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Authors
Clark, SW
Meyer, DI

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ACT3: A Putative Centractin Homologue in S. cerevisiae
Is Required for Proper Orientation of the Mitotic Spindle
Sean W. Clark and David I. Meyer
Department of Biological Chemistry and the Molecular Biology Institute, University of California, Los Angeles, California 90024

Abstract. As part of our ongoing efforts to understand the functional role of vertebrate centractins, we have identified a new member of the actin-related family of proteins in the yeast Saccharomyces cerevisiae using a PCR-based approach. Consistent with the current nomenclature for actin-related proteins in yeast, we propose to denote this locus ACT3. The primary amino acid sequence of Act3p is most similar to canine and human α-centractin (73% similarity/54% identity). The sequence of a genomic clone indicates ACT3 lies adjacent to and is transcribed convergently with respect to FUR1 on chromosome VIII. Molecular genetic analysis indicates ACT3 is represented by a single gene from which the corresponding mRNA is expressed at a low level compared to ACT1. Tetrad analysis of heterozygotes harboring a TRP1 replacement of the ACT3-coding region indicates ACT3 is nonessential for growth under normal conditions and at extremes of temperature and osmolarity. However, growth at 14°C indicates a spindle orientation defect similar to phenotypes recently described for yeast harboring mutations in actin, tubulin, or cytoplasmic dynein. Taken together, our data suggest that ACT3 is the S. cerevisiae homologue of vertebrate centractins.

Actin is a major component of the eukaryotic cytoskeleton. In its filamentous form, actin is responsible for muscle contraction, cytokinesis, maintenance of cell morphology, cell motility, and organelle movement (Pollard and Cooper, 1986). Even between diverse species, actins form a cohesive group of highly conserved proteins in regard to their primary sequence, length, and function. In recent years, a large number of proteins related in primary sequence to actin have been identified (Herman, 1993). Unlike conventional actins, these actin-related proteins (ARPs) form a heterogeneous group both in primary sequence, overall length, and the position of peptide insertions and deletions relative to actin. The location of ARP insertions have led some investigators to question the likelihood of ARP interaction with conventional actins and the possibility of polymer formation (Fyrberg and Fyrberg, 1993). Recently, actin was found to have a tertiary structure similar to that of HSC-70 and hexokinase (Flaherty et al., 1991; Bork et al., 1992). This has led to the suggestion, despite their distinct cellular roles, that these three proteins evolved from a common progenitor (Bork et al., 1992). Likewise, the diversity of the ARP family poses the question of whether ARPs share any functional relationship to conventional actins. It may be, that, aside from the conservation of residues involved in nucleotide binding and scaffolding for the tertiary structure, ARPs constitute a family of proteins in their own right, as distinct in function from conventional actins as HSC-70 and the sugar kinases. Accordingly, one focus of our research has been to clarify the functional relationship between certain actin-related proteins and conventional actins.

Unfortunately, the acquisition of functional details regarding this family of proteins has not progressed nearly as rapidly as new members and their homologues have been identified. ARPs have been found not only in fungi (Lees-Miller et al., 1992a; Schwob and Martin, 1992; GenBank Accession Number L21184; Harata et al., 1994; Plamann et al., 1994), but also in nematodes (J. Lees-Miller personal communication), fruit flies (Fyrberg and Fyrberg, 1993; Frankel et al., 1994), dogs (Clark and Meyer, 1992), cows (Tanaka et al., 1992; Paschal et al., 1993), and humans (Clark and Meyer, 1992; Lees-Miller et al., 1992b). In yeast, three ARPs have been identified, all of which, like conventional actin, are essential. In Saccharomyces cerevisiae, the product of the essential ACT2 gene has been proposed to act late in the cell cycle, perhaps at cytokinesis (Schwob and Martin, 1992). ARPs identified in two other genetically tractable organisms, Caenorhabditis elegans and Drosophila melanogaster, have yet to be functionally or phenotypically characterized (Fyrberg and Fyrberg, 1993; Frankel et al., 1994).

