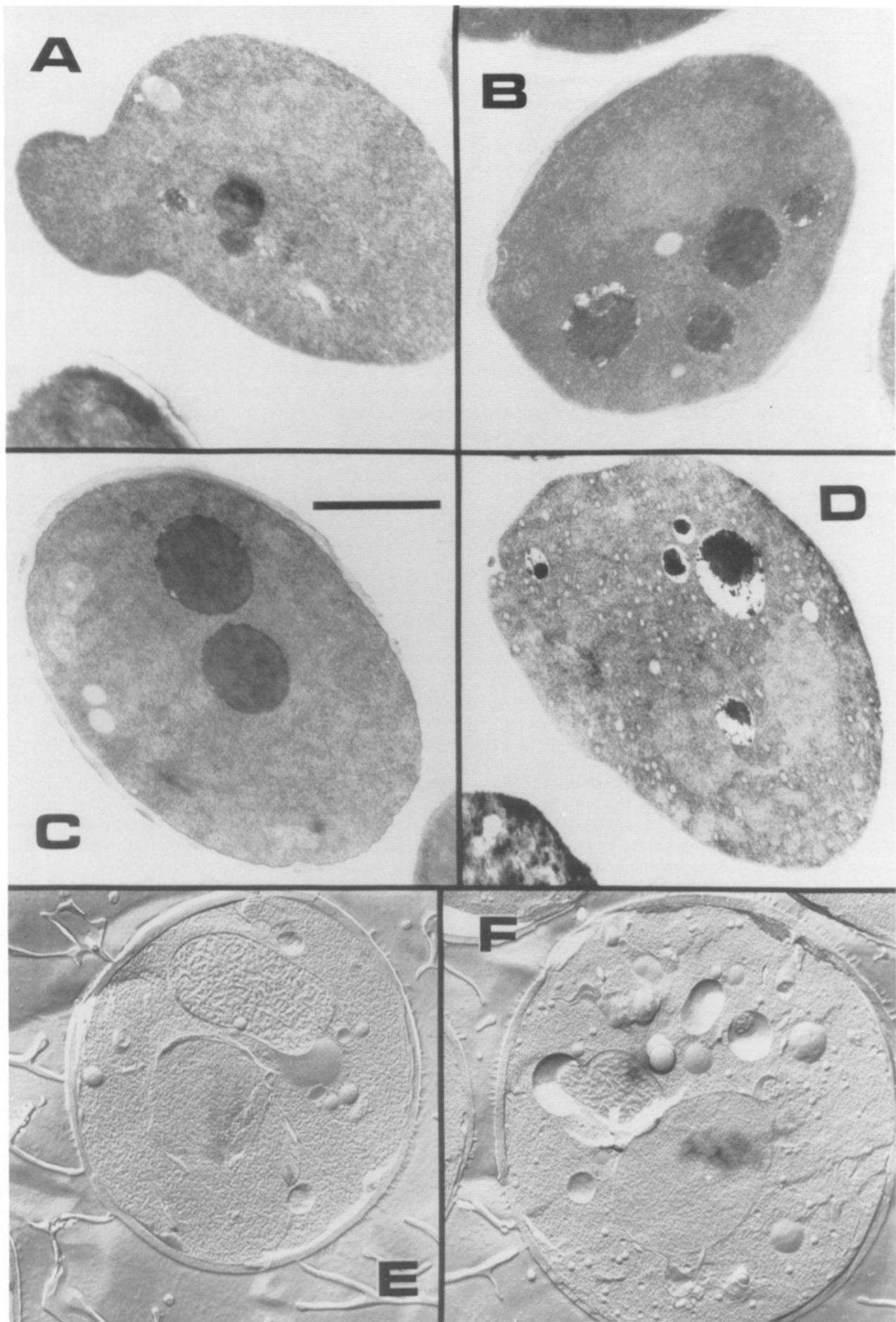


Figure 8. EM of mutant and wild-type cells. (A–D) Wild-type (A and C) or *myo2-66* mutant (B and D) cells were visualized by thin-section EM after growth at 23°C (A and B) or after 3 h at 36°C (C and D). (E and F) *myo2-66* mutant cells after growth at 23°C (E) or after 3 h at 36°C (F) were examined by freeze fracturing. Bar, 1 μm .

son and Botstein, 1982) and the mating pheromone α -factor (Bücking-Throm et al., 1973; Julius et al., 1984).

