Suppressors of Yeast Actin Mutations

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Manuscript received August 11, 1988
Accepted for publication December 10, 1988

ABSTRACT

Suppressors of a temperature-sensitive mutation (*act1-1*) in the single actin gene of *Saccharomyces cerevisiae* were selected that had simultaneously acquired a cold-sensitive growth phenotype. Five genes, called *SAC* (suppressor of actin) were defined by complementation tests; both suppression and cold-sensitive phenotypes were recessive. Three of the genes (*SAC1*, *SAC2* and *SAC3*) were subjected to extensive genetic and phenotypic analysis, including molecular cloning. Suppression was found to be allele-specific with respect to actin alleles. The *sac* mutants, even in *ACT1*$^+$ genetic backgrounds, displayed phenotypes similar to those of actin mutants, notably aberrant organization of intracellular actin and deposition of chitin at the cell surface. These results are interpreted as being consistent with the idea that the *SAC* genes encode proteins that interact with actin, presumably as components or controllers of the assembly or stability of the yeast actin cytoskeleton. Two unexpected properties of the *SAC1* gene were noted. Disruptions of the gene indicated that its function is essential only at temperatures below about 17° and all *sac1* alleles are inviable when combined with *act1-2*. These properties are interpreted in the context of the evolution of the actin cytoskeleton of yeast.

THE eukaryotic cytoskeleton consists of a number of different filamentous structures; each type of filament consists of one or two major protein subunits along with an unknown number of minor components or attachments, sometimes called "associated" proteins. The function of these elaborate filamentous structures is only incompletely understood. It is known that they are dynamic, in that their structure is regulated by a continuing balance between assembly and disassembly. The number and roles of the associated proteins is less clear, as most of these have been identified only by their association or binding *in vitro*.

In the hope of applying sophisticated genetic as well as biochemical analysis to the problem of cytoskeletal structure and function, we have undertaken a study of the cytoskeleton of a simple unicellular eukaryote, the budding yeast *Saccharomyces cerevisiae*. Two major classes of cytoskeletal filaments common to all other eukaryotes have been partially characterized in this yeast: microtubules (consisting mainly of α- and β-tubulin) and microfilaments (consisting mainly of actin). Previous studies have shown that actin and β-tubulin in yeast are each specified by a single essential gene (SHORTLE, HABER and BOTSTEIN 1982; NEFF *et al.* 1983), and that α-tubulin is specified by two genes, one of which is expressed but not essential (SCHATZ, SOLOMON and BOTSTEIN 1986). The yeast actin and tubulin genes specify proteins homologous to their mammalian counterparts: in particular, the amino acid sequence of yeast actin is 91% identical to that of chicken (NG and ABELSON 1980; GALLWITZ and SEIDEL 1980).

Yeast contains relatively little actin, only about 0.3% of the cell protein (GREER and SCHEKMAN 1982). The actin is arranged in a polarized, asymmetric fashion (ADAMS and PRINGLE 1984; KILMARTIN and ADAMS 1984). The growing portion of the cell, the bud, has a high concentration of actin arranged in cortical patches. The nongrowing portion, the mother cell, contains actin arranged into cables which are oriented approximately along the mother-bud axis. Since yeast has only a single essential actin gene, we have been able to construct conditional-lethal (temperature-sensitive) actin mutant alleles (SHORTLE, NOVICK and BOTSTEIN 1984). Phenotypic analysis of these actin mutants has suggested roles for actin in the organization and assembly of the yeast cell surface (NOVICK and BOTSTEIN 1985).

We have begun to apply pseudoreversion methods to our conditional-lethal yeast actin mutants in the hope of defining genes that specify proteins that interact with actin *in vivo*. The underlying hypothesis is that if two proteins interact, a deleterious mutation in one protein can be suppressed by a compensating mutation in the interacting protein. In this way, by starting with a mutation in one component of a system one can, in theory, identify genes specifying other components.

In practice, however, a suppressor which has no phenotype other than suppression has relatively little utility, since it is relatively difficult to distinguish

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interaction suppressors from informational suppressors and, in any case, cloning such genes is difficult compared to cloning a gene with a lethal phenotype in addition to the suppression phenotype. JARVIK and BOTSTEIN (1975) introduced the use of suppressors that have, in addition, a conditional-lethal phenotype as a way to avoid these difficulties. In their method, revertants of a conditionally lethal mutant are screened for a growth defect under a new condition. They reverted cold-sensitive mutants defective in assembly of phage P22, and screened for phage which were, in addition, heat sensitive. In this very simple case it was possible to demonstrate that the *Sup/Ts* loci so obtained were in genes encoding proteins that interact specifically with the product of the original mutant gene.

The same approach was used to study a much more complex system, nuclear division in yeast (MOIR *et al.* 1982). They were not able to demonstrate directly that the suppressors were in genes encoding physically interacting proteins. Nevertheless they did show that many of the conditionally lethal suppressors had similar phenotypes to the original mutant. If the new mutant has a phenotype at its restrictive condition which resembles the phenotype of the original mutant, one can then reasonably propose that the suppressor gene product is another component of the system of interest and not an informational suppressor.

In this paper we describe the isolation of suppressors of a temperature-sensitive lethal actin mutant that have, in addition, a cold-sensitive growth phenotype of their own. We show that their genetic and phenotypic properties support the idea that the new genes they define specify proteins that interact with actin and/or actin filaments.

MATERIALS AND METHODS

Strains and media: The yeast strains used in this paper are listed in Table 1. All genetic manipulations were done essentially as described by SHERMAN, FINK and LAWRENCE (1974); experimental designs and details are given in the text or tables. Media for growth and sporulation are described in SHERMAN, FINK and LAWRENCE (1974). Most growth tests were scored by spotting cell suspensions made in water using a 32-point inoculator. Genotypes of diploids were routinely tested by tetrad dissection.

Two supposedly independent alleles (*act1-1* and *act1-3*; SHORTLE, NOVICK and BOTSTEIN 1984) were used in this study, represented by strains DBY1195 and DBY1241, respectively (Table 1). DNA sequence analysis revealed that these are in fact the same, and, further, both had the same silent base change a few residues away, making it virtually certain that they are descended from the same original mutagenic event. Therefore we have renamed all of these "*act1-1*" for consistency. For the record, all the strains in Table 1 except DBY1195 derive from the mutation originally labeled "*act1-3*."

For manipulations in bacteria, *Escherichia coli* strain HB101 (BOYER and ROULLAND-DUSSOIX 1969) was used. Media are described in DAVIS, BOTSTEIN and ROTH (1980).

TABLE 1

Yeast strains

Strain	Genotype
DBY473	*MATα his4-619 mox1-1*
DBY877	*MATα his4-619*
DBY1195	*MATα his4 ura3-52 ade2 act1-1*
DBY1241	*MAT***a** *ura3-52 act1-3 MOX⁺ can1*
DBY1640	*MAT***a** *ura3-52 act1-3*
DBY1692	*MATα his4-619 act1-3 MOX⁺*
DBY1707	*MAT***a**/*MATα leu2-3, 112/leu2-3, 112 ura3-52/ura3-52 lys2-801/+*
DBY1710	*Mat***a** *kar1-1 ura3-52 tris 3-*
DBY1775	*Mat***a** *sac1-50::LEU2 leu2-13, 112 ura3-52* (orientation 1)[a]
DBY1776	*Mat***a** *sac1-51::LEU2 leu2-13, 112 ura3-52* (orientation 2)[a]
DBY1788	*MAT***a**/*MATα sac1-51::LEU2/sac1-51:LEU2 leu2-3, 112/leu2-3, 12, ura3-52/ura3-52, lys2-801/+*
DBY1836	*MAT***a** *ura3-52 his4-619*
DBY1837	*MAT***a** *ura3-52*
DBY1840	*MAT***a** *sac1-60 ura3-52 his4-619* (deletion)
DBY1885	*MAT***a** *sac1-6 ura3-52*
DBY1886	*MATα sac1-6 his4-619*
DBY1887	*Mat***a** *sac1-6 ura3-52 lys2-803*
DBY1888	*MATα sac1-6 ura3-52 lys2-803*
DBY1900	*MAT***a** *sac1-8 ura3-52*
DBY1901	*MATα sac1-8 his4-619*
DBY1918	*MAT***a** *sac2-1 MOX⁺ ura3-52*
DBY1919	*MATα sac2-1 MOX⁺ his4-619*
DBY1924	*MAT***a** *sac2-1 ura3-52*
DBY1926	*MAT***a** *sac2-1 MOX⁺ ura3-52*
DBY1936	*MAT***a** *sac2-2 MOX⁺ his4-619*
DBY1947	*MAT***a** *sac2-4 MOX⁺ ura3-52*
DBY1948	*MATα sac2-4 MOX⁺ his4-619*
DBY1952	*MATα sac3-1 his4-619*
DBY1953	*MAT***a** *sac3-1 ura3-52*
DBY1954	*MATα sac3-1 his4-619*
DBY1958	*MAT***a** *sac3-2 ura3-52*
DBY1959	*MATα sac3-2 his4-619*
DBY1963	*MAT***a** *sac3-2 ura3-52*
DBY1969	*MATα sac3-2 ura3-52*
DBY1998	*MATα his4-619 act1-3 mox1-1 can1*
DBY2003	*MAT***a** *ura3-52 MOX⁺*

[a] In orientation 1 the reading strands of both the *SAC1* and *LEU2* genes are the same.

Isolation of revertants: Spontaneous revertants of *act1-1* were selected by first plating dilutions of saturated cultures (about 0.6 to 3 × 10^8 cells/ml) of DBY1195, DBY1241 or DBY1998 to obtain many isolated single colonies on YEPD plates at 26°. After 3 days, isolated colonies were suspended in 1 ml sterile water and 0.2 ml (4–6 × 10^5 cells) was spread on YEPD plates that were then incubated at 37°.