In vertebrates, biochemical studies have found an ARP (centractin/actin-RPV, henceforth referred to as centractin) associated with two macromolecular complexes: the pl50stud/dynactin complex (Lees-Miller et al., 1992b; Paschal et al., 1993) and a cytoplasmic chaperonin (Melki et al., 1993).
The pl50<sup>Cd</sup>/dynactin complex affects the activity of cytoplasmic dynein while the cytoplasmic chaperonin appears to assist in the folding of centractin and γ-tubulin. Crude preparations of cytoplasmic dynein will bind vesicles and move them along microtubules (Schoer et al., 1989). However, when further purified, cytoplasmic dynein still binds to, but can no longer translocate, vesicles (Schoer and Sheetz, 1991). This assay has led to the biochemical characterization of a cytosolic complex capable of restoring vesicle movement to purified cytoplasmic dynein (Schoer and Sheetz, 1991). This dynein-activation complex has been designated as the pl50<sup>Cd</sup> (Holzbauer et al., 1991; Paschal et al., 1993) or dynactin (Gill et al., 1991) complex. The pl50<sup>Cd</sup>/dynactin complex consists of three major constituents having molecular masses of 45, 50, and 150 kD (Gill et al., 1991; Paschal et al., 1993). The 45-kD component is now known to be centractin (Lees-Miller et al., 1992b, Paschal et al., 1993) which contributes more than half of the complex's mass (Gill et al., 1991; Paschal et al., 1993). The 150-kD component has been cloned and partially characterized (Gill et al., 1991; Holzbaur et al., 1991). It is homologous to an essential Drosophila gene, Glued, which when mutant leads to pleiotropic developmental defects particularly evident in the eye (Meyerowitz and Kankel, 1978; Harte and Kankel, 1982; Garen and Kankel, 1983; Gill et al., 1991; Holzbaur et al., 1991). The 150-kD protein also shares similarity to CLIP-170, a microtubule-endocytic vesicle linkage protein (Pierre et al., 1992). The 50-kD protein has yet been characterized. In contrast to the heterogenous size distribution of actin filaments, the pl50<sup>Cd</sup>/dynactin complex, and hence centractin, is monodisperse (Paschal et al., 1993). Immunofluorescence data using antibodies against two of the components, centractin and pl50<sup>Cd</sup> indicates a localization of the complex to the centrosome and cytoplasmic vesicles (Clark and Meyer, 1992, 1993; Gill et al., 1992; Paschal et al., 1993), a localization overlapping with that of cytoplasmic dynein (Gill et al., 1992). However, the role of centractin in this regulator of cytoplasmic dynein has not been clarified.

Recently, cytoplasmic dynein has been identified in the yeast S. cerevisiae (Esthel et al., 1993; Li et al., 1993). Surprisingly, dynein is not an essential gene and the only phenotype thus far identified does not involve vesicular trafficking. Instead, yeast lacking dynein have defects in mitotic spindle orientation and thus nuclear migration (Esthel et al., 1993; Li et al., 1993). However, despite its proposed role in the poleward movement of chromosomes (Sawin and Scholey, 1991), chromosome segregation occurs normally in dynein mutants (Esthel et al., 1993; Li et al., 1993). A similar spin-dle misorientation phenotype is found in yeast harboring mutations in actin (Palmer et al., 1992) and tubulin (Huffaker et al., 1988; Palmer et al., 1992).

Clearly, the actin-related protein family has grown rapidly since the discovery of the first nonconventional actin in yeast by Schwob et al. (1988). The size of this family presents a problem when comparing ARPs among different species. Fyrberg and Fyrberg (1993) have suggested classifying ARPs based on peptide insertions relative to conventional actins. However, relationships contingent on sequence similarities are most meaningful when born out by functional similarity. Thus, we have initiated an effort to delineate the role of centractins through their characterization in the genetically-amanable organism, S. cerevisiae.

### Materials and Methods

#### Media

*S. cerevisiae* were grown in YPD medium (1% bacto-yeast extract/2% bacto-peptone/2% dextrose) for routine work (Guthrie and Fink, 1991). For growth curves, SD medium (0.67% yeast nitrogen-base/2% dextrose) (Guthrie and Fink, 1991) plus Nutrient Mix (0.4% adenine, 0.2% histidine, 0.3% leucine, 0.2% uracil, 0.2% tryptophan, 0.3% lysine) was used. When selection was necessary, SD medium was used containing Nutrient Mix minus the appropriate nutrients. For sporulation, SPO plates were used (Guthrie and Fink, 1991). For Escherichia coli strain XLI-Blue (Stratagene Inc., La Jolla, CA) LB medium was used (Sambrook et al., 1989) and when required, ampicillin, IPTG, and XGAL were added to final concentrations of 100 μg/ml, 0.3 mM, and 50 μg/ml, respectively. For *E. coli* strain Y1090, NZY medium was used containing 0.2% maltose. λgt11 phage were grown on lawns of Y1090 on NZY plates. 1.6% Bacto agar (Difco, Detroit, MD) was used to solidify all media as necessary, except NZY/100 agarose which was solidified with 1.6% agarose (GIBCO BRL, Gaithersburg, MD).

#### PCR Amplification of the ACT3 Fragment

Primers ARP-9 and ARP-10R (see Table I) were added at 50 pmol to a 100

### Table II. Plasmids

| Plasmid          | Description                                                                 |
|------------------|----------------------------------------------------------------------------|
| pBSckS-YS1       | 3.1-kb genomic clone of *ACT3* in pBluescriptKS+ at EcoRI                  |
| pBSckS-YS2       | 4.5-kb genomic clone of *ACT3* in pBluescriptKS+ at EcoRI                  |
| pBSckS-910       | 276-bp PCR amplified product of primers ARP-9 and ARP-10R in pBluescriptKS+ (ddT vector) at EcoRV |
| pBSKS-TRP1       | 1022 bp TRP1 SspI/SnuI fragment derived from plasmid YRP17, subcloned into pBluescriptKS+ at SmaI |
| pTRP17-20        | act3ΔA1-366::TRP1 disruption/replacement plasmid                           |
Table III. *Saccharomyces cerevisiae* Strains