Invertase is synthesized in two forms: an intracellular, nonsecreted form produced constitutively, and a secreted, extracellular form that is only synthesized under conditions of low glucose concentration. The invertase that is destined for export is processed through the ER and Golgi system to become packaged into vesicles and transported to the growing bud (Esmon et al., 1981). To measure invertase export, proliferating wild-type and *myo2-66* mutant cells were transferred to medium containing a low glucose concentration (0.1%). A portion of the population then was incubated at the restrictive temperature and invertase secretion was assessed. As shown in Fig. 7, *myo2-66* mutant cells retained significant capacity to secrete invertase even at the restrictive temperature.

The *myo2-66* mutant cells also secreted α -factor at the restrictive temperature. The yeast mating pheromones, *a*-factor and α -factor, are secreted by distinct mechanisms (Julius et al., 1984; McGrath and Varshavsky, 1989). Although *a*-factor export is mediated by a yeast homologue of the mammalian P-glycoprotein (McGrath and Varshavsky, 1989), α -factor is secreted by the same pathway that secretes invertase (Julius et al., 1984). To assess the activity of each of these pathways, we followed the procedure of McGrath and Varshavsky (1989). These assays showed that the secretion of α -factor by *MAT α* mutant cells via the classical secretion pathway, and of *a*-factor by *MAT α* mutant cells via the P-glycoprotein-mediated pathway, was in each case unimpeded (data not shown). These direct tests show that the secretion pathway remains functional in mutant cells. Indeed, the isotropic pattern of surface growth exhibited by arrested mutant cells also provides evidence that secretion continues at the restrictive temperature.

The unbudded phenotype and continued secretion shown by mutant cells suggests that it is the vectorial transport of secretory vesicles, necessary to localize secretion at the site of bud initiation, that is impaired. Indeed, thin sectioning and freeze fracturing showed that incubation of *myo2-66* mutant cells at the restrictive temperature resulted in the marked accumulation of vesicles within the cytoplasm (Fig. 8, *D* and *F*). At the restrictive temperature the delivery of vesicles to the cell surface thus is abnormal. However, the significant bulk secretion shows that secretory vesicles eventually arrive at the cell surface. These findings suggest that vesicles make their way to the plasma membrane of mutant cells, but slowly. The resultant longer transit time consequently leads to the accumulation of transport vesicles.

Discussion

Myosins belong to a class of proteins that provide mechanochemical forces along many cytoskeletal structures (Kiehart, 1990; Vale and Goldstein, 1990; Pollard et al., 1991). These force-generating molecules facilitate a wide variety of cellular movements. The *MYO2* gene described here encodes a novel myosin protein that possesses the globular head domain characteristic of all myosin proteins (Fig. 3, *B* and *C*), but a unique COOH-terminal tail unlike those found for myosin II molecules (Fig. 4 *A*). Despite the apparent novelty of the COOH-terminal domain within the *MYO2* protein, the pre-

dicted amino acid sequences within the COOH-terminal region contain features that may reflect *MYO2* protein function.

Immediately downstream from the highly conserved globular head domain is a region of net basic charge (+33) that encompasses amino acid residues 750–925. This region contains a motif (Fig. 9 *D*) that resembles the calmodulin (CAM)-binding site in neuromodulin, a neural-specific CAM-binding protein (Alexander et al., 1988). The putative *MYO2* CAM-binding sites also resemble a repeated region within the p190 myosin-like protein from vertebrate brain (Espreafico, E., R. Cheney, F. Spindola, M. Coelho, D. Pitta, M. Mooseker, and R. Larson. 1990. *J. Cell Biol.* 111:167a; Cheney, R., and E. Espreafico, personal communication). Indeed, a p190 fusion protein is capable of binding calmodulin only when at least one of the p190 repeating units is present (Cheney, R., and E. Espreafico, personal communication). These potential CAM-binding sites within the *MYO2* protein suggest that Ca^{2+} may play a role in *MYO2* activity. Mutations in another gene, *CDC24*, also bring about a *myo2*-like defect in bud development (Sloat et al., 1981), and several investigations have suggested that the *CDC24* protein interacts with Ca^{2+} (Ohya et al., 1986*a,b*; Miyamoto et al., 1987). Thus the *MYO2* and *CDC24* proteins may be involved in a common Ca^{2+} -mediated process of spatial organization for bud site selection and cell surface development.

