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Screens for Extragenic Mutations That Fail to Complement *act1* Alleles Identify Genes That Are Important for Actin Function in *Saccharomyces cerevisiae*

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ABSTRACT

Null mutations in *SAC6* and *ABP1*, genes that encode actin-binding proteins, failed to complement the temperature-sensitive phenotype caused by a mutation in the *ACT1* gene. To identify novel genes whose protein products interact with actin, mutations that fail to complement *act1-1* or *act1-4*, two temperature-sensitive alleles of *ACT1*, were isolated. A total of 14 extragenic noncomplementing mutations and 12 new alleles of *ACT1* were identified in two independent screens. The 14 extragenic noncomplementing mutations represent alleles of at least four different genes, *ANC1*, *ANC2*, *ANC3* and *ANC4* (Actin NonComplementing). Mutations in the *ANC1* gene were shown to cause osmosensitivity and defects in actin organization; phenotypes that are similar to those caused by *act1* mutations. We conclude that the *ANC1* gene product plays an important role in actin cytoskeletal function. The 12 new alleles of *ACT1* will be useful for further elucidation of the functions of actin in yeast.

STUDIES of the phenotypes of temperature-sensitive actin mutants (NOVICK and BOTSTEIN 1985; READ, OKAMURA and DRUBIN 1992) and of the intracellular organization of actin structures (ADAMS and PRINGLE 1984; KILMARTIN and ADAMS 1984) have implicated the *Saccharomyces cerevisiae* actin cytoskeleton in the establishment and maintenance of polarized secretion and cell growth. In budding yeast, as in all eukaryotic organisms, the cellular functions of actin are carried out with the help of an array of actin-binding proteins and regulatory factors [reviewed in DRUBIN (1990)]. A complete understanding of how cytoskeletal organization is controlled requires identification and characterization of the many proteins that modulate the functions of actin in the cell. Actin binding proteins and regulatory factors have been identified in yeast using a variety of approaches. For example, the actin filament stabilizing protein tropomyosin (LIU and BRETSCHER 1989a) was identified because it shares biochemical properties with tropomyosins previously identified in other species. Proteins such as Abp1p (DRUBIN, MILLER and BOTSTEIN 1988) and fimbrin (DRUBIN, MILLER and BOTSTEIN 1988; ADAMS, BOTSTEIN and DRUBIN 1989, 1991) were identified on the basis of their capacity to bind actin filaments *in vitro*. The phenotypes caused by deleting or overexpressing the genes that encode these actin binding proteins have provided direct evidence for the contribution of these proteins to actin cytoskeletal function *in vivo*.

Genes that encode actin binding proteins have also been identified in genetic selections designed to isolate mutations that interact genetically with alleles of the single actin gene *ACT1*. For example, alleles of the *SAC6* (Suppressor of ACtin) gene were identified as dominant suppressors of the temperature-sensitive phenotype caused by the *act1-1* allele (ADAMS and BOTSTEIN 1989). The product of the *SAC6* gene is yeast fimbrin, an actin filament bundling protein that was identified independently on the basis of its ability to bind actin filaments (DRUBIN, MILLER and BOTSTEIN 1988; ADAMS, BOTSTEIN and DRUBIN 1989, 1991). Genetic selections have also identified new genes encoding novel proteins that are important for cytoskeletal function. For example, alleles of the *SAC1*, *SAC2*, *SAC3*, *SAC4* and *SAC5* genes were identified as mutations that suppress the temperature-sensitive growth phenotype caused by the *act1-1* allele and simultaneously acquire a cold-sensitive growth phenotype (NOVICK, OSMOND and BOTSTEIN 1989). Alleles of the *SAC7* gene were isolated in a similar selection for mutations that suppress the temperature-sensitive phenotype caused by the *act1-4* allele (DUNN and SHORTLE 1990). Although the nature of the molecular interactions between the *SAC1-SAC5* and *SAC7* gene products and actin have not been determined, the phenotypes of the *sac1*, *sac2*, *sac3* and *sac7* mutant strains suggest that the *SAC1*, *SAC2*, *SAC3* and *SAC7* gene products participate in the functions of the actin cytoskeleton.

The isolation of the *SAC1–SAC7* genes not only highlights the success of genetics in identifying poten-

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tially interacting proteins, but demonstrates that employing different genetic strategies can often lead to the identification of mutations in different genes. Therefore, new genetic approaches may lead to the identification of novel components of the actin cytoskeleton. To take advantage of this potential, we screened for extragenic mutations that fail to complement the temperature-sensitive phenotype of *act1* alleles.

We chose this approach because extragenic mutations that fail to complement one another can reside in genes whose protein products participate in a common process or interact as components of a common structure [reviewed in FULLER *et al.* (1989)]. The isolation of extragenic noncomplementing mutations as a method of identifying interacting cytoskeletal gene products has been of interest since STEARNS and BOTSTEIN (1988) and HAYS *et al.* (1989) isolated mutations in α-tubulin genes that fail to complement mutations in β-tubulin genes. The α-tubulin and β-tubulin proteins physically associate to form a heterodimer that is the subunit of a microtubule. In addition to alleles of tubulin genes, mutations in nontubulin genes that behave as extragenic noncomplementing mutations were also isolated in Drosophila. The phenotypes caused by these mutations suggest that they might reside in genes that encode functional components of the tubulin cytoskeleton (REGAN and FULLER 1988, 1990). Isolation of extragenic noncomplementing mutations might therefore prove to be a generally useful approach for the identification of interacting gene products.

In this report we demonstrate that mutations in *SAC6* and *ABP1*, genes that encode proteins that bind to actin *in vitro* and affect actin organization and function *in vivo* (DRUBIN, MILLER and BOTSTEIN 1988; ADAMS, BOTSTEIN and DRUBIN 1989, 1991), fail to complement the temperature-sensitive phenotype caused by the *act1-4* allele. Screens for mutations that fail to complement the temperature-sensitive phenotype caused by *act1-1* and *act1-4* have identified multiple extragenic noncomplementing (*anc*, *a*ctin *n*on*c*omplementing) mutations and new alleles of *ACT1*. Mutations in one of these genes (*ANC1*) affect actin organization, demonstrating that the *ANC1* gene product participates in actin function *in vivo*.