| Genotype                                      | Source                        |
|-----------------------------------------------|-------------------------------|
| ABYS1 MATa pral prb1 prc1 cpsl ade           | Toy et al., 1988              |
| GPY278 MATa/MATa leu2/leu2 ura3/ura3 his3-D200/his3-D200 trp1-D901/trp1-D901 ade2/+ /lys2/+ | G. Payne (University of California at Los Angeles) 1991 |
| W303 MATa/MATa leu2-3,112/leu2-3,112 ura3-1/ura3-1 his3-11,15/his3-11,15 trp1-1/trp1-1 ade2-1/ade2-1 can1-100/can1-100 | Munn et al., 1991 |
| BY101 GPY278 act3A1-366::TRP1/+               | This study                    |
| BY103 GPY278 act3A1-366::TRP1/+               | This study                    |
| BY106 W303 act3A1-366::TRP1/+                | This study                    |
| BY108 W303 act3A1-366::TRP1/+                | This study                    |
| BY101-9A MATa leu2 ura3 his3-D200 trp1-D901 ade2 act3A1-366::TRP1 (segregant of BY101) | This study                    |
| BY101-9B MATa leu2 ura3 his3-D200 trp1-D901 (segregant of BY101) | This study                    |
| BY101-9C MATa leu2 ura3 his3-D200 trp1-D901 lys2 (segregant of BY101) | This study                    |
| BY101-9D MATa leu2 ura3 his3-D200 trp1-D901 ade2 lys2 act3A1-366::TRP1 (segregant of BY101) | This study                    |
| BY103-3A MATa leu2 ura3 his3-D200 trp1-D901 (segregant of BY103) | This study                    |
| BY103-3B MATa leu2 ura3 his3-D200 trp1-D901 act3A1-366::TRP1 (segregant of BY103) | This study                    |
| BY103-7C MATa leu2 ura3 his3-D200 trp1-D901 ade2 lys2 act3A1-366::TRP1 (segregant of BY103) | This study                    |
| BY103-7D MATa leu2 ura3 his3-D200 trp1-D901 ade2 lys2 (segregant of BY103) | This study                    |
| BY106-1A MATa leu2-3,112 1 his3-11,15 trp1-1 ade2-1 can1-100 (segregant of BY106) | This study                    |
| BY106-1B MATa leu2-3,112 1 his3-11,15 trp1-1 ade2-1 can1-100 act3A1-366::TRP1 (segregant of BY106) | This study                    |
| BY106-1C MATa leu2-3,112 1 his3-11,15 trp1-1 ade2-1 can1-100 act3A1-366::TRP1 (segregant of BY106) | This study                    |
| BY106-1D MATa leu2-3,112 1 his3-11,15 trp1-1 ade2-1 can1-100 (segregant of BY106) | This study                    |
| BY106-9B MATa leu2-3,112 1 his3-11,15 trp1-1 ade2-1 can1-100 act3A1-366::TRP1 (segregant of BY106) | This study                    |
| BY106-9C MATa leu2-3,112 1 his3-11,15 trp1-1 ade2-1 can1-100 (segregant of BY106) | This study                    |
| BY108-4B MATa leu2-3,112 1 his3-11,15 trp1-1 ade2-1 can1-100 (segregant of BY108) | This study                    |
| BY108-4C MATa leu2-3,112 1 his3-11,15 trp1-1 ade2-1 can1-100 act3A1-366::TRP1 (segregant of BY108) | This study                    |
| GX3087 MATa arg1                              | G. Payne                      |
| GX3088 MATa arg1                              | G. Payne                      |

μl reaction containing 100 ng ABYS1 genomic DNA, 2.5 U Taq DNA polymerase (The Perkin-Elmer Corp., Norwalk, CT), 200 mM dNTPs (Pharmacia LKB Nuclear, Gaithersburg, MD) in a buffer containing 10 mM Tris–Cl, pH 8.8, 50 mM KCl, 1 mM DTT, 1.5 mM MgCl2, and 0.001% gelatin. The reaction was cycled 35 times in a Perkin-Elmer Thermal Cycler at 94°C, 1 min (melting), 50°C, 1 min (annealing), 72°C, 30 s (polymerization). Reactions were started at 80°C, directly from ice. The amplified products were isolated from LMP-agarose (GIBCO BRL) gels using Gelase (Epiceric Technologies, Madison, WI) according to the manufacturer’s protocol. Gel-purified amplification products were sequenced directly to confirm their identity and determine the degree of heterogeneity using the Cyclist Taq DNA Sequencing System (Stratagene Inc.) with the annealing temperature equal to that used in the initial amplification. To create plasmids pBSKS-TRP1, the amplified products were isolated from agarose gels using a Spin-X column (Costar Corp., Cambridge, MA) (Volgelstein, 1987) and digested with the appropriate restriction enzyme. Plasmid pBSKS-TRP1 was digested at SacI and BamHI, gel purified and the P17/P18 amplification products were isolated from LMP-agarose (GIBCO BRL) gels using Gelase (Epiceric Technologies, Madison, WI) according to the manufacturer’s protocol. Gel-purified amplification products were sequenced directly to confirm their identity and determine the degree of heterogeneity using the Cyclist Taq DNA Sequencing System (Stratagene Inc.) with the annealing temperature equal to that used in the initial amplification. To create plasmids pBSKS-TRP1, the amplified products were isolated from agarose gels using a Spin-X column (Costar Corp., Cambridge, MA) (Volgelstein, 1987) and digested with the appropriate restriction enzyme. Plasmid pBSKS-TRP1 was digested at SacI and BamHI, gel purified and the P17/P18 amplification product was ligated in. The ligation product was transformed into XL1-Blue, dCTP (3000 Ci/mmol; NEN, Boston, MA) and purified away from unincorporated radiolineotide by spin chromatography through G-50 (Pharmacia LKB Nuclear) by standard methods (Sambrock et al., 1989).