After four days incubation, four revertant colonies were then picked from each plate and tested for growth at 17° as well as 37°; the best candidate of the four (*i.e.*, poor growth at the low temperature and good growth at the high temperature) was single-colony purified on YEPD plates at 26°. Two isolated colonies from each streak were retested at four temperatures: 37°, 26°, 17° and 14°.

Growth tests at this stage and in all subsequent genetic experiments were done with the 32-point inoculator.

Growth at 37° (interpreted as suppression of the *act1-1* mutation) was assessed after 3 days of incubation. Growth at 17° or 14° (cold-sensitivity) was assessed at various times between 3 and 7 days. Wild-type, *act1-1* and a cold-sensitive mutant control (usually a *sac1* strain) were carried on each plate.

Molecular cloning of the *SAC* genes: The *SAC1* and *SAC3* genes were cloned by "complementation" of the cold-sensitivity phenotypes of the corresponding mutants. Strains DBY1887 (*MATα sac1-6 lys2-803 ura3-52*) and DBY1958 (*MAT***a** *sac3-2 ura3-52*) were transformed by the method of ITO *et al.* (1983) with plasmid DNA from the yeast genomic library described by ROSE *et al.* (1987). This library was made in a centromere-containing shuttle vector (YCp50; C. MANN and R. W. DAVIS, unpublished data; MA *et al.* 1987) that carries the *URA3* gene (selectable in *ura3* mutants of yeast) and the *bla* gene (selectable in *E. coli* as ampicillin resistance). Transformants were selected on minimal plates lacking uracil at 30°. After colonies had appeared, the plates were replica plated to similar plates incubated at 14°. Total DNA was extracted from cultures of colonies that grew on these plates by the method of HOLM *et al.* (1986) and the complementing plasmids recovered in *E. coli* strain HB101 by selecting for the plasmid's ampicillin-resistance gene. The restriction maps of the complementing plasmids (pRB390 and pRB391 are shown in Figure 1).

SAC2 was cloned by a more elaborate procedure first devised by J. RINE (unpublished data). This was necessitated by the very poor transformation efficiency characteristic of the *sac2* mutants. The library was first introduced into a *kar1* strain (DBY1710: *MATα kar1-1 ura3-52 his3*) that has a high frequency of transformation. The plasmids were then transferred to a *sac2-1* strain (PNY39-10A: *MAT***a** *sac2-1 ura3-52 can*^{R} *cyh*^{R}) by replica-plating the Ura$^+$ transformants of DBY1710 to YEPD plates already seeded with PNY39-10A). "Cytoductants" (*i.e.*, PNY39-10A cells that had received the plasmid but not the nucleus from transformed DBY1710) were selected on plates lacking uracil but containing cycloheximide (5 μg/ml) and canavanine (50 μg/ml), taking advantage of the fact that both drug-resistances are recessive traits. One set of selective plates was incubated at 14° and another at 30°. Although Ura$^+$, Can^R and Cyh^R papillae appeared commonly in the replica-plated patches at 30°, only one patch had more than a single papilla at 14°. These cold-resistant, uracil-independent, double drug-resistant papillae were single-colony purified and shown to retain the *Mat***a** phenotype of the *sac2-1* parent. Plasmids were recovered into *E. coli* from these strains and reintroduced by transformation (the frequency, though too low for selection from a library, permits transformation by a single species of plasmid) into a *sac2-1* strain by selection of the *URA3* marker. All the transformants had lost their cold sensitivity, showing that the plasmid indeed contains a gene that complements *sac2-1*. The restriction map of the *SAC2* plasmid is shown in Figure 1 also.

Construction of integrating *SAC* plasmids: In the case of *SAC1*, an integrating plasmid was made by subcloning the *Bgl*II (shown to be external to the gene by subcloning experiments) to *Sal*I fragment (in the vector) into the integrating vector YIp5 (BOTSTEIN *et al.* 1979) cleaved with *Bam*HI and *Sal*I. This plasmid was then used to transform a *ura3 SAC1*$^+$ strain by integration and thus mark the *SAC1* locus with the *URA3* marker. Tetrad analysis of a diploid made by crossing such an integrant with a *ura3 sac1* strain confirmed that integration of the plasmid was at the *SAC1* locus because no *URA3 sac1* segregants were observed among 16 tetrads.

A *SAC2* integrating plasmid was made by cleaving pRB397 (Figure 1) with *Cla*I and *Sal*I, producing a fragment that carries the complementing region (as determined by subcloning experiments). This fragment was ligated into YIp5 cleaved with *Cla*I and *Sal*I. The resulting integrating plasmid was used in exactly the same way as its *SAC1* analogue to confirm that the plasmid contains DNA from the *SAC2* locus; *i.e.*, in a cross exactly analogous to the one described above for *SAC1*, no *URA3 sac2* segregants were observed among 22 complete tetrads.

A *SAC3* integrating plasmid was made by cleaving pRB391 (Figure 1) with *Sal*I and ligating the yeast-DNA-containing fragment into YIp5 that had been cleaved with *Sal*I and that had also had its ends dephosphorylated. The resulting integrated plasmid was used as above to confirm that the yeast DNA in pRB391 indeed derives from the *SAC3* locus. Again, no *URA3 sac3* segregants were found among 16 complete tetrads analyzed.

Disruptions of the *SAC1* gene: We partially localized the *SAC1* gene by subcloning experiments from pRB390 (Figure 1) into the vector (YCp50) followed by tests of ability to complement a *sac1* mutation; the results are shown in Figure 2. Briefly, these experiments suggested that the *Bgl*II site is external to the functional gene, the *Eco*RI site at the left may or may not be internal to the gene but is surely close to the end of the functional gene. The *Eco*RI site at the right is certainly internal to the gene.

This information was used in constructing the three kinds of disruptions shown below in Figure 3. The integrative disruption was produced by sub-cloning an internal fragment (*Hin*dIII to the *Eco*RI at the right; see also Figure 1) from pRB390 into YIp5 (BOTSTEIN *et al.* 1979) followed by integration of this plasmid; integration of this plasmid; integration was directed to the *SAC1* locus by cleavage with *Xho*I before transformation (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981): the allele is called *sac1-40*.

The insertion-disruption was produced by inserting the *LEU2* gene (a *Sal-Xho* fragment identical to the one in YEp24; see BOTSTEIN *et al.* 1979) into the *SAC1 Bgl*II-*Sal*I fragment cloned in YIp5 (described above). Both orientations were recovered and both were used to transform, as linear DNA, a *SAC1*$^+$ strain (ORR-WEAVER, SZOSTAK and ROTHSTEIN 1981). The resulting insertion alleles *sac1-50::LEU2* and *sac1-51::LEU2* differ only in the orientation of *LEU2* relative to *SAC1*.

The deletion was constructed by cleaving the aforementioned *Bgl*II-*Sal*I YIp5 clone with *Bam*HI and religating. The deletion clone was then used to replace the normal locus by "transplacement" (SCHERER and DAVIS 1979), producing the deletion allele *sac1-60*.

All the disruption alleles were tested by gel-transfer hybridization experiments (performed as described by DAVIS, BOTSTEIN and ROTH 1980) using genomic DNA (isolated by the method of HOLM *et al.* 1986) and probes derived from the *SAC1* DNA in pRB390.

Immunofluorescence and electron microscopy: Immunofluorescence microscopy and electron microscopy were done as described before (NOVICK and BOTSTEIN 1985). Actin was stained with affinity-purified rat anti-yeast actin antibody, a generous gift from JOHN KILMARTIN. Chitin was stained with Calcofluor White M2R, generously provided by Dr. John Pringle.

RESULTS

Isolation of suppressors of actin mutants: Spontaneous heat-resistant revertants of yeast strains carrying a conditional-lethal temperature-sensitive actin