MATERIALS AND METHODS

Media and strains: Media for yeast growth and sporulation were as described by (ROSE, WINSTON and HIETER 1990), with the exception of benomyl plates, which were as described by (STEARNS and BOTSTEIN 1988). Benomyl was a generous gift of E. I. du Pont de Nemours and Co., Inc. To test for an osmosensitive phenotype, cells were grown on YEPD plates containing either 0.9 M NaCl, 1.2 M KCl, or 1.8 M sorbitol. The yeast strains used in this study are derivatives of strain S288C and are listed in Table 1.

Genetic techniques and yeast transformation: Yeast mating, sporulation and tetrad analysis were performed as described by (ROSE, WINSTON and HIETER 1990). Growth on plates was scored by spotting suspensions of cells in water onto plates using a 32-point inoculator. Yeast cells were transformed with DNA by the lithium acetate method of (ITO *et al.* 1983) as modified by (SCHIESTL and GIETZ 1989). Transformants were plated onto SD plates supplemented with the appropriate nutrients to select for cells carrying the plasmid. Genetic distances were calculated using the formula: genetic distance in centiMorgan (cM) = 50(TT + 6NPD)/(TT + PD + NPD), where TT, PD and NPD are the number of tetratype, parental ditype and nonparental ditype tetrads, respectively.

Isolation of mutants: The procedure for isolating mutants was similar to that described by (STEARNS and BOTSTEIN 1988). Cells of the strain DDY5 (*act1-1* screen) or DDY186 (*act1-4* screen) were mutagenized using ethyl methanesulfonate (EMS) according to (ROSE, WINSTON and HIETER 1990). Aliquots of mutagenized cultures were plated onto YEPD plates to determine the percent viability of the cells. Aliquots with 10% or 20% viable cells were plated onto YEPD plates to give a density of approximately 150 colonies per plate, and these master plates were incubated at 20° for 7 days. Colonies were mated by replica plating to fresh lawns of strains DDY16 and DDY180 (*act1-1* screen) or DDY266 and DDY267 (*act1-4* screen) on SD plates supplemented with the appropriate nutrients to select for diploid cells. Plates were incubated at 20° for 20–24 hr to allow cells to mate, then incubated at 37°, the restrictive temperature.

At 36–48 hr, plates were examined for colonies that formed viable diploids when crossed to wild-type cells but not to *act1* mutant cells. These colonies were candidate noncomplementing mutants. Candidates were picked from the master plate and retested for their noncomplementing phenotype by spotting suspensions of cells in water onto tester lawns on SD plates as described above to select for diploid cells. Plates were incubated at 20° for 20–24 hr to allow cells to mate, transferred to 37°, and the noncomplementing phenotype was scored after 36–48 hr. All subsequent scoring of the noncomplementing phenotype was performed in this manner. Tester strains used for retesting and scoring were DDY190, DDY375, DDY194 and DDY208 for the *act1-1* screen, and DDY266, DDY267, DDY268, DDY269 for the *act1-4* screen.

Immunofluorescence: Yeast cells were grown to early log phase in YEPD media. Fixation and immunofluorescence procedures were carried out as described by DRUBIN, MILLER and BOTSTEIN (1988). The affinity purified antibodies that recognize yeast actin (DRUBIN, MILLER and BOTSTEIN 1988) were used at a 1:25 or 1:50 dilution. Fluorescein-conjugated anti-heavy and -light chain secondary antisera were obtained from Organon Teknika-Cappel (Malvern, PA).

RESULTS

Mutations in actin-binding protein genes fail to complement mutant alleles of *ACT1*: We tested whether null mutations in *ABP1*, *TPM1* (*tropomyosin*) and *SAC6*, genes that encode actin-binding proteins (DRUBIN, MILLER and BOTSTEIN 1988; LIU and BRETSCHER 1989a; ADAMS, BOTSTEIN and DRUBIN 1989), fail to complement two recessive temperature-sensitive (Ts^-) alleles of *ACT1*, *act1-1* and *act1-4*. The specific mutations tested were: *abp1::LEU2* (HOLTZ-

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source or Reference
DDY5	α, *his4-619, leu2-3,112*	This laboratory
DDY7	α, *ura3-52, his4-619, tub2-201*	This laboratory
DDY8	**a**, *ura3-52, his4-619, tub2-201*	This laboratory
DDY9	**a**, *ura3-52, his4-619, tub2-201*	This laboratory
DDY16	**a**, *ura3-52, his4-619, act1-1*	This laboratory
DDY17	α, *ura3-52, his4-619, act1-1*	This laboratory
DDY180 (DBY2055)	**a**, *ura3-52, his4-619*	BOTSTEIN Laboratory
DDY185 (DBY2060)	**a**, *leu2-3, 112*	BOTSTEIN Laboratory
DDY186 (DBY2061)	α, *ura3-52, leu2-3,112*	BOTSTEIN Laboratory
DDY190 (DBY2065)	**a**, *lys2-801am*	BOTSTEIN Laboratory
DDY194 (DBY2352)	α, *lys2-801am*	BOTSTEIN Laboratory
DDY197	**a**, *tpm1::URA3, his4, ura3*	LIU and BRETSCHER (1989b)
DDY198	α, *tpm1::URA3, lys2, ura3*	LIU and BRETSCHER (1989b)
DDY200	**a**, *tpm1::URA3, his3, leu2, ade2, ade3, can1, sap3, ura3*	LIU and BRETSCHER (1989b)
DDY207	α, *ura3-52, ade2-101, act1-4*	T. DUNN and D. SHORTLE
DDY208 (DBY1989)	α, *lys2-803, act1-1*	BOTSTEIN Laboratory
DDY216	α, *sac6::URA3, lys2, trp1, his3, leu2, ura3*	ADAMS, BOTSTEIN and DRUBIN (1989)
DDY217	**a**, *sac6::URA3, lys2, trp1, his3, leu2, ura3*	ADAMS, BOTSTEIN and DRUBIN (1989)
DDY263	**a**, *ura3-52, lys2-801am, leu2-3,112, abp1::LEU2*	This laboratory
DDY264	α, *ura3-52, lys2-801am, ade2-101, leu2-3,112, abp1::LEU2*	This laboratory
DDY266	**a**, *ura3-52, ade2-101*	This study[a]
DDY267	**a**, *ura3-52, ade2-101, act1-4*	This study[a]
DDY268	α, *ura3-52, ade2-101*	This study[a]
DDY269	α, *ura3-52, ade2-101, act1-4*	This study[a]
DDY271	**a**, *his4-619, leu2-3,112, act1-4*	This study[a]
DDY322	α, *ura3-52, leu2-3,112, his3Δ200, abp1::LEU2*	This laboratory
DDY354	**a**, *ura3-52, leu2-3,112, his3Δ200, cry1, tub2-201, ACT1::HIS3*	WERTMAN, DRUBIN and BOTSTEIN (1992)
DDY375	**a**, *lys2-803, act1-1*	This laboratory
DDY376	**a**, *ura3-52, leu2-3,112, act1-209*	This study
DDY377	**a**, *his3Δ200, leu2-3,112, ura3-52*	This laboratory
DDY378	**a**, *ura3-52, lys2-801am*	This laboratory
DDY379	α, *ura3-52, lys2-801am*	This laboratory
DDY380	α, *ura3-52, his4-619, act1-211*	This study
DDY381	**a**, *ura3-52, leu2-3,112, act1-211*	This study
DDY382	**a**, *ANC1:URA3:ANC1, ura3-52, his4-619*	This laboratory
DDY547	**a**/α, *ura3-52/ura3-52, his4-619/+, +/leu2-3,112*	This laboratory
DDY548	**a**/α, *anc1-1/anc1-1, ura3-52/ura3-52, his4-619/+, +/leu2-3,112*	This laboratory
DDY555	**a**/α, *anc1Δ1::HIS3/anc1Δ1::HIS3, ade2/+, can1/can1, +/cry1, leu2-3, 112/leu2-3,112, ura3-52/ura3-52*	This laboratory
DDY556	**a**/α, *act1-1/act1-1, ura3-52/ura3-52, his4-619/his4-619*	This laboratory