Downstream from the putative CAM-binding sites in the *MYO2* protein is a region of potential α -helical structure. The distribution of amino acid residues along these predicted α helices (Fig. 9, *A–C*) suggests by analogy that these potential α -helical domains in *MYO2* could interact with similar domains to form coiled-coil configurations. This α -helical coiled-coil structure results from the periodicity of amino acids in a seven-amino acid (“heptad”) repeat unit; in this heptad repeat (*abcdefg*) positions *a* and *d* are occupied by hydrophobic residues (Cohen and Perry, 1986). As shown in Fig. 9, two adjacent regions within the COOH-terminal portion of the *MYO2* protein, residues 926–981 and 1010–1086, contain heptad repeats. Both of these regions show striking periodicity, with hydrophobic residues concentrated at the *a* and *d* positions (Fig. 9 *A*). The 19 heptad repeats within these two regions also display marked periodicity in the distribution of charged residues, with negatively charged residues excluded from positions *a* and *d* and confined mainly to positions *g* and *c* (Fig. 9 *B*). The clustering of positively charged residues is similar but less pronounced. The two putative heptad-repeat regions are separated by a 28-amino acid spacer that may serve as a hinge region. This short region of potential α -helical structure also is reflected in the pattern of alignments in the dot-matrix plot displayed in Fig. 4 *A*. Over an amino acid region encompassing residues 926–1086, the COOH-terminal region of the *MYO2* protein reflects sequence similarities with the α -helical tail domain of myosin II from *Dictyostelium* (Fig. 4 *A*), as well as from nematode and *Acanthamoeba* (data not shown). The limited extent of these potential coiled-coil domains in the *MYO2* protein clearly distinguishes this myosin isoform from the well-studied myosin II isoforms from a number of systems as well as the myosin encoded by the yeast *MYO1* gene (Watts et al., 1987). Nonetheless, the presence of the heptad repeat domains in *MYO2* suggests that the *MYO2* protein may self-associate.

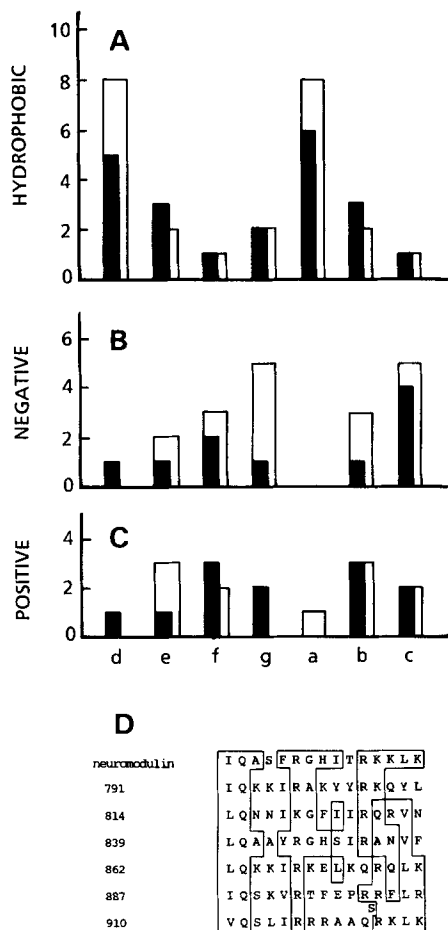


Figure 9. Amino acid distributions in the MYO2 COOH-terminal region. (A-C) The positions of hydrophobic (A), acidic (B) and basic (C) amino acids in positions a-g are shown for residues 925-981 (solid bars) and 1010-1086 (open bars). (D) MYO2 residues identical or similar to amino acids in the neuromodulin CAM-binding site are boxed. The amino acid position is indicated to the left of each MYO2 segment.

The predicted amino acid sequence of the COOH-terminal region of the MYO2 protein also displays identity (28%) to the COOH-terminal region of the recently described p190 myosin-like protein from vertebrate brain (Espreafico, E., R. Cheney, F. Spindola, M. Coelho, D. Pitta, M. Mooseker, and R. Larson. *J. Cell Biol.* 111:167a. 1990; Cheney, R., and E. Espreafico, personal communication). This region of the MYO2 gene also contains nucleotide sequences that could indicate mRNA splicing. The sequence GTATGT suggests that a potential 5'-splice junction (Langford et al., 1984) could be located within codon 1286 (Fig. 3). In addition, the downstream sequence TATTAACC, at nucleotide 4772, closely resembles the TA(C/T)TAACA consensus recognition signal for splicing in yeast (Parker et al., 1987). Use of the 3'-proximal PyAG at nucleotide 4792 as the 3'-splice boundary would generate a truncated polypeptide extended only 11 amino acids beyond the splice junction, whereas use of the next PyAG site at position 4886 would generate an extension of only eight amino acids. However, the similarity of the predicted amino acid sequences of the MYO2 and p190 pro-

teins makes it unlikely that uniform splicing of the mRNA takes place. The novel COOH-terminal domain is undoubtedly involved in functions specific to this MYO2 myosin isoform.