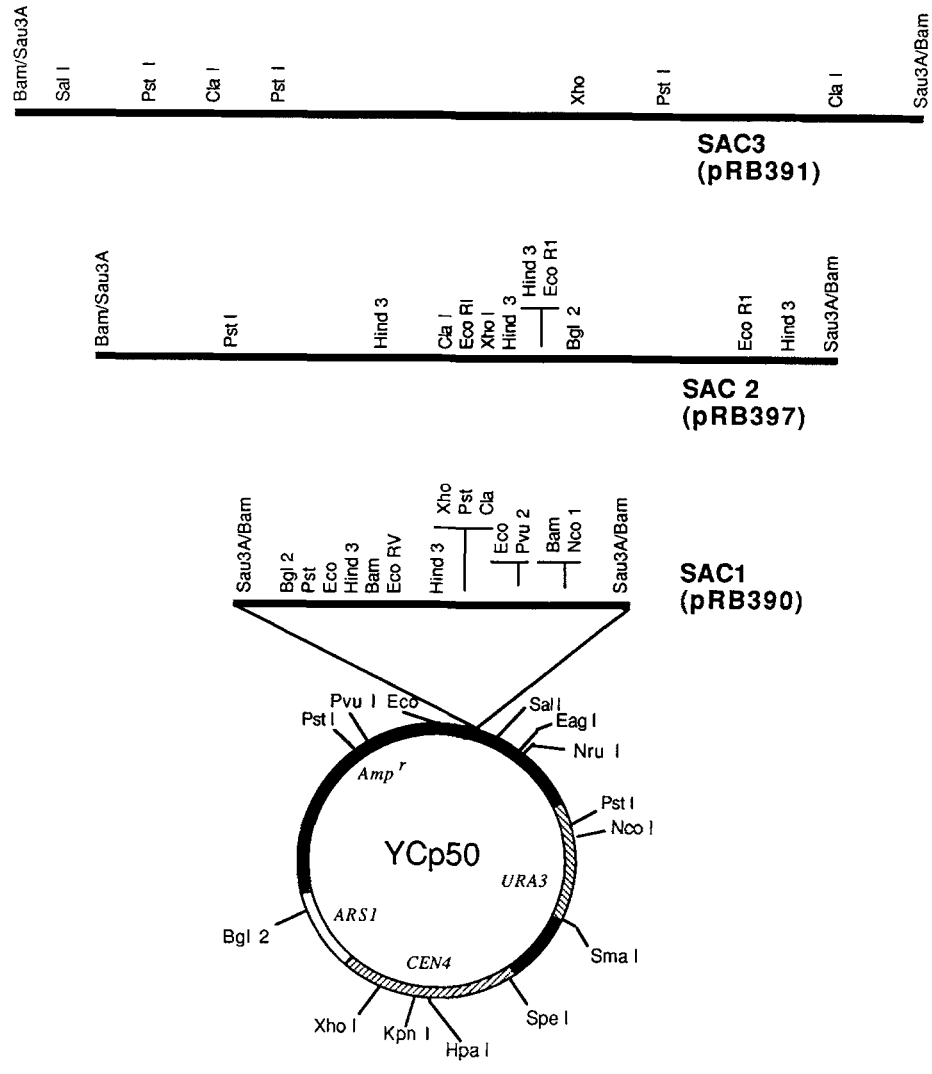

FIGURE 1.—Restriction maps of plasmids containing the *SAC1*, *SAC2* and *SAC3* genes. The isolation of the plasmids from the genomic library described by ROSE *et al.* (1987) is described in the text. The Bam/Sau3A sites at the ends of the inserts were not checked explicitly; the positions of the remaining restriction sites were determined by standard methods (DAVIS, BOTSTEIN and ROTH 1980).

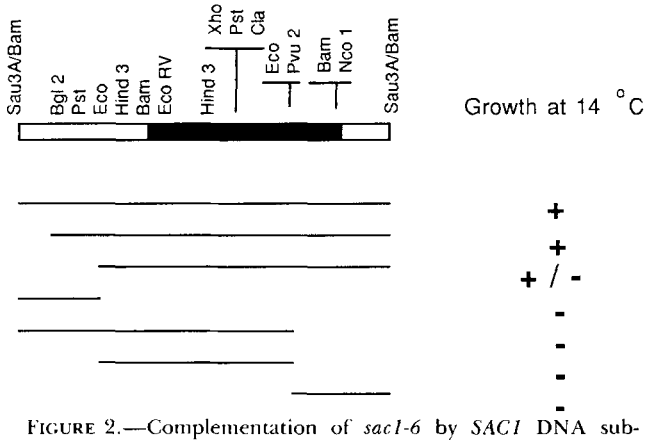

FIGURE 2.—Complementation of *sac1-6* by *SAC1* DNA subclones. Various subclones were constructed in the YCp50 vector and introduced into a *sac1-6 ura3* strain by selecting Ura$^+$. Transformants were tested for growth at 14°. The filled bar indicates the region deleted in *sac1-60* (see below).

mutation were selected at 37° on YEPD plates. Colonies appearing after 4 days were picked and tested for their ability to grow at 14°. The two mutations used (*act1-1* and *act1-3*) are in reality the same, changing residue 32 from Pro to Leu (SHORTLE, NOVICK and BOTSTEIN 1984) and henceforth will be referred to as *act1-1*. Independence of the revertants was assured because each reversion plate was made from a culture begun with an isolated colony; only one candidate was saved from each plate. A total of 33 independent temperature-resistant, cold-sensitive isolates were obtained from about 671 revertant colonies screened. Two additional revertants were isolated independently by D. SHORTLE, bringing the total to 35.

Suppression and cold sensitivity are both caused by a mutation unlinked to the *ACT1* locus: All the revertants were crossed to wild-type strains to assess linkage of the suppressor with the mutation conferring the cold sensitivity (Cs) and with the original *act1* heat-sensitive (Ts) mutation. Seven of the 35 revertants exhibited poor sporulation and/or spore viability in this test and were not studied further. In each of the remaining 28 revertants the Cs determinant proved to be unlinked to the *ACT1* locus. Further-

more, in every case the temperature-resistant (Ts^+) phenotype proved to be due to a suppressor also unlinked to the original *act1* mutation. In these crosses, preliminary evidence for the linkage of the mutations determining the suppressor (Ts^+) and Cs phenotypes was obtained in the failure to observed any meiotic products that are simultaneously Ts and Cs. A more definitive test of linkage between the determinant of the Cs and suppressor phenotypes was carried out by crossing a strain bearing *act1-1* and the suppressor mutation with another strain carrying only *act1-1*. The results in every case were 2 Cs, Ts^+:2 Ts, Cs^+ spores, *i.e.*, cosegregation as a single locus for the Cs and suppressor phenotypes; at least 7 complete tetrads were tested for each case. Thus the 28 independent mutants all appear to contain a single mutation (Sup/Cs) unlinked to the actin locus that simultaneously confers the phenotypes: suppression of the heat sensitivity due to *act1-1* and cold sensitivity.

A final test of the hypothesis that a single locus is responsible for both suppression and cold sensitivity was carried out by reconstituting the original genotype of the revertants (*i.e.*, *act1-1*, Sup/Cs) from strains in which the two mutations had been separated. In each case it was observed that introduction of the cold-sensitivity determinant resulted in suppression of the heat sensitivity of *act1-1*.

Tests of dominance and complementation: The Sup/Cs revertants were crossed to *act1-1* and $ACT1^+$ strains to determine whether the mutations were recessive or dominant. The two phenotypes, cold sensitivity and ability to suppress the heat sensitivity caused by the *act1-1* mutation, were tested separately. The cold-sensitive phenotype was recessive: only strains homozygous for the Sup/Cs mutation showed the cold-sensitive phenotype, regardless of the genotype at the actin locus (*i.e.*, in ACT^+/ACT^+, *act1-1*/ACT^+ and *act1-1/act1-1* diploids although most tests were made in diploids heterozygous at the actin locus). The suppression was, somewhat surprisingly, also recessive in every case: when tested as *act1-1/act1-1* diploids, only those strains homozygous for a Sup/Cs mutation grew at 37°, the nonpermissive temperature for *act1-1*. It turned out that homozygous *sac* diploids sporulate very poorly, so the diploids were not dissected. The significance of recessive suppression is addressed in the DISCUSSION.

The fact that both phenotypes of the Sup/Cs mutations are recessive makes it possible to define complementation groups in two ways: by suppression or by cold sensitivity. To define groups by cold-sensitivity Sup/Cs strains (usually containing an intact $ACT1^+$ gene) were crossed to each of the original revertants (*i.e.*, putative genotype *act1-1* Sup/Cs) and the resulting diploids were tested for growth at 14°, the common nonpermissive temperature. Five groups [*SAC1* (15 members), *SAC2* (4 members), *SAC3* (4 members), *SAC4* (5 members), and *SAC5* (1 member)] were defined by this approach. To define complementation groups by suppression several representative Sup/Cs strains carrying the *act1-1* mutation were crossed and the diploids were tested for growth at 37°. Growth of the diploid at the elevated temperature indicates noncomplementation. The results of this analysis confirmed the results of the test based on cold sensitivity. Since the suppression by *sac4* and *sac5* mutations is relatively weak, they were not studied further in any detail.

A modifier of *sac2*: In crosses of *sac2 act1-1* strains to certain ACT^+ strains (DBY473) but not others (DBY877) and again in crosses to certain *act1-1* strains (DBY1998) but not others (DBY1692), meiotic products were sometimes found which are both Cs and Ts in phenotype. This observation was first noticed in the experiments described above. Because the phenomenon is completely strain dependent, it seemed clear that it did not mean that any of the *sac2* isolates contained a complex mutation. Instead we supposed that there must exist, in the genetic background common to strains DBY473 and DBY1998, a modifier that interferes with the suppression normally caused by *sac2* mutations. Further crosses established that there appears to be a single modifier locus (*MOX1*) common in our strains unlinked to either the *ACT1* or *SAC2* loci. The recessive allele (*mox1-1*) prevents expression of the *sac2* suppression phenotype; the cold-sensitivity phenotype remains unchanged. Suppression by *sac1* and *sac3* mutations is unaffected by the state of the *MOX1* locus. We subsequently have obtained preliminary evidence that at least some *sac4* and *sac5* mutations fail to suppress in the presence of *mox1-1*. This phenomenon has not been studied further, and the experiments involving *sac2* described below were done with *MOX1* (*i.e.*, those that allow suppression) strains.

Allele specificity: Suppressors can function by several mechanisms. Bypass suppressors function by creating or activating an alternative pathway and thereby eliminating or reducing the need for the defective gene product. By their nature, bypass suppressors are not allele specific, they suppress virtually all recessive defects. We tested *sac1*, *sac2* and *sac3* mutations for their ability to suppress the temperature-sensitivity of a second allele of actin, *act1-2*, that changes residue 58 in the protein from ala to thr (SHORTLE, NOVICK and BOTSTEIN 1984). Two members of each of the three *SAC* groups were crossed to an *act1-2* strain and the temperature sensitivity of the meiotic products was assessed. The results of these experiments are summarized in Table 2.

In the cases of *sac1-6* and *sac1-8* many inviable spores resulted from the crosses. The pattern of invi-

TABLE 2

Allele specificity of interactions between *sac* point mutations and *act1* mutations

sac allele	Phenotype of *sac-act* double mutants		
	act1-1	*act1-2*	*act1-4*
sac1-6	Ts^+ (Sup)Cs	Inviable (16/16)	Ts (9/10)
sac1-8	Ts^+ (Sup)Cs	Inviable (13/13)	–
—			
sac2-1	Ts^+ (Sup)Cs	Ts Cs (9/9)	–
—			
sac2-4	Ts^+ (Sup)Cs	Ts Cs (10/10)	–
—			
sac3-1	Ts^+ (Sup)Cs	Ts Cs (9/12)[a]	–
—			
sac3-2	Ts^+ (Sup)Cs	Ts Cs (5/7)[a]	–
—			

Crosses were made between strains carrying the indicated mutations and tetrads dissected; viability (except for the *sac1-act1-2* crosses) was excellent (>95%). Data for *sac-act* double mutations are derived from tetrads that either contained all four spores viable or, in the *sac1-act1-2* crosses, NPD or TT tetrads in which the assignment of the *act-sac* genotype was unambiguous.