[a] Derived from crossing strain DDY207, obtained from T. DUNN and D. SHORTLE, to DDY185.

MAN, YANG and DRUBIN 1993), which causes no noticeable phenotype; *sac6::URA3* (insertion #26, ADAMS, BOTSTEIN and DRUBIN 1991), which causes a recessive Ts⁻ phenotype; and *tpm1::URA3* (LIU and BRETSCHER 1989b), which causes slow growth and a recessive Ts⁻ phenotype. The *abp1* (DDY322, DDY263, DDY264), *sac6* (DDY216, DDY217), and *tpm1* (DDY197, DDY198, DDY200) strains were mated to *act1-1* (DDY16, DDY17), *act1-4* (DDY269, DDY271) and *ACT1* (DDY354, DDY377, DDY379) strains and the resulting diploids were tested for growth at the nonpermissive temperature (37°). The results are shown in Figure 1. Both the *sac6::URA3* and the *abp1::LEU2* mutations complement *act1-1* and fail to complement *act1-4*. The *tpm1::URA3* mutation, despite causing more severe growth defects than the *sac6::URA3* or *abp1::LEU2* mutations, complements *act1-1* and weakly complements *act1-4*.

The above results indicate that screening for extragenic mutations that fail to complement temperature-sensitive actin alleles should lead to the isolation of mutations in known cytoskeletal genes, and might lead to the identification of new cytoskeletal genes. Furthermore, the allele specificity of the extragenic noncomplementing interaction suggests that screens for extragenic mutations that fail to complement different *act1* alleles might lead to the identification of mutations that reside in different genes.

Isolation of noncomplementing mutations: Two independent genetic screens similar to that employed by STEARNS and BOTSTEIN (1988) were used to isolate mutations that fail to complement *act1-1* and *act1-4*

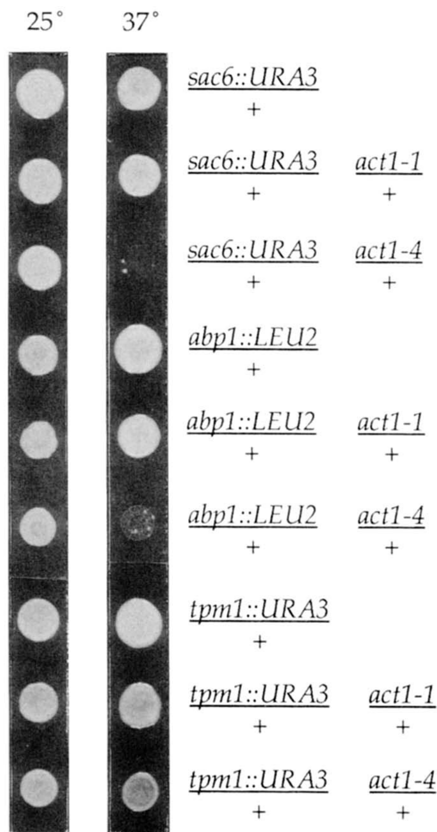

FIGURE 1.—Null mutations in genes that encode actin binding proteins fail to complement the Ts⁻ phenotype caused by the *act1-4* allele. The *sac6::URA3* and *abp1::LEU2* mutations fail to complement *act1-4* and complement *act1-1*, and the *tpm1::LEU2* mutation complements both *act1-1* and *act1-4*. The *abp1::LEU2*, *sac6::URA3* and *tpm1::LEU2* mutant strains were mated to *act1-1* and *act1-4* strains and the resulting diploids were spotted onto YEPD plates. Spots were photographed after 40–50 hr at 37°.

(see MATERIALS AND METHODS). Briefly, EMS mutagenized haploid yeast colonies were replica plated onto lawns of *ACT1* and *act1* cells, allowed to mate at the permissive temperature (20°), and then shifted to the nonpermissive temperature (37°). Colonies that formed viable diploids at 37° when crossed to the *ACT1* but not to the *act1* cells were candidate noncomplementing mutants. Because these candidate mutants form viable colonies at 37° when crossed to *ACT1* cells, all Ts⁻ mutations that were isolated (see below) are recessive for the Ts⁻ phenotype.

The results of the screens are summarized in Table 2. Approximately 29,000 colonies were screened for mutations that fail to complement *act1-1* and 60 noncomplementing mutant strains were identified. Independently, 26,000 colonies were screened for mutations that fail to complement *act1-4* and 125 noncomplementing strains were identified. These 185 noncomplementing strains were then backcrossed to wild type strains to separate the *anc* (actin *n*on*c*omplementing) mutations from other EMS induced mutations and to determine whether the Anc⁻ phenotype was due to the effects of a single nuclear mutation. After sporulating the resulting diploids and dissecting tetrads, 27 of the 185 mutant strains–11 from the *act1-1* screen, 15 from the *act1-4* screen–showed 2:2 segregation of the Anc⁺:Anc⁻ phenotypes (data not shown), indicating that the Anc⁻ phenotype is caused by a single nuclear mutation. Some of the remaining 159 noncomplementing mutant strains displayed either very poor sporulation efficiency or poor spore viability. Others failed to show 2:2 segregation of the Anc⁺:Anc⁻ phenotypes, suggesting that the Anc⁻ phenotype was caused by the combinatorial effects of more than one mutation.