MYO2 Affects the Actin Cytoskeleton

The MYO2 protein probably interacts directly with actin. The MYO2 protein exhibits a typical actin-binding region in the NH₂-terminal domain and impaired MYO2 function, brought about by incubation of *myo2-66* mutant cells at a restrictive temperature, brings about marked disorganization of the actin cytoskeleton and delocalized distribution of actin cortical patches (Fig. 5 F). The rapid loss of actin cables and the presence of actin bars, as well as the random distribution of cortical actin patches, are the same effects caused by mutations in the actin gene itself (Novick and Botstein, 1985), and mutations in other genes encoding various actin-binding proteins (Drubin et al., 1988; Liu and Bretscher, 1989; Haarer et al., 1990).

MYO2 Is Necessary for Vectorial Secretion

Proper morphogenesis for the budding yeast requires directed and localized secretion to the area of the cell surface undergoing rapid growth, specifically the bud. Despite the dependence of morphogenesis on an intact secretory pathway, mutations that affect morphogenesis do not necessarily block secretion. For example, the *myo2-66* mutation described here does not prevent bulk secretion of either invertase, α -factor, or general cell surface components. Actin mutants have a similar phenotype (Novick and Botstein, 1985).

We presume that the accumulation of intracellular vesicles in *myo2-66* mutant cells (and in actin mutant cells; Novick and Botstein, 1985) reflects a decreased efficiency of vesicle transport. In wild-type cells with an active secretory pathway secretory vesicles are rarely seen (Novick et al., 1980), which is thought to reflect the rapidity with which these vesicles are transported to the cell surface (Novick et al., 1981). A mutation that slows this transport could therefore lead to an accumulation of these vesicles, as vesicles are produced at normal rates but are then slower at reaching the cell surface. A defect in efficient vectorial transport of secretory vesicles along actin cables may then liberate these vesicles to embark on a slower course of undirected diffusion to the cell surface.

The enrichment scheme that allowed the isolation of the original *myo2-66* mutant depends on the ability of mutant cells to enlarge (Prendergast et al., 1990). We show that the *myo2-66* mutation does not compromise cell growth, but does impair the selective partitioning of newly produced cell material to the developing bud, resulting in unbudded mother cells that are abnormally large and new daughter cells that are abnormally small. This enrichment scheme also allowed the isolation of many new mutant alleles of the *CDC24* gene (Prendergast et al., 1990), another gene implicated in polarized secretion and localized cell surface growth (Sloat et al., 1981). Indeed, an enlarged and unbudded cell morphology and random distribution of actin that characterizes the *myo2-66* mutation are also features of *cdc24* mutations (Sloat et al., 1981). Thus the further use of this enrichment scheme may be a profitable way to identify additional genes involved in morphogenesis.

Temporal Regulation of Bud Formation

In addition to the spatial regulation of cell growth that involves the localized delivery of new cell constituents to the site of bud development, the timing of bud formation also is regulated in the mitotic cell cycle. This temporal regulation of bud formation is a function of the central cell cycle regulatory event START (Hartwell, 1974; Hartwell et al., 1974; Singer et al., 1984). One component of this regulatory activity is a highly conserved protein kinase, p34^{cdc2}, that for the budding yeast is encoded by the *CDC28* gene (Beach et al., 1982; Draetta et al., 1987). For the budding yeast, activation of p34^{cdc2} protein kinase instigates processes that form a bud (Wittenberg and Reed, 1988).

The MYO2 protein described here contains a putative p34^{cdc2} phosphorylation site (Peter et al., 1990a), TPLK, at residue 1097 (Fig. 3). This putative phosphorylation site is within the unique COOH-terminal domain of the MYO2 protein, 10 amino acid residues from the potential coiled-coil domain. Interestingly, two conserved p34^{cdc2} phosphorylation sites that are modified in lamin also are found next to an α -helical domain; phosphorylation at these sites disrupts lamin protein interactions (Heald and McKeon, 1990; Peter et al., 1990b; Ward and Kirschner, 1990), one of the early changes associated with the onset of mitosis (see Murray, 1989; Moreno and Nurse, 1990; Nurse, 1990). By a similar mechanism, alteration by phosphorylation of the interactions between the MYO2 "motor" protein and its cellular cargo, the vesicles destined for the site of bud formation, may initiate and coordinate bud development.

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