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# Genetic and Biochemical Analysis of the Adenylyl Cyclase-Associated Protein, cap, in *Schizosaccharomyces pombe*

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We have identified, cloned, and studied a gene, *cap*, encoding a protein that is associated with adenylyl cyclase in the fission yeast *Schizosaccharomyces pombe*. This protein shares significant sequence homology with the adenylyl cyclase-associated CAP protein in the yeast *Saccharomyces cerevisiae*. CAP is a bifunctional protein; the N-terminal domain appears to be involved in cellular responsiveness to RAS, whereas loss of the C-terminal portion is associated with morphological and nutritional defects. *S. pombe* cap can suppress phenotypes associated with deletion of the C-terminal CAP domain in *S. cerevisiae* but does not suppress phenotypes associated with deletion of the N-terminal domain. Analysis of *cap* disruptants also mapped the function of cap to two domains. The functional loss of the C-terminal region of *S. pombe* cap results in abnormal cellular morphology, slow growth, and failure to grow at 37°C. Increases in mating and sporulation were observed when the entire gene was disrupted. Overproduction of both cap and adenylyl cyclase results in highly elongated large cells that are sterile and have measurably higher levels of adenylyl cyclase activity. Our results indicate that cap is required for the proper function of *S. pombe* adenylyl cyclase but that the C-terminal domain of cap has other functions that are shared with the C-terminal domain of *S. cerevisiae* CAP.

## INTRODUCTION

Genetic and biochemical studies have revealed many details about the role of adenylyl cyclase and its regulation in the budding yeast *Saccharomyces cerevisiae*. In this yeast, adenylyl cyclase is not absolutely essential, but attenuation of its activity severely inhibits cell growth (Matsumoto *et al.*, 1982; Toda *et al.*, 1987; Wigler *et al.*, 1988). In *S. cerevisiae*, RAS proteins are required to stimulate adenylyl cyclase activity (Toda *et al.*, 1985). Strains that express the activated $RAS2^{val19}$ protein have constitutively high levels of adenylyl cyclase activity (Toda *et al.*, 1985). Such strains exhibit various phenotypes, including failure to arrest in G1 phase on nutrient starvation and inability to survive either nitrogen starvation or moderate heat shock (Kataoka *et al.*, 1985; Toda *et al.*, 1985; Sass *et al.*, 1986).

Many components of the RAS-cAMP pathway in *S. cerevisiae* have been identified (Defeo-Jones *et al.*, 1983; Kataoka *et al.*, 1984, 1985; Powers *et al.*, 1984; Wigler *et al.*, 1988), but the question of whether RAS acts directly on adenylyl cyclase or on another protein to indirectly stimulate adenylyl cyclase activity remains unanswered. At least one other protein, named CAP, is associated with adenylyl cyclase and has been implicated in its regulation (Fedor-Chaiken *et al.*, 1990; Field *et al.*, 1990). CAP has two distinct functional domains; expression of the N-terminal portion appears sufficient for full cellular responsiveness to RAS proteins, and this domain presumably interacts with adenylyl cyclase, whereas loss of the C-terminal portion causes morphological and nutritional defects that appear unrelated to adenylyl cyclase or RAS (Field *et al.*, 1990; Gerst *et al.*, 1991). These latter defects are suppressed by overproduction of profilin, an actin and phospholipid-binding protein (Vojtek *et al.*, 1991). The defects that result from loss of the C-terminal domain are also suppressed by the expression of just the C-terminal portion of CAP

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itself. Hence, the N-terminal and C-terminal portions of CAP appear to act independently. These domains are connected by a proline-rich stretch of amino acids that appear to be functionally silent.