Linkage of *anc* mutations to *ACT1*: To test whether any of the *anc* mutations were genetically linked to *ACT1*, we determined whether they were linked to the *tub2-201* mutation, which is tightly linked to *ACT1* (1 cM; THOMAS, NEFF and BOTSTEIN 1985). The *anc* mutants were crossed to *tub2-201* mutants (DDY7, DDY8, DDY9) and the segregation of Anc⁻ and benomyl resistant (Ben^R, conferred by *tub2-201*) phenotypes was followed in tetrad analysis (Table 3). A total of 12 strains (seven containing mutations isolated in the *act1-1* screen, five containing mutations isolated in the *act1-4* screen) contained *anc* mutations that are very tightly linked to *tub2–201* (from 0 to 3.2 cM for 11 of the mutations, and 7.9 cM for an exceptional case, *act1-211*, which is discussed in the footnotes of Table 3) and are therefore most likely alleles of *ACT1*. These new actin alleles were designated (and from here on will be referred to as) *act1-200* through *act1-211*. The remaining 14 *anc* mutations are not linked *tub2–201* (Table 3) and are therefore extragenic noncomplementing mutations.

Temperature-conditional phenotypes of *anc* mutations and new *act1* alleles: The growth of haploid *anc* and *act1* strains was examined at 14° for a Cs⁻ phenotype, at 20° (the permissive temperature) and at 37° for a Ts⁻ phenotype. Five *anc* mutations confer recessive temperature-conditional phenotypes. Two of these (*anc2-1* and *anc4-1*) confer only recessive Ts⁻ phenotypes, and three (*anc1-1*, *anc1-2* and *anc3-1*) confer recessive Ts⁻/Cs⁻ (temperature-sensitive and cold-sensitive) phenotypes (Table 4), although the Cs⁻ phenotypes of *anc1-1* and *anc1-2* mutants and the Ts⁻ phenotype of *anc3-1* mutants are characterized by slow growth rather than lack of growth at the restrictive temperature (Table 4). The remaining nine extragenic mutations (*nc50* through *nc58*) do not cause a temperature-conditional phenotype and from here on are designated *nc* instead of *anc* because they were not placed into linkage groups (see below). Of the 12 *act1* mutations isolated, three (*act1-200*, *act1-201*, *act1-211*, Table 4) confer a Ts⁻ phenotype and none confer a Cs⁻ phenotype. Backcrossing and tetrad analysis confirmed that the temperature-conditional phenotypes are recessive and that the mutations that

TABLE 2

Results of screens for mutations that fail to complement *act-1* and *act1-4*

	act1-1	*act1-4*	Total
Colonies screened	29,000	26,000	55,000
Mutations isolated	11	15	26
Mutations in *ACT1*[a]	7 (1 Ts⁻)	5 (2 Ts⁻)	12 (3 Ts⁻)
Extragenic mutations[a]	4 (2 Ts⁻/Cs⁻)	10 (2 Ts⁻, 1 Ts⁻/Cs⁻)	14 (2 Ts⁻, 3 Ts⁻/Cs⁻)

[a] The number of mutations in each category that cause a temperature conditional phenotype is listed in parentheses. Ts⁻ means no growth or slow growth at 37°, Cs⁻ means no growth or slow growth at 14°, and Ts⁻/Cs⁻ means no growth or slow growth at both 37° and 14°.

TABLE 3

Linkage data from crosses of *act1* and *anc* mutants to *tub2-201* mutants

act1 mutation	Tetrad type[a] PD	TT	NPD	*anc* mutation[c]	Tetrad type[a] PD	TT	NPD
act1-200	41	0	0	*anc1-1*	5	9	5
act1-201	41	2	0	*anc1-2*	3	7	1
act1-202	40	2	0	*anc2-1*	2	6	0
act1-203	44	1	0	*anc3-1*	1	3	2
act1-204	36	2	0	*anc4-1*	2	4	2
act1-205	32	1	0	*nc50*	1	5	0
act1-206	26	1	0	*nc51*	1	4	0
act1-207	29	2	0	*nc52*	5	7	0
act1-208	35	3	0	*nc53*	1	5	2
act1-209	37	0	0	*nc54*	0	8	0
act1-210	28	2	0	*nc55*	2	8	1
act1-211[b]	32	6	0	*nc56*	2	4	2
				nc57	0	7	0
				nc58	2	5	0

[a] Tetrad types isolated from sporulated *act1*/+ +/*tub2-201* or *anc*/+ +/*tub2-201* diploid cells are listed. The *act1* and *anc* mutations were identified because they cause an Anc⁻ or a temperature-conditional phenotype. The *tub2-201* mutation was identified because it causes resistance to the microtubule destabilizing drug benomyl (Ben[R]). PD is parental ditype, TT is tetratype, NPD is nonparental ditype.

[b] For *act1-211*, most of the TT tetrads were produced from the first backcross of the heavily mutagenized *anc* strain to the *tub2-201* strain (first backcross: 9 PD, 5 TT, 0 NPD). Very few TT tetrads were produced from the second backcross to the *tub2-201* strain (second backcross: 23 PD, 1 TT, 0 NPD), indicating that this mutation is tightly linked to *ACT1*. Since this *anc* mutation causes a recessive Ts⁻ phenotype, the *anc* mutant strains DDY380 and DDY381 were transformed with the plasmid pKFW28, a YCp50 (centromere based) plasmid that contains a single copy of the *ACT1* gene (K. WERTMAN, unpublished results). The plasmid pKFW28 rescues the Ts⁻ phenotype caused by the *anc* mutation (data not shown). This finding taken together with the linkage data indicates that this *anc* mutation is most likely an allele of *ACT1*.

[c] The *nc50, nc51, nc52, nc53, nc54, nc55, nc56, nc57* and *nc58* mutations were designated *nc* instead of *anc* because they were not placed into linkage groups (see RESULTS and Table 5).