**Isolation and Sequencing of the ACT3 Genomic Clones, YC1 and YC2**

A S. cerevisiae λgt11 genomic library (YLI001b; Clontech, Palo Alto, CA) was screened with the cloned PCR product, pBSCKS-910, using standard methods (Sambrock et al., 1989). Two lambda phage clones were isolated and large scale preps made by the plate lysis method (Sambrock et al., 1989). Purified phage DNA was digested with EcoRI and the liberated fragments of 3.1 and 4.3 kb were subcloned into pBluescriptKS+ producing plasmids pBSKS-YC1 and pBSKS-YC2, respectively (Table II). Nested exonuclease III deletions (Sambrock et al., 1989) were produced and both clones were sequenced entirely by double-stranded sequencing on both the sense and antisense strands. Clone pBSKS-YC1 was found to be encompassed by clone pBSKS-YC2, thus only the latter was used for further study.

**Construction of the act3Δ1-366::TRP1 Disruption/Replacement Plasmid, pTRP1-20**

Sequences flanking the region of ACT3 to be deleted were amplified by PCR from plasmid pBSCKS-YC2. The primers were designed so as to use naturally occurring restriction sites where possible and otherwise incorporate new sites with minimal changes in the nucleotide sequence (see Fig. 5). For the 5' flanking region the primers were P19a and P20 (see Table I). Amplification parameters were 1 min 94°C, 1 min 50°C, 30 s 72°C for 25 cycles. The amplification products were isolated from agarose gels using a Spin-X column (Costar Corp., Cambridge, MA) (Volgelstein, 1987) and digested with the appropriate restriction enzyme. Plasmid pBSKS-TRP1 was digested at SacI and BamHI, gel purified and the P17/P18 amplification product was ligated in. The ligation product was transformed into XL1-Blue,
then plasmid DNA was resolyed by boiling, lysis, confirmed by restriction digest, and cleaved with EcoRI and XhoI for insertion of the P19A/P20 amplification product. The completed plasmid, pTRP17-20, was isolated using a Midi Prep column (QIAGEN Inc., Chatsworth, CA). Plasmid pTRP17-20 was cleaved at SacI and XhoI, then isolated from a LMP-agarose gel. The fragment was quantitated using a TKO 100 DNA fluorometer (Hoefer Sci. Instr., San Francisco, CA).

Disruption of the ACT3 Locus

Replacement was accomplished by the one-step method (Guthrie and Fink, 1991). Strains W303 and GPY278 (see Table III) were transformed by the LiOAc method (Ito et al., 1983) using 5 μg of the pTRP17-20 plasmid SacI/XhoI fragment.

Southern and Northern Blotting

DNA was separated on 1% agarose gels in 1× TAE. RNA was added to three volumes of 5% formaldehyde/1.2× MOPS/15% formaldehyde, heated 5 min at 68°C, then separated on 15% formaldehyde/1% agarose gels in 1× MOPS. Blotting was conducted by capillary transfer to ZetaProbeGT membranes (Bio Rad Labs, Hercules, CA). Gel preparation, transfer, blot fixation, hybridization, and striping on were carried out according to the manufacturer. Blots were washed twice for 5 min at 65°C in 0.2× SSC/5%SDS then twice for 30 min at 65°C in 0.2× SSC/1%SDS. Blots were exposed to X-Omat film (Kodak) with a single intensifying screen at -80°C.

Isolation of Genomic DNA, Total RNA, and Poly(A)+ RNA

Genomic DNA and total RNA were prepared according to Rose et al. (1990). Poly(A)+ RNA was selected on oligo-dT Cellulose (Boehringer Mannheim Corp., Indianapolis, IN) according to Sambrook et al. (1989).

Genetic Methods

All yeast growth was at 30°C unless otherwise noted. Sporulation and tetrad dissection were according to (Guthrie and Fink, 1991). Temperature sensitivity was scored by streaking on YPD plates at 14, 30, or 37°C; osmotic sensitivity was scored on YPD/1.5M KCl plates and grown at 30°C. Mating was tested by complementation using strains GX3087 or GX3088. Growth was measured by both absorbance at 600 nm (A600) and directly by hemocytometer. Cultures were maintained below At0o = 1.1 by dilution into fresh media and grown until the density doubled. The completed plasmid, pTRP17-20 was cleaved at SacI and XhoI, then isolated from a LMP-agarose gel. The fragment was quantitated using a TKO 100 DNA fluorometer (Hoefer Sci. Instr., San Francisco, CA).