[a] The indicated numbers showed very weak suppression, while the remainder were viable and showed no suppression at all.

ability indicated that the double mutants (*i.e.*, *sac1-6 act1-2* and *sac1-8 act1-2*) are inviable even at 26°. Therefore, instead of observing suppression (*i.e*, growth at the nonpermissive temperature) we observed, with both *sac* alleles, inviability of the double mutants even at permissive temperature. This is not only an instance of allele specificity, it is also an instance of "synthetic lethality" (DOBZHANSKY 1946; STURTEVANT 1956). Synthetic lethality occurs when the combination of two mutations, neither by itself lethal, causes lethality. Like allele-specific suppression, synthetic lethality is a useful genetic indication of a possible interaction of gene products. HUFFAKER, HOYT and BOTSTEIN (1987) provide additional instances affecting the yeast cytoskeleton. Table 2 also summarizes the results of similar crosses between *sac1-6* and yet another actin mutation, *act1-4*. The double mutations with *act1-4* are viable and show a Ts as well as a Cs phenotype, indicating no suppression of the actin mutation.

Crosses between *sac2-1* or *sac2-4* and *act1-2* gave good spore viability. The double mutants could again be recognized by their Ts and Cs phenotype: they appeared at the frequency expected from unlinked determinants. The double mutants did not show improved growth at 37°. Similarly, crosses between *sac3-1* or *sac3-2* and *act1-2* also gave good spore viability. The double mutants could readily be scored, appeared at the expected frequency and showed little or no change in their growth properties at the elevated temperature. Thus the suppression exerted by *sac2* and *sac3* is apparently also allele specific, since *act1-1* but not *act1-2* is suppressed by each of the alleles tested.

Molecular cloning of *SAC* genes: Three of the *SAC* genes (*SAC1*, *SAC2* and *SAC3*) were cloned by complementation of the mutant defects that cause cold sensitivity in each case. As described in MATERIALS AND METHODS, a library of cloned fragments in the low copy centromere vector YCp50 which includes the selectable marker *URA3* (C. MANN and R. W. DAVIS, unpublished data; MA *et al.* 1987) was used. Strains carrying the *sac1*, *sac2* or *sac3* mutation as well as the nonreverting phenotype. In the cases of *SAC1* and *SAC3* it sufficed to select for Ura^+ transformants followed by screening of these transformants for growth at 14° to find candidates. Unfortunately, *sac2* mutants proved to be poorly transformable, and therefore the library plasmids had to be introduced indirectly by the method suggested to us by J. RINE. As described in detail in MATERIALS AND METHODS, the library was introduced into a strain bearing the *kar1-1* mutation, after which the plasmids were passed to the *sac2 ura3* strain using the Ura^+ selection. After this first screening, it was found that the transformability of *sac2* strains, though insufficient for screening libraries, was adequate for introducing individual candidate plasmids to confirm the cotransfer of the Ura^+ and Cs^+ phenotypes.

In each case, plasmids that could confer both the Ura^+ and Cs^+ phenotypes were found: six in the case of *sac1* and one each of *sac2* and *sac3*. These plasmids were subjected to analysis with restriction endonucleases and simple subcloning experiments; from these a plasmid containing a minimal insert that still contained the intact *SAC* gene was chosen for further work. The structures of these plasmids are summarized in Figure 1.

The finding of a plasmid that can complement a mutation is not sufficient experimental support for a claim to have cloned the corresponding gene. Thus in each case a subclone was made into an integrating yeast vector (YIp5) which was then used to integrate, by homology, the subclone and its *URA3* marker into the genome. Integration was directed to the *SAC* locus by cutting the subclone with an enzyme that cleaves only in the insert (*i.e.*, *Xho*I for *SAC1* and *SAC3*; *Bgl*II for *SAC2*; Figure 1). The strains used for the integration were SAC^+ *ura3-52*; after integration they should carry the $URA3^+$ gene at the *SAC* locus. This expectation was tested by crossing each with a strain of genotype *sac ura3-52* and observing, upon tetrad analysis, complete linkage of the Ura^+ and Cs^+ phenotypes.

Mapping *SAC1* and *SAC3*: The *SAC1* gene was mapped first by the 2-μm plasmid-directed integration method of FALCO and BOTSTEIN (1983), which localized the gene to the right arm of chromosome *XI*. A three point cross was then performed by crossing a *sac1 ura1* strain with a *trp3* strain. In 29 complete

tetrads, there were no tetratypes with respect to *sac1* and *trp3*; only 7 tetratypes with respect to *sac1* and *ura1* were observed. This means that the *SAC1* locus lies about 12 cM from *URA1* and very close (2 cM or less) to *TRP3*. No previously reported conditional-lethal mutations have been mapped to this vicinity.

The *SAC3* map position was found first fortuitously in a cross between *sac3* and *sec7* (known to be on chromosome *IV*) that indicated strong linkage: 31 PD:0 NPD: 1TT (*ca.* 1.6 cM). To determine the map position more accurately, crosses were made between a *sac3-2* strain and a strain carrying the linked markers *sec5-24 sec7-1 aro1 hom2* with the result that *sac3-2* failed to recombine with *hom2*. There were 6 tetratypes with respect to *sac3* and *aro1* and 1 tetratype with respect to *sac3* and *sec5*. Thus *SAC3* lies about 8 cM from *ARO1* between that gene and *SEC5*. In order to ascertain whether the *sac3* mutations might in fact be alleles of *SEC7*, *SEC5* or *SEC1* (which has been mapped to a position nearby), strains carrying mutations in each of these genes were transformed with the plasmid (pRB398) bearing the *SAC3* gene DNA: none of the mutations was affected by the presence of the plasmid, indicating that *SAC3* is a new gene in this region of chromosome *IV*.

Using pulsed-field gel electrophoresis separation followed by gel-transfer hybridization, we have found that *SAC2* lies on chromosome *IV* also. Genetic mapping on this genetically largest of the yeast chromosomes is in progress.

Disruption of the *SAC1* gene: Gene disruption can be used to obtain a null allele of a gene. Three approaches have been used to disrupt yeast genes. The first utilizes a fragment of the gene which is internal, *i.e.*, missing essential information from both ends. The fragment is subcloned on an integrating plasmid. Upon integration a partial duplication is formed. One copy is deleted at the amino terminus and the second copy is deleted at the carboxy terminus. The second approach involves a construction in which a selectable marker has been inserted into the gene. The third approach involves integration of a plasmid carrying a deleted form of the gene. In a second step the plasmid is excised from the chromosome leaving the deletion on the chromosome. We used all of these approaches to construct null alleles of *SAC1*. The structures of the disrupted genes are shown in Figure 3.

With all three disruptions, we made the surprising observation that the *sac1*-disruption phenotype is cold-sensitivity. This observation was fortified in each case by the failure of strains carrying the disruption to complement the spontaneous *sac1* mutations derived by reversion of actin mutations. In each case the cold sensitivity was recessive. In the third disruption scheme, an intermediate is a duplication caused by integration of a plasmid with a deletion: the loss of the plasmid can result either in a deletion allele or a wild-type allele. The former were always Cs, while the duplication and the latter were always cold resistant. These results show that the *SAC1* gene product is essential, but only at low temperatures.

Suppression of *act1-1* by *sac1*-disruption alleles: The finding that a *sac1*-disruption allele is recessive for cold sensitivity allowed us to test the possibility that suppression of *act1-1* by the *sac1* Sup/Cs alleles could be the result of simple loss of gene function. To this end, crosses were made between *act1-1* strains and strains carrying one of the disruptions shown in Figure 3. In all four disruption mutations (the integrative disruption *sac1-40*; the two oppositely oriented insertions *sac1-50*::*LEU2* and *sac1-51*::*LEU2*; and the deletion *sac1-60*) and the *sac1-6* mutation (as a control) were crossed to *act1-1* and *act1-2* strains. Table 3 shows the phenotypes of the *sac-act* double-mutants; as before only tetrads that contained four viable spores or those that were unambiguously non-parental ditype or tetratype were used to score for viability and suppression.

Some aspects of the results were straightforward, but others were unexpectedly complicated. The straightforward result was that the disruption mutations had in common with the *sac1-6* and *sac1-8* mutations inviability as haploids when combined with *act1-2*. This inviability would therefore appear to be a property of null as well as point mutations: normal *SAC1* function is absolutely required when *act1-2* actin is the only actin available.

In contrast, the *sac1*-disruption mutations did not behave similarly when tested for their ability to suppress *act1-1*. The deletion allele (the most likely to be a true null mutation) failed to suppress, but the other disruptions did suppress. There was even heterogeneity between the two *LEU2*-insertion alleles: one orientation suppressed well, while in the other case only about half the double-mutant spores managed to grow at the temperature nonpermissive for *act1-1*. This set of results leaves open two kinds of interpretation: one alternative is that the true null phenotype is failure to suppress (indicated by the behavior of the deletion) and the others all manage to provide some activity because they can express part of the *SAC1* gene; the other, somewhat more complicated alternative is that the true null phenotype is ability to suppress *act1-1* and the *sac1-60* deletion fails to suppress because a neighboring gene that also affects *act1-1* has been damaged.