TABLE 4

Temperature-conditional phenotypes of new *act1* alleles and *anc* mutations

Allele	Growth at[a] 14°	20°	37°	Tetrad types[b] PD	TT	NPD
act1-200	+	+	−	20	0	0
act1-201[c]	+	+	−	29	4	0
act1-211	+	+	−	41	0	0
anc1-1	±	+	−	39	0	0
anc1-2[c]	±	+	−	39	2	0
anc2-1	+	+	−	30	0	0
anc3-1[c]	−	+	±	40	3	0
anc4-1[c]	+	+	−	34	3	0

[a] + means wild type growth, ± means slow growth and − means no growth or very slow growth. Growth 14° was scored after 5–7 days. Growth at 20° was scored after 3 days. Growth at 37° was scored after 40–50 hr.

[b] This column lists tetrad types derived from the sporulation of diploid strains that are heterozygous for *act1* or *anc* alleles. The tetrad types refer to the segregation of the Ts⁻ and Anc⁻ phenotypes. The *act1* mutants were identified by scoring their Anc⁻ phenotype. For Ts⁻/Cs⁻ mutations, the mutations that cause the Cs⁻ phenotype segregate with the mutations that cause the Ts⁻ phenotype in each of at least 30 tetrads tested.

[c] For *act1-201, anc1-2, anc3-1* and *anc4-1*, all TT tetrads were derived from the first or second backcross of heavily mutagenized strains to wild-type strains. *act1-201* first backcross: 6 PD, 4 TT, 0 NPD, subsequent backcrosses: 23 PD, 0 TT, 0 NPD; *anc1-2* first backcross: 3 PD, 2 TT, 0 NPD, subsequent backcrosses: 36 PD, 0 TT, 0 NPD; *anc3-1* first backcross: 5 PD, 3 TT, 0 NPD, subsequent backcrosses: 35 PD, 0 TT, 0 NPD; *anc4-1* first backcross: 4 PD, 1 TT, 0 NPD, second backcross: 17 PD, 2 TT, 0 NPD, third backcross: 13 PD, 0 TT, 0 NPD.

cause the temperature-conditional phenotypes are very tightly linked to the *act1* and *anc* mutations (Table 4).

Linkage grouping of *anc* mutations: To determine the number of genes defined by the *anc* and *nc* mutations, we tested these mutations for genetic linkage to one another. Linkage analysis was facilitated by the cloning of the *ANC1* gene (M. WELCH and D. DRUBIN, unpublished results) and creation of the haploid strain DDY382 that has a *URA3* gene integrated at the *ANC1* chromosomal locus (*ANC1:URA3:ANC1*, M. WELCH and D. DRUBIN, unpublished results). The *ANC1:URA3:ANC1* strain was crossed to *anc* and *nc* mutant strains, the resulting diploids were sporulated, and the segregation of Ura⁺ (*ANC1:URA3:ANC1*), Ts⁻ or Cs⁻ (*anc2-1, anc3-1, anc4-1*) and Anc⁻ (*nc50* through *nc58*) phenotypes was followed in tetrad analysis (Table 5). As expected, the *anc1-1* and *anc1-2* mutations are very tightly linked to *ANC1:URA3:ANC1*. The *anc2-1, anc3-1, anc4-1* and *nc50* through *nc58* mutations are not linked to *ANC1:URA3:ANC1* and therefore are not alleles of *ANC1*. To determine whether the *anc2-1, anc3-1* and *anc4-1* mutations are alleles of the same gene, these *anc* mutant strains were crossed to each other in all

TABLE 5

Linkage of *anc* mutations to *ANC1:URA3:ANC1*, *anc2-1* and *anc4-1*

	Tetrads derived from a cross to:								
	ANC1:URA3:ANC1			*anc2-1*			*anc4-1*		
Mutation	PD	TT	NPD	PD	TT	NPD	PD	TT	NPD
anc1-1	45	0	0		Not determined			Not determined	
anc1-2	34	0	0		Not determined			Not determined	
anc2-1	1	4	3		Not determined		4	9	3
anc3-1	4	8	1	0	5	3	1	6	1
anc4-1	2	8	4	4	9	3		Not determined	
nc50	2	3	0	4	3	0	5	6	4
nc51	2	3	0		Low viability[a]			Low viability[a]	
nc52	2	10	2	3	4	1		Not scorable[b]	
nc53	1	4	3		Unlinked[c]		1	1	6
nc54	1	5	2	4	4	0	1	5	2
nc55	1	4	1	2	3	2	1	4	2
nc56	1	8	8	4	3	1	1	3	4
nc57	2	11	0	3	4	1	2	5	1
nc58	1	3	2	2	2	0		Not scorable[b]	

The Ura^+ phenotype was used to identify the *ANC1:URA3:ANC1* mutation. The Ts^- phenotype was used to identify the *anc1-1*, *anc1-2* and *anc4-1* mutations. The Cs^- phenotype was used to identify the *anc3-1* mutation. The Anc^- phenotype was used to identify the *nc50*, *nc51*, *nc52*, *nc53*, *nc54*, *nc55*, *nc56*, *nc57* and *nc58* mutations. Each mutant strain was backcrossed at least three times before linkage crosses were performed.

[a] Low viability means that extensive spore inviability made it impossible to determine whether the two *anc* mutations were linked.

[b] Not scorable means that it was difficult to simultaneously score the less severe Anc^- phenotype caused by the *nc52* and *nc58* mutations and the more severe phenotype caused by the *anc4-1* mutation because the difference between the less severe Anc^- phenotype and the Anc^+ phenotype was obscured.

[c] Tetrad types could not be exactly determined because some spores were nutritionally prototrophic and spores need to be auxotrophic to test for the Anc^- phenotype (see MATERIALS AND METHODS). One is a PD tetrad (four spores scored), one is a PD or TT tetrad (three spores scored, all are Anc^-), four are TT or NPD tetrads (three spores scored, one is Anc^+).

pairwise combinations, the resulting diploids were sporulated and the segregation of Ts^- and Cs^- phenotypes was followed in tetrad analysis (Table 5). These mutations are not tightly linked to one another and therefore define three genes–*ANC2*, *ANC3* and *ANC4*. Although we anticipated that alleles of *SAC6* and *ABP1* might have been isolated in the *act1-4* screen, none of the *anc1*, *anc2*, *anc3* or *anc4* mutations are alleles of *SAC6* or *ABP1* (VINH *et al.* 1993).