Analysis of Yeast Morphology

For analysis of the yeast vacuole and endocytosis we utilized two methods. First we took advantage of the naturally fluorescent ade2 fluorophore (Weisman et al., 1987; Guthrie and Fink, 1991). Wild type and actΔ haploids were grown to stationary phase in YPD to accumulate the fluorophore, then cultures were shifted to the appropriate temperature by dilution into fresh media and grown until the density doubled, the fluorophore was allowed to accumulate 2 h at 30°C. Yeast were then washed three times in 1 ml ice-cold 50 mM sodium succinate/20 mM sodium azide, pH 5.0, then resuspended in 10 μl wash buffer according to the method of (Guthrie and Fink, 1991). Lucifer Yellow CH was visualized with the B2 filter set. TriX-400 and Ektachrome 400 (Kodak) were used to photograph yeast. Yeast cultures were scored for the percentage of large-budded cells, nuclear position, and spindle orientation. Nuclear position/spindle orientation was scored as abnormal if two separated nuclei were present in the mother of budded cells which were not along the mother/bud axis or if a large-budded cell had a single nucleus, not adjacent to the mother/bud neck. All other configurations were scored as normal. Unbudded cells were additionally scored for the presence of anucleate and multinucleate cells.

Results

Cloning of ACT3

We employed a PCR-based approach to identify new actin-related proteins in yeast. The primers were based on sequences which were previously shown to amplify a centrin-like sequence from Pneumocystis carinii by L. Fletcher, R. Tidwell, and C. Dykstra (GenBank Accession Number L21184). Using S. cerevisiae genomic DNA as a template, we amplified two products. Direct sequencing of the smaller fragment indicated that it represented 24% of the ACT3 coding region (see Fig. 1). The larger PCR product was not further characterized. The smaller amplification product was subcloned, then used to probe an S. cerevisiae genomic library. Two clones were obtained and sequenced, one completely encompassing the other. The larger, 4.5-kb clone was found to contain several open reading frames including ACT3 and the previously-identified FUR1 gene which has been mapped to chromosome VIII (Kern et al., 1990). ACT3 and FUR1 are conversely transcribed with the predicted polyadenylation signal of FUR1 residing in the COOH-terminal coding region of ACT3 (see Fig. 2).
ACT3 Is Most Related to the Centractin Class of ARPs

To determine the relationship of ACT3 to known ARPs, we considered both the percent identity, determined by the program BESTFIT, as well as dendrograms and alignments created by the PILEUP program (see Figs. 3 and 4). Most relevant to this study, two ARPs recently identified in the fungi (Edman et al., 1988) P. carinii (GenBank Accession Number L21184) and N. crassa (Plamann et al., 1994), which by sequence similarity would belong to the centractin class, may be better represented as a class of their own. If conventional actins are taken as a standard for comparison, centractins have a small peptide deletion near residue T229 in Actlp (see Fig. 4). In contrast, P. carinii and N. crassa centractin-like proteins have small peptide insertions near the position of the centractin deletion (see Fig. 4).

Based on percent identity and similarity, ACT3 is most closely related to the centractin class of ARPs. However, the dendrogram, which represents the clustering of similarity scores considered pairwise, places ACT3 both outside the centractin and conventional actin classes. This placement in a unique class is supported by the longer primary sequence of ACT3 (384 residues) compared to both centractins (376 residues) and conventional actins (375 residues) as well as the distribution of peptide inserts. Specifically, ACT3 does not share the deletion, relative to conventional actin, found in all centractins near Actlp residue T229 (see Fig. 4). The precise point of the centractin deletion is subjective as this region is divergent from actin. Unlike ACT1 and ACT2, introns were not found in the ACT3 coding region.

ACT3 Is a Single Copy Gene

Southern hybridization was used to determine if ACT3 has repeated or related sequences elsewhere in the genome. Genomic DNA prepared from the haploid strain, ABYS1, was independently cleaved with EcoRI and XbaI. With a probe representing the ACT3 coding region, a single hybridizing fragment was obtained in each case (Fig. 5). Neither of these fragments comigrated with those hybridizing to an ACT3 probe. These results are consistent with a single locus representing ACT3.

Replacement of the ACT3 Locus with TRPI

To eliminate the expression from the ACT3 locus, a gene replacement experiment was performed (Fig. 2). A disruption plasmid was created by subcloning the regions flanking the ACT3 coding sequence into plasmid pBSKS-TRPI containing the TRPI gene. The 3' flanking region was selected so as to leave the FUR1 polyadenylation signal intact (Kern et al., 1990), diminishing the possibility of inadvertently affecting FUR1 expression. This disruption replaced all but the 18 carboxy-terminal residues of Act3p (see Figs. 1 and 2), act3A1-366::TRP1 DNA was introduced into two diploid strains, GPY278 and W303. Genomic DNA was prepared from four transformants of each strain and correct integration of the disrupted plasmid construction and do not occur in the genome sequence.

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tion was verified by Southern blotting (Fig 6 A). In wild-type genomic DNA, cleavage with XbaI yields a 3.1-kb fragment hybridizing to an ACT3 probe (see Fig. 5, lane 4). When TRP1 replaces ACT3 (see Fig. 2), there is only a small decrease in size of the genomic region, but a new XbaI site is introduced, cleaving the 3.1-kb fragment into two smaller fragments (2.4 and 0.7 kb). These fragments hybridize only weakly to the ACT3 probe, as all but the flanking sequences have been replaced with TRP1. Thus, Fig. 6 A, lanes 1, 2, and 4 contain correct integrants whereas lane 3 does not. Confirmed diploids were sporulated and dissected. Two representative transformants from each strain were sporulated and tetrads were scored for segregation of auxotrophic markers including the Trp÷-marked ACT3 replacement. All transformants sporulated and in 28 tetrads, the Trp + phenotype (see Fig. 7).