The *SAC* genes affect sporulation: During the genetic analysis of the sac mutants we observed a sporulation defect in homozygous diploid strains. This defect was quantitated by the technique of SIMCHEN, PINON and SALTS (1972). As shown in Table 4, homo-

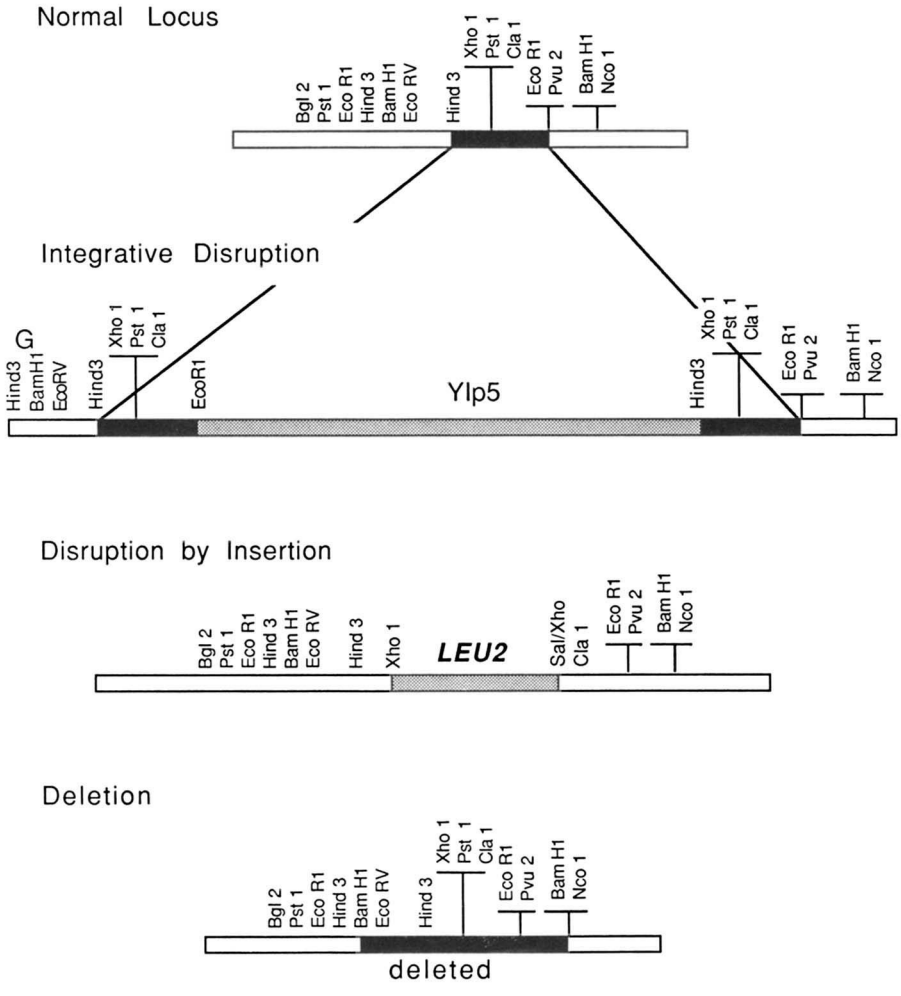

FIGURE 3.—Disruptions of the *SAC1* gene. The genomic restriction pattern at the *SAC1* locus before and after disruption by integration, insertion and deletion are shown. The constructions are described in MATERIALS AND METHODS. The map of the normal locus and each of the disruptions was checked by gel-transfer hybridization using fragments of the gene derived from pRB390 (Figure 1) as probe.

TABLE 3

Allele specificity of interactions between *sac*-disruption mutations and *act1* mutations

sac allele	Phenotype of *sac-act* double mutants	
	act1-1	*act1-2*
sac1-6 (point mutation)	Ts^+ (Sup)Cs (12/12)	Inviable (16/16)
sac1-40 (integrative disruption)	Ts^+ (Sup)Cs (12/12)	Inviable (13/13)
sac1-50::LEU2	Ts^+ (Sup)Cs (19/19)	Inviable (23/23)
sac1-51::LEU2	Ts^+ (Sup)Cs (7/12) Ts Cs (5/12)	Inviable (10/10)
sac1-60 (deletion)	Ts Cs (18/18)	Inviable (24/24)

The crosses were performed and data extracted as described in the legend to Table 2.

zygous *sac1* diploids showed a nearly complete block, while *sac2* and *sac3* mutants varied from allele to allele. The actin mutant when homozygous also showed a partial defect at 26° and a more severe defect at higher temperatures.

Mutations in the *SAC* genes affect the actin cytoskeleton: If the assembly of the actin cytoskeleton involves the *SAC* genes or their products, we would predict that the *sac* mutants would, at their restrictive temperature, affect the appearance of the actin cytoskeleton even in cells carrying a normal *ACT1* gene. We tested this prediction by growing *sac* strains at 30° then shifting them to 14° for 24 hr before fixing and staining the cells for immunofluorescence microscopy with anti-actin antibody. Figure 4 shows examples of what one sees after examination of these preparations. Wild-type cells under these conditions (Figure 4A) show an asymmetric pattern, with patches of actin near the surface of the bud and cables of actin in the mother cell mainly oriented along the mother-bud axis (KILMARTIN and ADAMS 1984; ADAMS and PRINGLE 1984; NOVICK and BOTSTEIN 1985).

Strains carrying either a *sac1* Sup/Cs allele (*sac1-6* is shown in Figure 4C) or any of the *sac1* null alleles show faint cables at 26°; following the shift to 14° there is a loss of visible cables and the cortical patches normally localized to the bud become randomly distributed between the bud and mother (Figure 4B). This staining pattern is similar to that seen in the temperature-sensitive actin mutant, *act1-1*, the mutant whose phenotype the suppressing *sac* alleles suppress (NOVICK and BOTSTEIN 1985).

Strains carrying the *sac2-1* allele show randomly

TABLE 4

Sporulation of *sac* mutants

Strains crossed	Diploid genotype	Sporulation (%) 26°	30°
DBY877/DBY2003	Wild type	75	70
DBY1640/DBY1692	*act1-3/act1-3*	32	14
DBY1885/DBY1886	*sac1-6/sac1-6*	<1	
DBY1900/DBY1901	*sac1-8/sac1-8*	<1	
DBY1918/DBY1919	*sac2-1/sac2-1*	15	
DBY1947/DBY1948	*sac2-4/sac2-4*	3	
DBY1953/DBY1952	*sac3-1/sac3-1*	37	
DBY1958/DBY1959	*sac3-2/sac3-2*	6	

Sporulation was assessed in diploids after 5 days on sporulation medium as described by SHERMAN, FINK and LAWRENCE (1974).

distributed actin patches, and occasional heavy actin bars after incubation at 14° (Figure 4D), whereas reasonably normal patterns are observed at 26° (Figure 4E). This mutant phenotype is similar to that of *act1-2*, which also shows heavy bars at the permissive temperature (NOVICK and BOTSTEIN 1985).

Strains carrying *sac3-1* or *sac3-2* alleles show thick cables at 30° (*sac3-1* is shown in Figure 4F) which develop into heavy bars upon a shift to 14° (Figure 4, G and H). Patches, normally concentrated in the bud at the permissive temperature, become essentially randomly distributed at the restrictive temperature as well.

The altered actin staining pattern in each of the *sac* strains suggests an involvement of the *sac* gene products in actin assembly. However it could be an indirect effect of the growth defect. To explore this possibility we treated wild-type cells in various ways to mimic different growth defects. Cells were shifted to media containing cycloheximide, to mimic defects in protein synthesis; media containing no glucose, to mimic metabolic defects; or media containing sodium azide but no glucose, to mimic defects in energy metabolism, the last treatment is sufficient to lower ATP levels severely. Staining of the treated cells with anti-actin antibody (Figure 5) revealed some alteration in all cases. Cells starved for glucose (Figure 5B) showed both patches and cables as in control cells, however the patches were not exclusively in the bud portion of the cells, and the cables were somewhat fainter than in the control. This trend became more accentuated in the cycloheximide and azide treated cells (Figure 5, C and D). In these cases cables were rarely seen and the patches were randomly distributed. This phenotype is reminiscent of that of *act1-1* and the *sac1* mutants. In no case, however, did we observe the heavy bars of actin visible in *act1-2*, *sac2-1* or *sac3-2*. These results suggest that although the effect of *sac1* on actin assembly might be explained as an indirect effect of a metabolic defect, the alterations in *sac2* and *sac3* mutants cannot be explained as simply. The drug studies do indicate that the altered actin staining patterns must be interpreted cautiously since actin assembly is very dynamic and can be controlled by a variety of parameters.

Altered chitin distribution in *sac* mutants: Actin mutants show altered deposition of chitin (NOVICK and BOTSTEIN 1985). Normally only the bud scar stains with the fluorescent dye Calcofluor, in the actin mutants the scars are abnormally large and upon incubation at the restrictive temperature generalized staining of the cell surface is seen. We stained the *sac* mutants with Calcofluor following a shift to 14°. The most dramatic result (Figure 6) was seen with *sac1* strains. Although they stain normally when grown at 30°, following the shift bright patches are seen on the cell surface. These patches are frequently seen on the bud, though they can also be found on the mother cell or covering the neck region. No difference could be seen between the *sac1::LEU2* and the *sac1* Sup/Cs alleles with respect to this phenotype. The *sac2* and *sac3* mutants also showed an altered staining pattern. At the restrictive temperature generalized staining of the cell surface was seen. This delocalized distribution of chitin is less distinctive than the patches seen on *sac1* strains and may be a nonspecific result of slow growth (ROBERTS *et al.* 1983).

Examination of *sac* mutant phenotypes by thin section electron microscopy: The actin mutants display, as part of their phenotype, a partial block in invertase secretion and a buildup of secretory vesicles at their restrictive temperature (NOVICK and BOTSTEIN 1985). We examined the *sac* mutants for these phenotypes. The mutants were grown at 30°, a portion of the culture was fixed for electron microscopy and the remainder of the culture was shifted to 14° for 8 hr, and then fixed. Figure 7 shows examples of what was observed in the electron microscope. Two control images (*sac3-1* [Figure 7A] and *sac2-1* [Figure 7B], both grown at 30° (permissive temperature) are shown that look like wild type. The most prominent organelles are the nucleus, the vacuole, and mitochondria; only short stretches of endoplasmic reticulum (ER) and a few vesicles are seen. No obvious Golgi structure is seen in wild-type cells. A series of 10-nm filament rings line the plasma membrane in the region of the neck. The appearance of wild type is not altered by growing them at 14° (not shown).