Finally, experiments were performed to determine whether the *nc50* through *nc58* mutations (which do not cause a temperature-conditional phenotype) are alleles of the *ANC2* or *ANC4* genes (the *ANC3* gene was not tested). We crossed *nc50* through *nc58* mutant strains to *anc2-1* and *anc4-1* mutant strains in all pairwise combinations, sporulated the diploids, and followed the segregation of the Anc^- phenotype in tetrad analysis (Table 5). The *nc50*, *nc52*, *nc53*, *nc54*, *nc55*, *nc56*, *nc57* and *nc58* mutations are not tightly linked to and are therefore not alleles of *ANC2*, and similarly the *nc50*, *nc53*, *nc54*, *nc55*, *nc56* and *nc57* mutations are not tightly linked to and therefore are not alleles of *ANC4*. For some crosses, diploids failed to produce viable spores (marked "low viability," Table 5) and for other crosses, the difference in severity of the Anc^- phenotype caused by different *anc* mutations made it impossible to follow the segregation of two *anc* mutations in a cross (marked "not scorable," see footnotes to Table 5). Because of these difficulties we did not determine whether the *nc51* mutation is an allele of *ANC2*, or whether the *nc51*, *nc52* or *nc58* mutations are alleles of *ANC4*.

Complementation behavior of new *act1* mutations with *act1-1* and *act1-4*: To test whether the new *act1* alleles identified in the *act1-1* noncomplementation screen also failed to complement *act1-4*, and vice versa, *act1-nc* (*act1* alleles isolated in this study) strains were mated to *act1-1*, *act1-4*, and *ACT1* tester strains and growth at 37° was evaluated. Of the 12 *act1* alleles isolated in the two screens, 11 fail to complement both *act1-1* and *act1-4*. *act1-1* and *act1-4* also fall into this group since they fail to complement one another. The remaining *act1* allele, *act1-209*, which was isolated in the *act1-4* noncomplementation screen, partially complements *act1-1* (Figure 2).

Mutations in *ANC1*, like mutations in *ACT1*, cause actin cytoskeletal defects and osmosensitivity: To determine how actin is organized in an *anc1-2* strain (DDY548) and in a control isogenic wild type strain (DDY547) grown at 25° and at 37°, immunofluorescence experiments were performed. The actin cytoskeleton in wild type yeast cells is organized asymmetrically at both 25° (Figure 3a) and 37° (Figure 3b) as described previously (ADAMS and PRINGLE 1984; KIL-

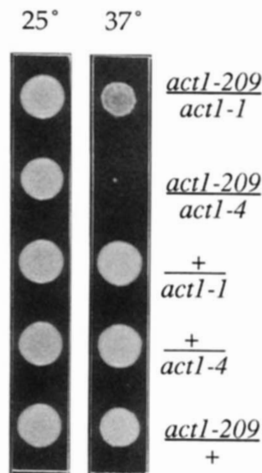

FIGURE 2.—The *act1-209* allele exhibits allele specific non-complementation. *act1-209/act1-1* (DDY376 × DDY16), *act1-209/act1-4* (DDY376 × DDY267), *act1-209/+* (DDY376 × DDY378), *+/act1-1* (DDY379 × DDY16) and *+/act1-4* (DDY185 × DDY207) diploids were spotted onto YEPD plates. Spots were photographed after 40–50 hr. The *act1-209/act1-1* diploid grows (although more slowly than the *act1-1/+* diploid) at 37° whereas the *act1-209/act1-4* diploid does not grow, indicating that *act1-209* partially complements *act1-1* but not *act1-4*.

MARTIN and ADAMS 1984). In budding cells cortical actin patches are concentrated in growing buds, and at the region of septum formation during cytokinesis. Cytoplasmic actin cables are oriented along the mother cell to bud axis. In a subpopulation of unbudded cells, cortical patches are concentrated at presumptive bud sites. Actin organization in *anc1* mutants was indistinguishable from wild type organization at 25° (Figure 3c). However, actin was disorganized in *anc1* mutants shifted to 37° (Figure 3d). Strikingly, cortical patches were delocalized in 54% of budded *anc1* mutant cells compared to 3% of budded wild-type cells at 37° (bottom two cells in Figure 3d). Cortical patches were also delocalized in 59% of unbudded *anc1* mutants compared to 32% of unbudded wild-type cells at 37°. In those budded cells with delocalized patches, cytoplasmic actin cables appeared to be oriented randomly rather than along the mother cell-bud axis.

We also tested whether mutations in *ANC1*, like mutations in *ACT1* (NOVICK and BOTSTEIN 1985; CHOWDHURY, SMITH and GUSTIN 1992), cause sensitivity to high osmotic strength growth media at 25° (osmosensitivity or Osm^s). Growth of wild type (DDY547), *act1-1* (DDY556), *anc1-1* (DDY548) and *anc1Δ1::HIS3* (a null mutation in *ANC1*, M. WELCH and D DRUBIN, unpublished results; DDY555) strains on YEPD and YEPD + 0.9 M NaCl was observed (Figure 4). Wild type, *act1-1*, *anc1-1* and *anc1Δ1::HIS3* strains grew at similar rates on YEPD (Figure 4, upper panel). However, *anc1-1* and *anc1Δ1::HIS3* strains, exhibiting growth defects similar to the *act1-1* strain, grew much more slowly than the wild-type strain on YEPD+NaCl (Figure 4, lower panel). The growth rate of *anc1-1* and *act1-1* strains on YEPD+NaCl were very similar, whereas the *anc1Δ1::HIS3* mutant strain grew more slowly than either. Similar but less dramatic growth defects were observed on YEPD + KCl and YEPD + sorbitol plates (data not shown).

DISCUSSION

Mutations in known actin-binding protein genes fail to complement actin mutations: We demonstrated that extragenic mutations that fail to complement mutations in the *S. cerevisiae ACT1* gene can reside in genes whose products are actin binding proteins. The *sac6::URA3* mutation (ADAMS, BOTSTEIN and DRUBIN 1991) and the *abp1::LEU2* mutation (HOLTZMAN, YANG and DRUBIN 1993) fail to complement the temperature-sensitive phenotype of the *act1-4* mutation. *SAC6* and *ABP1* encode proteins that bind to actin filaments *in vitro* and *in vivo*, and affect actin organization and function *in vivo* (DRUBIN, MILLER and BOTSTEIN 1988; ADAMS, BOTSTEIN and DRUBIN 1989, 1991). The fact that null alleles of these genes fail to complement *act1-4* shows that the actin cytoskeleton is sensitive to either the levels or stoichiometry of actin and actin-binding proteins.