Northern analysis (Fig. 6, C and D). The TRP1 replacement of ACT3 was verified by Southern blotting (Fig 6 A). In wild-type genomic DNA, cleavage with XbaI yields a 3.1-kb fragment hybridizing to an ACT3 probe (see Fig. 5, lane 4). When TRP1 replaces ACT3 (see Fig. 2), there is only a small decrease in size of the genomic region, but a new XbaI site is introduced, cleaving the 3.1-kb fragment into two smaller fragments (2.4 and 0.7 kb). These fragments hybridize only weakly to the ACT3 probe, as all but the flanking sequences have been replaced with TRP1. Thus, Fig. 6 A, lanes 1, 2, and 4 contain correct integrants whereas lane 3 does not. Confirmed diploids were sporulated and dissected. Two representative transformants from each strain were sporulated and tetrads were scored for segregation of auxotrophic markers including the Trp÷-marked ACT3 replacement. All transformants sporulated and in 28 tetrads, the Trp + phenotype (see Fig. 7).

Genomic DNA and poly(A)+ RNA prepared from tetrad segregants was further evaluated by Southern (Fig. 6 B) and northern analysis (Fig. 6, C and D). The TRP1 replacement of ACT3 was found in two of the four spores, as evident by the cleavage of the wild-type 3.1-kb fragment to 2.4- and 0.7-kb disrupted fragments, confirming normal segregation of the disrupted allele. Northern blotting of RNA from segregants uncovered three transcripts hybridizing to the ACT3 probe (Fig. 6 C). The smallest transcript most likely represents FUR1 as this sequence is represented in the probe and the transcript corresponds well with the published mRNA size (Kern et al., 1990). Considering that the FUR1 transcript signal is different in each of the four lanes and that ethidium bromide staining of northern RNA had indicated unequal RNA loading (not shown), we made a qualitative assessment of loading differences by reprobing the northern blot with ACT3 (Fig. 6 D). A comparison of the shorter exposure of the ACT3 northern with the ACT3 northern confirms that FUR1 mRNA expression levels are not disturbed by the act3 disruption. The two remaining transcripts are 1.3 kb and ~2.5 kb. The larger of these transcripts, upon longer exposure, is present in all four segregants of the tetrad and when compared with the ACT3 RNA signal, is equally represented. This transcript was also found on long exposure when probed with ACT3 (not shown). As it migrates near the residual 18S rRNA, we probed Northerns prepared with total RNA but found no increased signal strength (data not shown). As the remaining transcript is missing even on long exposures in the segregants which are phenotypically Trp+ and which carry the additional restriction site of TRP1, we conclude this transcript represents ACT3 which is wholly eliminated by the TRP1 replacement.

**Phenotypic Analyses**

**Cell Growth.** Trp+ segregants were found to be competent for mating as judged by replica plating onto a lawn of tester cells. Segregants were inspected for growth on solid media at 14, 30, and 37°C and on hyperosmotic media (1.5 M KCl). Growth rates were also examined in liquid culture at 14, 30, and 37°C (BY103-3A, BY103-8B). Fig. 8 represents the growth of wild-type and act3Δ cells at 30 and 14°C. Even after more than four doubling times at 14°C, there is no apparent difference in growth between wild-type and act3Δ cells. Growth was also monitored by direct hemocytometer counts and compared with the A600. At 14°C the number of cells per unit A600 was 1.5 × 107 (ACT3) and 1.3 × 107 (act3Δ). These values did not change over the course of the experiment, thus absorbance at 600 nm accurately reflects the increase in cell number for both wild-type and act3Δ cells. At all three temperatures, no significant difference in

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**Figure 5.** ACT3 is a single copy gene. A southern analysis of haploid wild-type strain, ABYS1, was made by independent digestion with two restriction enzymes (EcoRI, lanes 1 and 3; and XbaI, lanes 2 and 4). The blot was probed with ACT3 (lanes 1 and 2) or ACT3 (lanes 3 and 4) and washed at high stringency (0.2× SSC, 65°C). The ACT3 EcoRI fragment in lane 3 is ~7.1 kb while the ACT3 XbaI fragment in lane 4 is ~3.1 kb.

**Figure 6.** Southern and Northern analysis of parental heterozygous disruptants and their dissected tetrads. (A) representative transformants from disruption of parental diploid, W303, cleaved with XbaI and probed with ACT3. Note that one transformant (lane 3) is not integrated properly. (B-D) lanes 1 and 4 represent the disrupted members of the tetrad. (B) genomic DNA prepared from representative tetrad, (BY101-9A, BY101-9B, BY101-9C, BY101-9D) cleaved with XbaI and probed with ACT3. (C and D) poly(A)+ RNA prepared from the same tetrad as (B) probed with ACT3 (C) or ACT3 (D). Note that the smallest transcript, representing FUR1, and the largest transcript (†) are not altered by the disruption when compared to ACT3 mRNA (D). The intermediate-sized transcript, representing ACT3, is not present in the Trp+ segregants (lanes 1 and 4). ACT3 blots were exposed for 4 h (short exposure) or 35 h (long exposure). ACT3 blots were exposed for 18 h (short Northern exposure), 26 h (long Northern exposure), or 20 h (Southern).
doubling times was found between haploid strains harboring ACT3 or act3Δ-1-366::TRP1 (see Table IV). However, a subtle but reproducible difference in colony color was evident between ACT3 and act3Δ segregants harboring ade2 (e.g., BY106-1A, BY106-1B, BY106-1C, and BY106-1D). In 18 out of 20 tetrads, two were dark red, two were light red. The light red color cosegregated with Trp⁺ in each case. The two remaining tetrads had three dark red and one light red segregant which cosegregated with Trp⁺.