As shown in Figure 7C, *sac1-6* showed only a subtle change at 14°. Membrane-bounded structures were seen at low frequency that resembled the Golgi-related structure seen in the secretory mutant *sec7-1* (NOVICK, FIELD and SCHEKMAN 1980). The 10-nm filaments seen in the neck region of wild-type cells are clearly present in the *sac1* strains at both 30° and 14°.

The *sac2-1* and *sac2-2* strains showed a more dra-

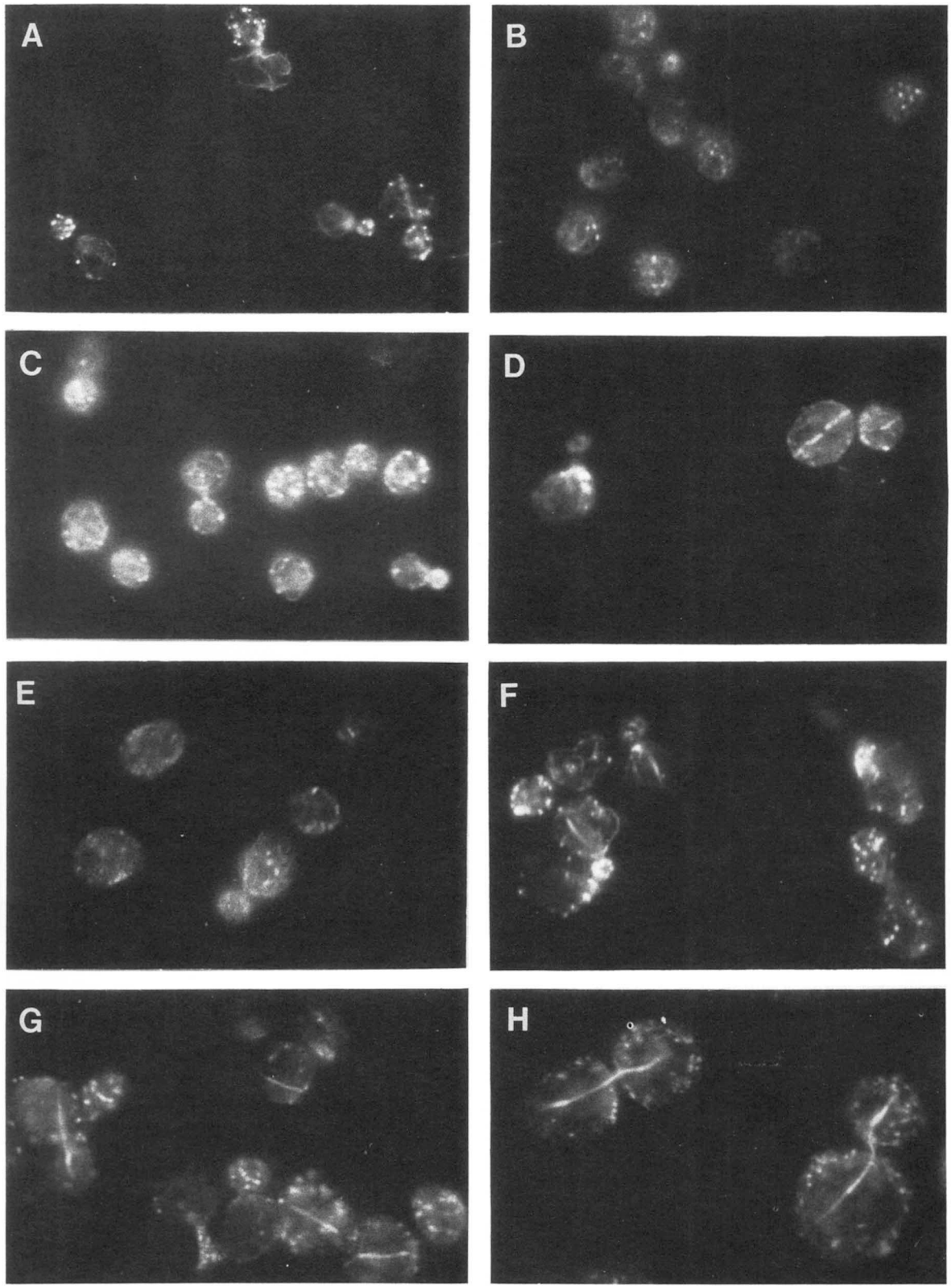

FIGURE 4.—Immunofluorescence localization of the actin in wild-type and *sac* mutants. Cells were grown at 26° in YPD medium and either shifted to 14° for 16 hr and fixed in formaldehyde or fixed directly. Following fixation cells were prepared for immunofluorescence and stained with anti-actin antibody. A) Wild-type strain, DBY1836, 14°; B) *sac1-6* strain, DBY1888 14°; C) *sac1-6* strain, DBY1888, 26°; D) *sac2-1* strain DBY1924, 14°; E) *sac2-1* strain, DBY1924, 26°; F) *sac3-1* strain, DBY1954, 26°; G) *sac3-1* strain, DBY1954, 14°; H) *sac3-2* strain, DBY1969, 14°.

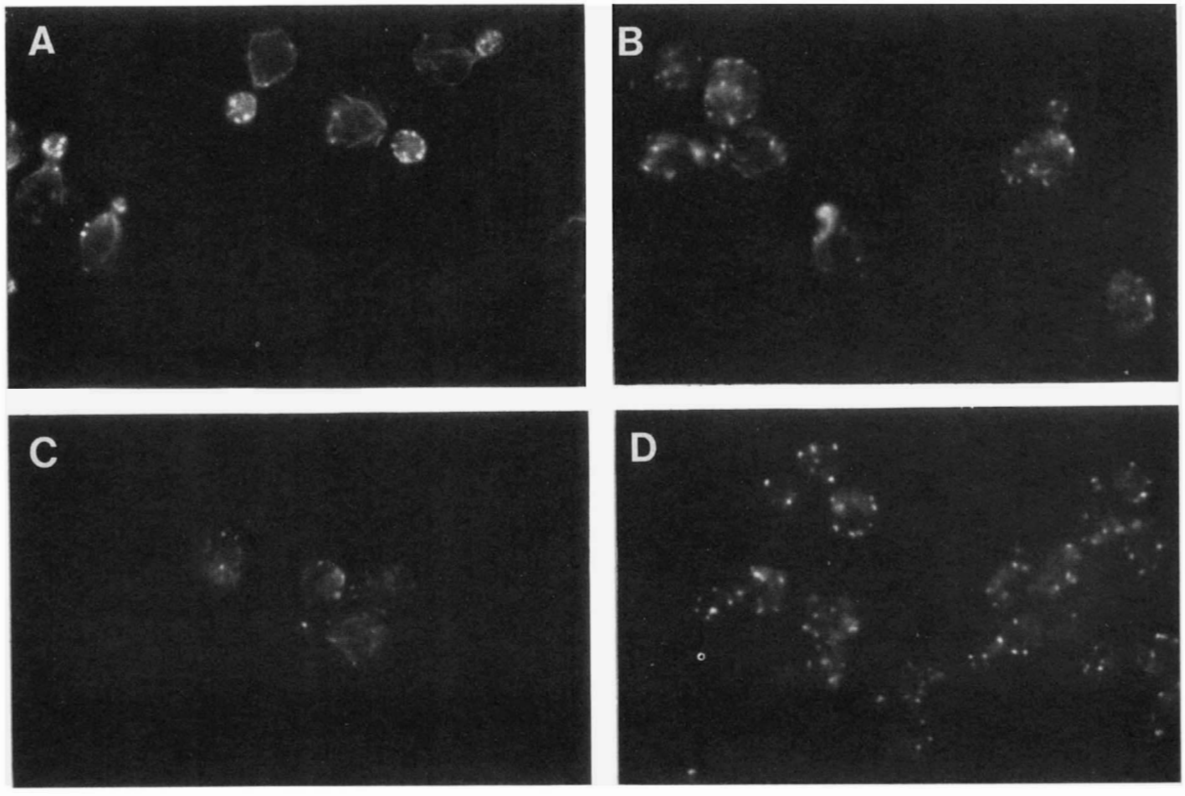

FIGURE 5.—Immunofluorescence localization of actin in wild-type cells following various treatments. Wild-type strain, DBY877, was grown at 30° in YPD and then treated for 2 h as described below, fixed and processed for immunofluorescence. A) Cells grown in YPD medium; B) cells shifted to YP medium (no glucose); C) cells shifted to YPD medium containing cycloheximide, 50 μg/ml; D) cells shifted to YP medium containing 10 mM NaN_3.

matic phenotype. At 30° both strains were found to have a low level accumulation of vesicles as well as more elaborate membrane structures (Figure 7B shows *sac2-1*). At 14° (Figure 7, D and E) the accumulation of membrane structures became more exaggerated. Vesicles and long flattened cisternae were seen. Many of the flattened cisternae appeared to contain a darkly staining granular substance. This dark granular staining is associated with the vacuole in wild-type cells. In some sections these darkly staining flattened cisternae appear to be contiguous with the endoplasmic reticulum. In some cells the flattened cisternae appear to be organized into stacks reminiscent of Golgi.

The *sac3-1* and *sac3-2* strains appeared to be normal at 30°. After incubation at 14° membrane structures were seen to develop in a fraction of the cells (Figure 7F shows *sac3-2*). These structures resembled endoplasmic reticulum. The significance of these structures is not clear due to their low frequency.