The failure of mutations in actin cytoskeletal genes to complement *act1* alleles is a specific phenomenon. Although the *sac6::URA3* and *abp1::LEU2* mutations fail to complement *act1-4*, they complement *act1-1*. In addition, the *tpm1::URA3* mutation (LIU and BRETSCHER 1989b) complements both *act1-1* and *act1-4*. This is significant because *tpm1::URA3* mutants have much more severe growth defects than *sac6::URA3* mutants, and *abp1::LEU2* mutants have no phenotype that we have been able to detect. This gene specificity of noncomplementation suggests that the extragenic noncomplementing interaction does not result from a general deterioration of cytoskeletal function, but rather from the loss of certain combinations of proteins that make specific contributions to cytoskeletal function.

Screens for extragenic noncomplementing mutations: The above observations suggested that screening for mutations that fail to complement *act1* alleles should lead to the isolation of null alleles of *SAC6*, *ABP1* and possibly mutations in other genes that encode actin cytoskeletal proteins. In the hope of identifying new cytoskeletal genes, we performed screens designed to isolate mutations that fail to complement two temperature-sensitive alleles of *ACT1*, *act1-1* and *act1-4*. Examination of the structure of the actin monomer (KABSCH *et al.* 1990) shows that *act1-1* (Pro-32 to Leu; SHORTLE, NOVICK and BOTSTEIN 1984) and *act1-4* (Glu-259 to Val; DUNN and SHORTLE 1990) change residues that are located in different domains of the actin molecule. These changes are therefore

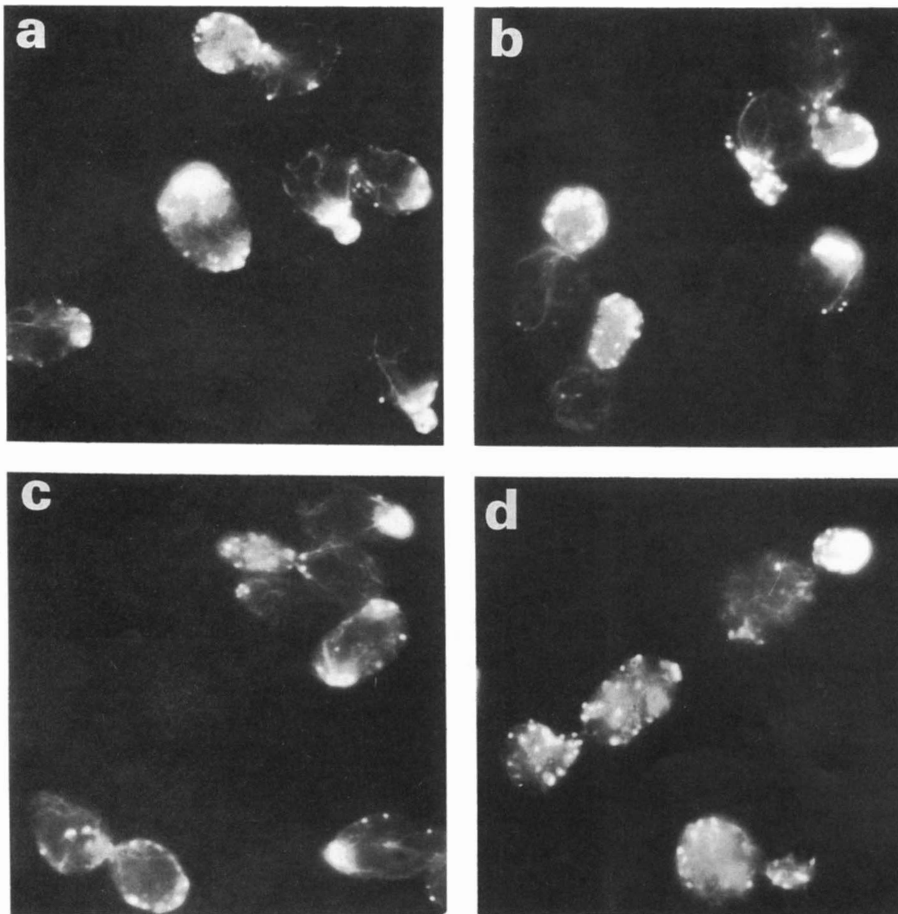

FIGURE 3.—*anc1* mutants have defects in actin cytoskeletal organization. Cortical actin structures, visualized by anti-actin immunofluorescence, are organized asymmetrically in wild-type cells at 25° (a) and 37° (b). Actin organization in *anc1* mutants is indistinguishable from wild-type actin organization at 25° (c). However, actin is disorganized in *anc1* mutant cells grown at 37° (d).

likely to have different effects on the assembly properties of actin monomers and on interactions with monomer binding proteins. Furthermore, in the atomic model of the actin filament proposed by (HOLMES *et al.* 1990), Pro-32 is located near the exposed surface of the actin filament and may be important for interactions with actin filament-binding proteins, whereas Glu-259 is located proximal to the interface between filament strands and may not directly influence interactions with actin-binding proteins. The observation that the *act1-1* and *act1-4* mutations cause unique phenotypes (DUNN and SHORTLE 1990; M. WELCH, D. VINH and D. DRUBIN, unpublished observations) supports the conclusion that each affects different aspects of actin function. Therefore, independent screens for mutations that fail to complement *act1-1* and *act1-4* are likely to identify genes that contribute to different aspects of actin function. For example, we would expect to isolate null alleles of *ABP1* and *SAC6* in the *act1-4*, but not the *act1-1* noncomplementation screen.

The screens are not likely to have identified all of the genes in which mutations that fail to complement *act1-1* or *act1-4* can be isolated. Firstly, we anticipated that temperature-conditional alleles of *SAC6* that fail to complement *act1-4* might have been isolated. However, none of the temperature-conditional mutations isolated in either the *act1-1* or *act1-4* screens are alleles of *SAC6*, *ABP1* or *TPM1* (VINH *et al.* 1993). It remains possible that *nc50*, *nc51*, *nc52*, *nc53*, *nc54*, *nc55*, *nc56*, *nc57* and *nc58* are alleles of *SAC6* or *ABP1*. Secondly, multiple mutations (2) were isolated in only one gene, *ANC1*. These facts suggest that there may be a large number of genes that can be mutated to give an Anc^- phenotype.