**Endocytosis and Vacuolar Morphology.** We also examined morphological features of the dissected tetrads grown at 30°C. These included vacuolar morphology/inheritance, visualized by the natural ade2 fluorophore, and fluid-phase endocytosis visualized by exogenously added Lucifer Yellow. When cells harboring ade2 are grown in low adenine medium they accumulate a fluorescent polymer in the vacuole (Weisman et al., 1987). Using this natural marker, we examined vacuolar morphology in wild-type and act3Δ cells (e.g., BY103-7C, BY103-7D, BY106-9B, BY106-9C, BY108-4B, and BY108-4C). We found no difference in gross vacuolar morphology nor in the inheritance of vacuoles (not shown). As a time, temperature, and energy-dependent marker of fluid-phase endocytosis, and as a secondary measure of vacuolar morphology, we monitored accumulation of Lucifer Yellow in wildtype and act3Δ segregants (e.g., BY103-3A and BY103-8B). As with the ade2 fluorophore, no differences were found in vacuolar morphology. More importantly, cells lacking Act3p had as strong a vacuolar fluorescence as wildtype indicating a similar accumulation of Lucifer Yellow (data not shown).

**Nuclear Position and Spindle Orientation.** To examine nuclear position in act3Δ and ACT3 strains at 14 and 30°C, log-phase cultures, grown at 30°C, were shifted to 14°C for at least 24 h or left at 30°C. Samples were removed, fixed, and stained with DAPI to visualize nuclei. At 30°C, nuclear positioning and division appeared normal in wildtype segregants (e.g., BY103-3A, BY101-9B, and BY106-1D) whereas act3Δ segregants had an increased number of abnormally positioned nuclei (e.g., BY103-9A, BY103-8B, and BY106-1C) (see Table V). At 14°C it was readily apparent that act3Δ, but not ACT3 segregants, had an increased percentage of large-budded cells with separated nuclei still in the mother (see Fig. 9 and Table V). The decreased temperature nearly doubled the percentage of cells with abnormal nuclear position. Surprisingly though, anucleate and multinucleate cells were not prevalent under any conditions (see Table V). More interestingly, immunofluorescence with anti-tubulin antibodies indicated that the spindle separating the nuclei was often perpendicular to, or otherwise not in line with, the mother/bud axis (see Fig. 9). In addition, astral microtubules often extended around the periphery of the cell. In other cases, spindle microtubules were fully extended causing the spindle to bend to fit within the confines of the mother cell.

**Discussion**

Considering that S. cerevisiae ACT1 and ACT2, Schizosaccharomyces pombe act2, and S. cerevisiae ARP (Harata et al., 1994) are all essential genes, it is most surprising that ACT3 is nonessential; yet, this is consistent with the phenotype of yeast lacking cytoplasmic dynein (Eshel et al., 1993; Li et al., 1993; Plamann et al., 1994; and see below). The fortuitous location of ACT3, adjacent to the FUR1 locus has allowed us to locate ACT3 on chromosome VIII. As both ACT1 (chromosome VI) and ACT2 (chromosome IV) reside on other chromosomes, apparently these actin-related pro-

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**Table V. Comparison of Nuclear Migration in ACT3 and act3Δ Strains**

| Segregant | Growth temperature | Total cells | Large budded cells | Biciliate cells | Anucleate cells | Cells with abnormal nuclear position | Cells with abnormal nuclei |
|-----------|--------------------|-------------|-------------------|----------------|----------------|------------------------------------|--------------------------|
| ACT3      | 30°C               | 498         | 79                | 1              | 0              | 2                                  | 3%                       |
| act3Δ     | 30°C               | 679         | 151               | 0              | 1              | 28                                 | 22%                      |
| ACT3      | 14°C               | 857         | 188               | 0              | 1              | 5                                  | 3%                       |
| act3Δ     | 14°C               | 1,384       | 220               | 0              | 3              | 89                                 | 40%                      |

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teins did not arise by simple duplication at a single locus. Assignment based solely on percent identity and similarity suggest ACT3 is the yeast homologue of vertebrate centractins (52% identity with human centractin). However, two pieces of evidence make such an assignment appear tenuous. First, all known centractins are 376 residues in length and have a bent spindle while in other cells, the astral microtubules were greatly extended (not shown).

Figure 9. Abnormal spindle orientation and morphology in act3Δ segregants. Yeast were grown at 30°C then shifted to 14°C for 24 h. Fixed yeast were stained with a monoclonal antibody against yeast tubulin and with DAPI to visualize nuclei. The spindle morphology of a wild-type segregant is shown for comparison wherein the spindle extends through the bud neck with a single DAPI-stained region in both the mother and bud. The cells labeled act3Δ also have elongated spindles separating the DAPI-stained regions, but the entire structure is contained within the mother cell. In some cases, the spindle microtubules extended to their full lengths, resulting in a bent spindle while in other cells, the astral microtubules were greatly extended (not shown).