Invertase secretion in *sac* mutants: Strains carrying *sac* mutations were tested for invertase secretion and accumulation. The cells were grown in 2% glucose at 30° then shifted to 0.1% glucose at 14°. At time points aliquots were removed and cells were assayed for internal and external invertase levels. Wild-type cells secrete invertase with little change in the internal level (Table 5). This result indicates that the transit time of invertase at 14° is short compared to the derepression time. As shown in Table 5, the *sac1-6* strain reproducibly shows partial derepression of external invertase even in media containing 2% glucose (the 0 hr time point). This property was seen in several other alleles tested. At 14° complete derepression of the external invertase is seen. The internal pool rises somewhat beyond the level found with wild type, suggesting that there may be a small increase in the transit time of invertase in this mutant. The *sac2-1* strain shows very little derepression of the external form, however the internal level rises less than twofold, suggesting that the low external level is the result of low synthesis rather than a block in secretion. Other possibilities are that invertase accumulates in an inactive form or that the invertase is degraded. The *sac3-1* and *sac3-2* strains also showed low levels of secretion without accumulation of active invertase.

DISCUSSION

We used pseudoreversion analysis to identify genes whose products are somehow involved in the structure, assembly or function of the actin cytoskeleton of yeast. We began with a mutation in the gene specifying

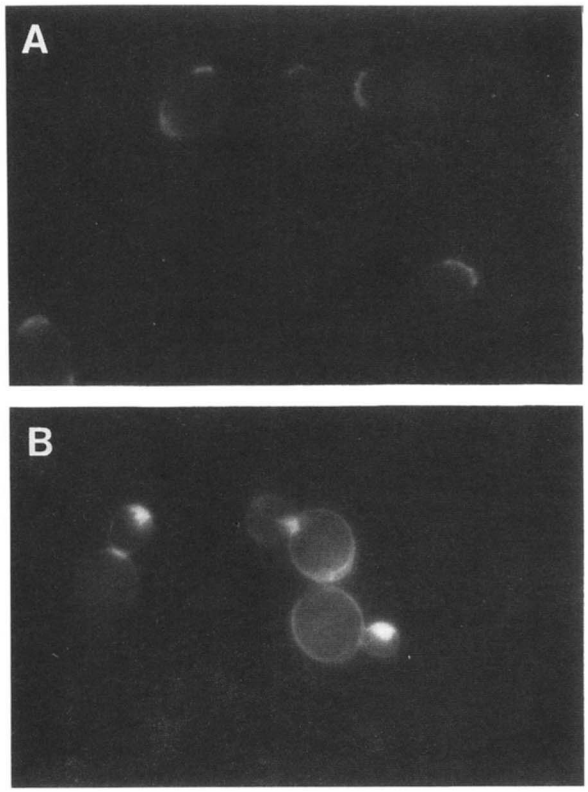

FIGURE 6.—Fluorescence localization of chitin in wild-type and *sac1* cells. Cells were grown at 26° in YPD medium then shifted to 14° for 24 hr. Cells were then processed for chitin localization. A) Wild-type diploid strain, DBY1707; B) *sac1*::*LEU2*/*sac1*::*LEU2* diploid strain, DBY1788.

actin, the central component of this system, and then sought mutations in genes specifying other components by isolating suppressors of the actin mutation. Because we restricted our attention to suppressors of our temperature sensitive actin mutant that simultaneously acquired a new phenotype (cold-sensitive growth; JARVIK and BOTSTEIN 1985; MOIR *et al.* 1982), the details of the phenotype of the new mutations could readily be studied, even in the absence of the original actin mutation, by means of temperature shift experiments to the new nonpermissive temperature. In addition, the new cold-sensitive phenotype made straightforward the selection of molecular clones of the genes in which the suppressor mutations had arisen.

The suppressor mutations we found here define five new genes (called *SAC* genes). Each of these gave rise to one or more mutations that suppress the *act1-1* mutation and simultaneously acquired a new cold-sensitive (Cs) phenotype. The suppression itself does not constitute a persuasive argument for the idea that there is an interaction between actin and the *SAC* gene products. For this reason we have, for three of the genes (*SAC1*, *SAC2* and *SAC3*) provided two other lines of evidence for such an interaction. First, we showed that each of the suppressor mutations is allele-specific: *i.e.*, it suppresses at least one, but not all actin mutations. Second, by examining these *sac* mutants' Cs phenotypes in the absence of any actin mutation, we show defects resembling those of the actin mutations themselves. Most significantly, we could show, for mutations in *SAC1*, *SAC2* and SAC3, characteristic defects in the spatial organization of actin within the cell.

Allele specificity: Assessment of the allele specificity of suppression was straightforward in the cases of *sac2* and *sac3*. In each case the double-mutant *sac act1-2* displayed both a Ts and a Cs phenotype, indicating failure of suppression. This suggests specificity in the relationship between actin and the *SAC2* or *SAC3* gene products, consistent with (but certainly not proving) a physical interaction.

The results with *SAC1* were more complicated. All *sac1* mutants tested (including both spontaneous and constructed disruption mutations) are lethal when combined with *act1-2*. We discuss this phenomenon separately below. Nevertheless, we could show allele specificity for a *sac1* mutation by using another actin mutation: the double mutant *sac1-6 act1-4* has both the Ts and Cs phenotypes.

Some *sac* phenotypes are similar to *act1* phenotypes: The strongest argument for the involvement of the *SAC* gene products in the actin cytoskeleton depends on the similarity between the consequences of the *sac* and *act1* mutations. The *sac* phenotypes were assessed in strains containing only wild-type actin genes yet they nevertheless closely resembled the most important actin mutant phenotypes.

Three phenotypes are especially significant: the immunofluorescent staining pattern revealed with antiactin antibody, the pattern of chitin deposition revealed by fluorescent staining with Calcofluor and the alteration of internal membrane structures revealed by thin section electron microscopy.

Probably the most compelling single piece of evidence for a direct role for the *SAC* gene products in the actin cytoskeleton is the altered pattern seen with antiactin antibody in the *sac* mutants. Mutations in *sac1* mimic the changes seen in *act1-1*, while mutations in *sac2* and *sac3* mimic the changes seen in *act1-2*.

The chief limitation to this line of argument is that the various alterations of the actin staining pattern may not be particularly specific phenotypes. Inhibition of protein synthesis, starvation for carbon source or treatment with metabolic poisons cause a loss or partial loss of the cables seen in wild-type cells and a randomization of the normally polarized pattern of cortical patches similar in some respects to the phenotype of *sac1* mutants. A decrease in the rate of protein synthesis or energy metabolism in *sac1* could thus serve to explain, at least in part, the observed changes in the staining pattern.

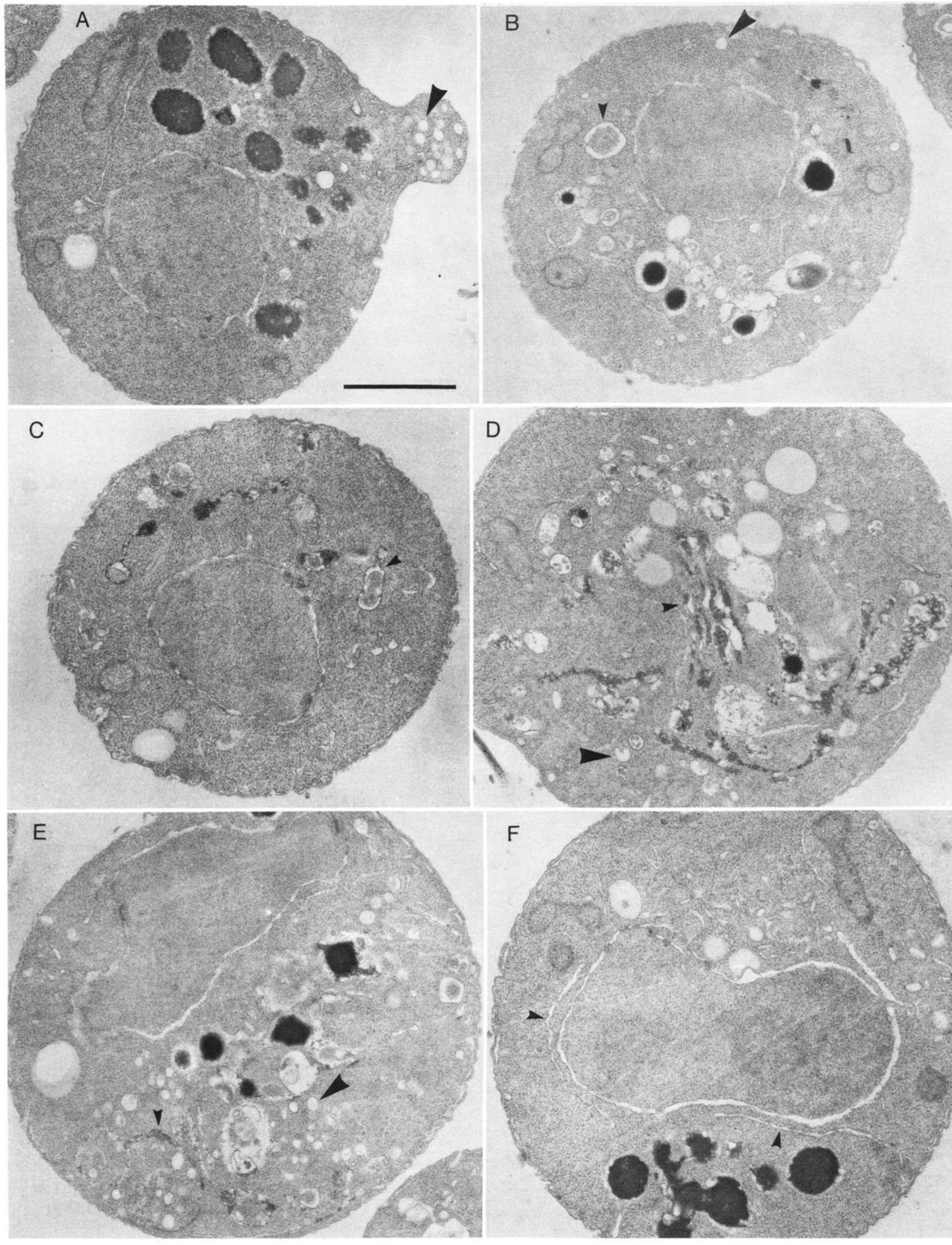

FIGURE 7.—Thin section analysis of *sac* cells. Cells were grown at 26° then shifted to 14° for 8 hr. Cells were then fixed and processed for electron microscopy. A) *sac3-2* strain, DBY1958, grown at 30°; B) *sac2-1* strain, DBY1926, 30°; C) *sac1-6* strain, DBY1885, 14°; D) *sac2-2* strain, DBY1936, 14°; E) *sac2-1* strain, DBY1926, 14°; F) *sac3-2* strain, DBY1958, 14°. The bar is 1 μm; the arrows indicate vesicles and other notable membranous structures.