A high proportion of the noncomplementing mutations isolated in this study are extragenic. In the *act1-1* screen, 4 of 11 (36%) of the mutations isolated are extragenic, and in the *act1-4* screen, 10 of 15 (67%) are extragenic. A similar proportion of extragenic (approximately 50%) relative to intragenic mutations was isolated in a screen for extragenic mutations that fail to complement the Cs^- phenotype of mutations in tubulin genes (STEARNS and BOTSTEIN 1988). These results suggest that for yeast structural proteins in general, a high proportion of the mutations isolated in screens for noncomplementing mutations are likely to be extragenic.

Mutations in *ANC1* cause defects in actin organization and sensitivity to high osmolarity: The actin cytoskeleton is disorganized in *anc1* mutant cells grown at the nonpermissive temperature. This defect

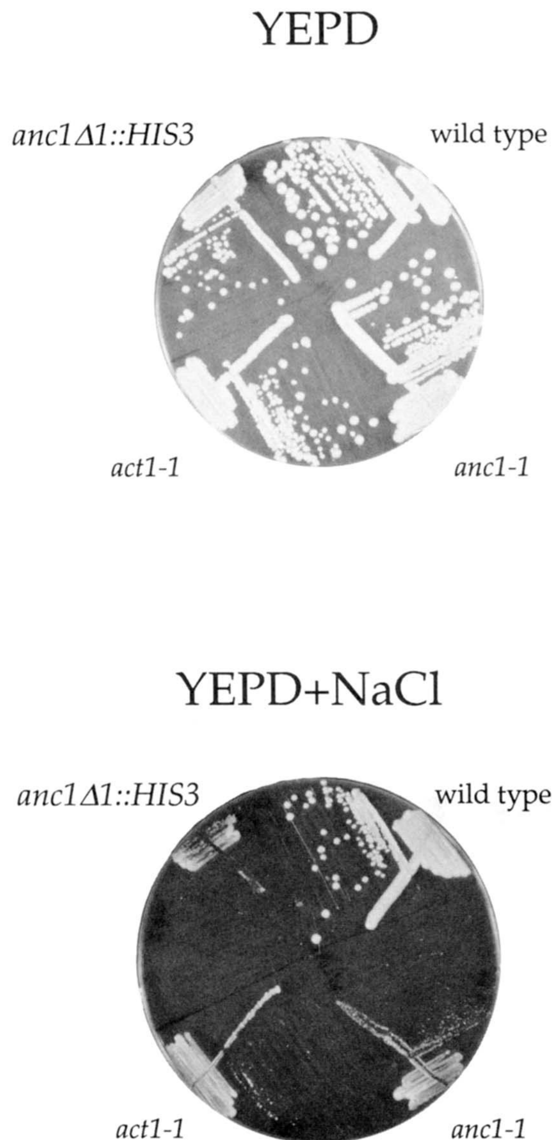

FIGURE 4.—*anc1* mutants, like *act1* mutants, are sensitive to high osmolarity. Wild type, *act1-1*, *anc1-1*, and *anc1Δ1::HIS3* strains were streaked and grown on YEPD and YEPD + 0.9 M NaCl plates at 25°. Growth on YEPD was photographed after 6 days and growth on YEPD + NaCl was photographed after 9 days.

in actin organization is similar to that caused by *act1* mutations (NOVICK and BOTSTEIN 1985), by mutations in the actin-binding protein genes *SAC6* (fimbrin; ADAMS, BOTSTEIN and DRUBIN 1991), *CAP1* and *CAP2* (capping protein α and β subunits; AMATRUDA *et al.* 1990, 1992), *PFY1* (profilin; HAARER *et al.* 1990), and by mutations in the actin suppressor genes *SAC1-SAC3* (NOVICK, OSMOND and BOTSTEIN 1989). Furthermore, mutations in *ANC1* cause an osmosensitive phenotype similar to that caused by *act1* mutations (NOVICK and BOTSTEIN 1985; WERTMAN, DRUBIN and BOTSTEIN 1992; CHOWDHURY, SMITH and GUSTIN 1992). The fact that *anc1* mutants exhibit two phenotypes that are similar to the phenotypes of *act1* mutants indicates that the *ANC1* gene product affects actin cytoskeletal function. The DNA sequence of *ANC1* indicates that it does not encode a component of the actin cytoskeleton that was identified by other genetic screens or by biochemical approaches (M. WELCH and D. DRUBIN, unpublished observations). Therefore, screens for extragenic mutations that fail to complement *act1* alleles can be used to identify new genes that are important for actin function.

New *act1* alleles: The screens for mutations that fail to complement *act1-1* and *act1-4* also identified 12 new *act1* alleles (seven from the *act1-1* screen and five from the *act1-4* screen), designated *act1-200* through *act1-211*. Three of these new *act1* alleles, *act1-200*, *act1-201*, and *act1-211* cause a recessive Ts^- phenotype. The *act1* alleles isolated in the noncomplementation screens add to the collection of mutations isolated by *in vitro* mutagenesis (SHORTLE, NOVICK and BOTSTEIN 1984; DUNN and SHORTLE 1990; JOHANNES and GALLWITZ 1991; WERTMAN, DRUBIN and BOTSTEIN 1992; COOK *et al.* 1993 and the references cited therein) and increase the number of actin alleles available for structure-function studies.

We have begun to examine the defects caused by the *act1-200* through *act1-211* alleles by testing whether these alleles complement *act1-1* and *act1-4*. One example of intragenic complementation was found. The *act1-209* allele, which causes no phenotype on its own, partially complements *act1-1* but not *act1-4*. One possible explanation for intragenic complementation is that the two mutant actins (*act1-1* actin and *act1-209* actin) co-assemble to form functional hybrid filaments. An alternative but less likely possibility is that the two mutant actins do not co-assemble, and that the two mutant filament types mediate complementary sets of cellular processes. The molecular basis for genetic interactions such as the complementation of *act1-1* and *act1-209* can be determined by characterizing the biochemical properties of purified mutant actin *in vitro* and the phenotypes caused by these mutations *in vivo*. Such experiments are likely to provide novel insights into the relationship between the structure and function of actin filaments.

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