As segregation of nutrient markers and their complementation by mating proved normal, we conclude there are no mutations (Novick and Botstein, 1985; Wertman et al., 1992). Thus, in this context, centractins play a role in the regulation of cytoplasmic dynein. The role of cytoplasmic dynein has recently been investigated in the yeast S. cerevisiae by disruption (Eshel et al., 1993; Li et al., 1993), where it was found to be nonessential, yet produced a nuclear migration/spindle orientation phenotype. In addition, the elimination of a novel gene, JNMI, has also been found to produce a defect similar to dynl/dhcl. Jnmp1 is found at the spindle pole, the yeast equivalent of the centrosome, and is proposed to be a component of the dynein microtubule motor (McMillan and Tatchell, 1994). It is interesting to note that a N. crassa centrin-like gene has also been reported to have an abnormal nuclear distribution (Plamann et al., 1994). Accordingly, we examined nuclear positioning in act3Δ strains and found a striking similarity with the dhcl/dynl phenotype. In cells lacking Act3p, the spindle elongates normally, but entirely within the mother cell, sometimes bending to adopt its full-length. Initially the spindle is not oriented along the mother/bud axis but must eventually pass through the bud neck prior to cytokinesis, in that anucleate and multinucleate cells are not common. Thus, it may well be that despite differences in the overall sequences of Act3p and centractins, these proteins may nonetheless be functionally homologous.

It is worth noting that a similar nuclear positioning phenotype is found in yeast harboring mutations in actin or tubulin; however, there are differences. Specifically, the act1-4, temperature-sensitive mutation leads to the creation of a large percentage of binucleate cells at the nonpermissive temperature, a phenotype not found in ACT3 mutants. Tub2-401 leads to a selective loss of astral microtubules at 18°C (Sullivan and Huffaker, 1992) and a spindle misorientation phenotype; however, this produces multinucleate and anucleate cells with a concomitant increase in chromosome loss not found in dynl/dhcl nor act3 mutants.

An important difference between mutations in ACT1, TUB2, DYN1/DHCL, JNMI, and ACT3 is that cells lacking Act3p do not have an increased doubling time relative to wild-type cells. In the case of act1 and tub2, it is clear that cytokinesis does not pause for the correction of spindle orientation and binucleate/anucleate cells are produced (Palmer et al., 1992; Sullivan and Huffaker, 1992). On the other hand mutations in DHCL/DYN1 or JNMI do not result in binucleate/anucleate cells, but, at least in the case of JNMI, accumulate large-budded cells. In this case cytokinesis apparently pauses while the spindle reorients, leading to an increased doubling time in mutant cells (McMillan and Tatchell, 1994). act3 is similar to dhcl/dynl and jnml in that anucleate and binucleate cells are not prevalent. Thus, it can be inferred that the cells must eventually reorient their spindle prior to cytokinesis. Surprisingly, however, act3Δ cells
do not have an increased doubling time as compared with wildtype and large budded cells do not accumulate. A similar situation has been found in yeast cells lacking dynein by one group (Eshel et al., 1993) but a second group has found conflicting data (Li et al., 1993). How a population of cells with misoriented spindles can have a normal doubling time without producing anucleate or binucleate cells is not clear at this time.

Considering also that the vertebrate endocytic pathway requires cytoplasmic dynein and microtubules (Bomsel et al., 1990; Aniento et al., 1993) and that yeast vacuolar integrity depends on intact microtubules (Guthrie and Wickner, 1988), ARPs which interact with dynein or the cytoskeleton might lead to defects in endocytosis or vacuolar morphology when absent. Analysis of fluid phase endocytosis and vacuolar morphology failed to reveal any differences in strains lacking Act3p. This may be a reflection of the use of actin, not tubulin, for endocytosis in yeast (Kubler and Riezman, 1993). The subtle color change we noted in ade2act3Δ segregants may reflect a change in vacuolar morphology or function but we believe it is more likely to be due to the biochemical interaction of the tyrophitin and adenine biosynthetic pathways. It is conceivable that the presence of TRP1 in act3Δ segregants could drain substrates, such as phosphoribosylpyrophosphate, from the adenine biosynthesis pathway (Jones and Fink, 1982) decreasing the accumulation of the fluorophore, poly(ribosylaminomimidazole).

The prospect of a yeast centractin homologue opens new avenues of investigation concerning centractin function. Making the most of the genetic tractability of yeast, homologues of the p50/twist/dynactin complex polypeptides or other interacting proteins can be identified. Furthermore, genetic interaction with the actin and tubulin cytoskeletons as well as cytoplasmic dynein can be analyzed. It is worth noting that synthetic lethality is not observed between dhcl/dyml and jmnl, yet double mutants harboring either jmnl or dhcl/dyml and cin8, a kinesin-like protein, do result in lethality (McMillan and Tatchell, 1994). Finally, it will be most interesting to determine, through biochemistry and immunocytochemistry, if Act3p, like vertebrate centractins, is found primarily in a 20S cytosolic complex or associated with the spindle pole body.

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