TABLE 5

Invertase secretion and accumulation by *sac* mutants

Strain	Genotype	External activity at hr:			Internal activity at hr:		
		0	6	8	0	6	8
DBY1837	*SAC*$^+$	0.002	0.162	0.311	0.034	0.055	0.058
DBY1840	*sac1-6*	0.097	0.239	0.317	0.036	0.059	0.080
DBY1918	*sac2-1*	0.012	0.041	0.044	0.029	0.054	0.046
DBY1953	*sac3-1*	0.006	0.040	0.066	0.034	0.038	0.034
DBY1963	*sac3-2*	0.003	0.054	0.092	0.032	0.034	0.039

Assay of internal and external invertase assay was performed as described by NOVICK and BOTSTEIN (1985).

The heavy actin bars seen in *sac2* and *sac3*, in contrast, are not seen in glucose starved or cycloheximide treated wild-type cells. Nevertheless, this phenotype could still somehow be the indirect result of a change in another cellular condition. This kind of reservation must therefore restrain interpretation of these data until a better understanding at the molecular level of the significance of the staining patterns seen by light microscopy is obtained.

Another similarity in the phenotypes of the *act1* and *sac* mutants is the aberrant pattern of chitin deposition. While chitin is normally confined to the neck and bud scars, in these mutants generalized staining or patches of staining is seen. As in the case of the actin staining pattern, the specificity of this phenotype can be questioned. ROBERTS *et al.* (1983) have argued that any mutation or treatment that results in slowed growth can result in generalized chitin deposition. The patches of chitin seen in *sac1* mutants and in *act1-2* appear however to be a more specific phenotype than the generalized staining seen in *sac2* and *sac3*.

The third similarity between a *sac* mutant phenotype and an *act1* mutant phenotype is the dramatic accumulation of intracellular vesicles we found in both *sac2* alleles we examined. This phenotype is restricted to *sac2* mutants; only subtle morphological differences were found in *sac1* and *sac3* mutants.

Not all the actin mutant phenotypes were found among the *sac* mutant phenotypes, however. We had found previously that secretion of the periplasmic protein invertase is slow in the actin mutants (NOVICK and BOTSTEIN 1985). The enzyme which accumulates intracellularly is the mature, post-Golgi form. Presumably vesicles accumulate in the actin mutants as a result of this slowing of the secretory pathway. We examined the *sac* mutants for these phenotypes and failed to find a comparable defect in invertase secretion.

One surprising finding is that mutations in *sac1* result in partial derepression of invertase in repressing media (2% glucose) suggesting that in this mutant glucose transport or subsequent metabolism may be altered. However, no accumulation of invertase is seen in *sac1* mutants upon derepression at the restrictive temperature and there is no clear accumulation of internal membrane structures. Mutations in *sac2* show little secretion or accumulation of invertase at the restrictive temperature. This is somewhat surprising considering the dramatic accumulation of membrane-bounded structures seen in these mutants. Perhaps invertase is accumulated in an inactive form or it is degraded. Alternatively, the lowered level of secretion may simply reflect a more general defect in protein synthesis or metabolism. These possibilities are being studied further.

Mutations in *sac3* lower the level of invertase secretion with no accumulation of an internal pool and no dramatic accumulation of membrane structures. This observation could again be explained by a general defect in protein metabolism. Thus, with the possible exception of *sac2*, the partial secretory block seen in the actin mutants is not reproduced in the *sac* strains.

In sum, the phenotypic data support, but of course cannot prove, the idea that the *SAC* gene products are involved quite directly in actin function. They could be components of the actin cytoskeleton, binding directly to actin itself; alternatively they could be controllers of actin filament assembly or stability. We cannot rule out the possibility that the effect of the *SAC* gene functions on actin is indirect and that suppression is the result of a subtle change in the intracellular environment. A definitive understanding of the cellular role of these genes must await the identification of their protein products. The cloning of the wild-type alleles of these genes will facilitate these studies, which are now in progress.

Recessiveness of the *SAC* genes: In the course of studying the *SAC* genes, we have made several surprising observations that deserve comment. The first surprise is that all the mutations studied here are recessive, both for their suppression and cold-sensitive growth phenotypes. MOIR *et al.* (1982) found that all of the Sup/Ts revertants of several Cs cell-division-cycle (*cdc*) mutants were recessive for the Ts phenotype, but dominant for suppression. That result was expected, since one easily can rationalize the dominance of suppression as a gain of function (*e.g.*, restoration of normal conformation of a protein by im-

proved binding of another) and the recessiveness of the new phenotype as a loss of function (*i.e.*, failure of the second protein to function at the new nonpermissive temperature).

These arguments made the recessive suppression we observed unexpected. Recessiveness suggests loss of function, and thus it is attractive to propose that the actin mutations' defect is a consequence of interactions with other proteins whose reduced functionality or absence can result in restoration of function. This notion leads to the expectation that the *SAC/sac* heterozygote would fail to suppress. The fact that the actin mutations themselves show phenotypes that include aberrant actin structures (*e.g.*, the bars seen in *act1-2*) is consistent with this kind of view. The further observation that such aberrant structures are seen in the *sac* mutants (*sac2* and *sac3* also display bars) is consistent with this idea: the bars involve interaction between the *ACT1* and *SAC* genes. Recessive suppression could also be explained as a result of copolymer formation. If the suppressor gene product coassembles with actin, then mixing suppressing and nonsuppressing forms of the gene product on the same filament of mutant actin may result in the failure of the entire structure. The overall effect is that the suppressor would appear to be recessive.

The recessiveness of all the suppressors stimulated a search for dominant suppressors of *act1* mutations. As described in the accompanying paper (ADAMS and BOTSTEIN 1989) this search yielded a new gene, *SAC6*, that yielded only dominant suppressing alleles, and failed to reveal any dominant alleles of the five *SAC* genes defined here.

The null phenotype of *SAC1*: The second genetic surprise was that one of the *SAC* genes (*SAC1*) appears to be essential to yeast cells only at low temperatures. At first, this observation might seem to contradict the idea that the *SAC1* gene product might be a component of the actin cytoskeleton, a structure very likely to be essential, since mutations in actin are lethal (SHORTLE, HABER and BOTSTEIN 1982; SHORTLE, NOVICK and BOTSTEIN 1984).

One simple way to understand this conditional requirement for *SAC1* is to imagine that actin filament assembly, stability or function has a natural cold sensitivity that would restrict the ability of organisms like yeast to grow below a certain temperature (*e.g.*, 14°). If this were so, a tremendous advantage would accrue to organisms that had evolved a protein that could extend the useful temperature range of actin filaments. On such a hypothesis, the *SAC1* gene has become an integral part of the actin cytoskeleton simply because it allows the cytoskeleton to function over a wider range of temperature and thus allows the organism to occupy a wider range of ecological niches. A similar argument was developed by HUANG, RAMANIS and LUCK (1982) to account for the nonessentiality for function of major structures in Chlamydomonas flagellae.

The finding that some putative null alleles of *sac1* suppress *act1-1* but that the deletion does not suggests that the suppressing disruptions might indeed not be true null mutations. Sequence analysis of the gene now in progress should shed considerable light on this possibility. The fact that the disruptions show suppression does, however, suggest that *SAC1* product interacts with actin (at least in the *act1-1* mutant) even at the high temperature at which it is not required. Alternatively, the effect of the *sac1* mutations may be quantitative, with lesser amounts suppressing while normal amounts (or complete absence) does not. Another possibility is that the *SAC1* gene product serves to regulate polymerization of actin by binding to the monomer form, as does the actin binding protein profilin in animal cells. Disrupting this gene would have the effect of increasing the concentration of free actin and therefore drive the assembly reaction toward further polymerization. If the actin defect affected polymerization such a change would give better function.

Synthetic lethality: The third genetic surprise was the discovery that all *sac1* alleles tested were inviable in combination with the *act1-2* allele. In order to try to interpret this instance of "synthetic lethality," it is important to take into account that this phenomenon extends to all alleles (including the disruption mutations) but applies only to one of the three actin alleles; it is also significant that the *sac1*-null phenotype in all likelihood is failure to grow at temperatures below 14°.

A relatively simple explanation might be that the *act1-2* allele degrades the assembly, stability or function of actin filaments in the same way that low temperature does. In that event it might well be that the viability of cells depending on this mutant actin has become dependent on normal *SAC1* function, just as wild-type cells require this function below 14°.

We thank T. DUNN and D. SHORTLE for sending us *act1-4* before publication. We thank our colleagues at MIT for useful discussions; particular thanks are due A. ADAMS for comments on the manuscript. Research was supported by grants from National Institutes of Health (GM21253 and GM18973) and the American Cancer Society (MV90). P.N. was supported by Helen Hay Whitney Fellowship.

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Communicating editor: M. CARLSON