To understand better the evolution and conservation of the RAS and adenylyl cyclase pathways, we began studying the fission yeast *Schizosaccharomyces pombe*. In *S. pombe*, both ras1 and adenylyl cyclase are involved in the sexual differentiation pathways that lead to conjugation and sporulation (Fukui *et al.*, 1986; Nadin-Davis *et al.*, 1986; Kawamukai *et al.*, 1991), but ras1 does not appear to regulate *S. pombe* adenylyl cyclase (Fukui *et al.*, 1986; Nadin-Davis *et al.*, 1986; Kawamukai *et al.*, 1991). Nevertheless, the *S. pombe* adenylyl cyclase shares significant homology with the *S. cerevisiae* adenylyl cyclase (Yamawaki-Kataoka *et al.*, 1989; Young *et al.*, 1989), both in the catalytic domain and in the repeated leucine-rich motif domain that is required for the RAS responsiveness of the *S. cerevisiae* enzyme (Colicelli *et al.*, 1990; Suzuki *et al.*, 1990). The *S. cerevisiae* and *S. pombe* adenylyl cyclases also appear to share some aspects of their regulation (Kawamukai *et al.*, 1991).

The *S. cerevisiae* CAP protein is complexed with adenylyl cyclase, appears to be required for RAS responsiveness, and is involved in maintenance of cell morphology. We sought a homologous protein in *S. pombe* to determine whether any CAP functions have been conserved in evolution. We report here the identification of a *S. pombe* protein that is both structurally and functionally related to *S. cerevisiae* CAP and appears to be required for adenylyl cyclase function and normal cellular morphology.

## MATERIALS AND METHODS

### *Strains and Media*

The genotypes of all yeasts strains used in this study are listed in Table 1. *S. pombe* strains were grown in either YEA-rich (0.5% yeast extract, 3% glucose 75 mg/l adenine) medium or PMA synthetic medium supplemented, when necessary, with 75 mg/l leucine and/or uracil (Mitchison, 1970). *S. cerevisiae* strains were grown in YPD-rich medium (1% yeast extract, 2% peptone, 2% dextrose) or SC synthetic medium with appropriate auxotrophic supplements (Rose *et al.*, 1990). The lithium acetate method was used for yeast transformation (Ito *et al.*, 1983). General genetic methods used for *S. pombe* have been described previously (Gutzet *et al.*, 1974; Moreno *et al.*, 1990).

### *DNA Manipulation and Analysis*

Procedures used for DNA manipulation and analysis (i.e., purification, restriction mapping, electrophoresis, transformation, colony hybridization, etc.) were previously described (Maniatis *et al.*, 1982). The DNA sequences of both strands of sequenced clones were determined by a modification (Biggins *et al.*, 1983) of the dideoxy-chain termination method (Sanger *et al.*, 1977). Polymerase chain reactions were performed as previously described (Saiki *et al.*, 1988).

### *Plasmids*

pAL, pIRT5, and pALY1 were previously described (Kawamukai *et al.*, 1991). YEp13M4 was previously described (Nikawa *et al.*, 1987). pWH5 is a shuttle vector used to construct a *S. pombe* genomic library (the gift of D. Beach, Cold Spring Harbor Laboratory). pT7.CAP and pYCYR were previously described (Field *et al.*, 1990). pSC2 was isolated from the *S. pombe* cDNA library, constructed in pADANS as described in the section below. pCAP2-1 and pCAP7-3 contain *S. pombe* cDNA clones in pBluescript SK- (Stratagene, La Jolla, CA). They were derived from lambda ZAP II clones and isolated from the *S. pombe* cDNA library described below by the in vivo excision procedure. pYCL1 was constructed by inserting a 2.5-kb *Not* I fragment containing the *S. pombe cap* cDNA from pCAP7-3 into the *Not* I site of the *S. cerevisiae* expression vector pADNS (Colicelli *et al.*, 1989). pACL1 was constructed by inserting the 2.5-kb *Not* I fragment en-

**Table 1.** Yeast strains used in this study

| | |
|---|---|
| *S. pombe* | |
| SP870 | $h^{90}$ *leu1-32 ade6-210 ura4-d18* |
| SP826 | $h^{+N}/h^{+N}$ *leu1-32/leu 1-32 ade6-210/ade6-216 ura4-d18/ura4-d18* |
| MK7 | $h^{90}$ *leu1-32 ade6-210 ura4-d18 cyr1::LEU2* |
| MK141 | $h^{90}$ *leu1-32 ade6-210 ura4-d18 cyr1::pALY4(LEU2)* |
| MK1009 | $h^{90}$ *leu1-32 ade6-210 ura4-d18 cap::ura4*$_{(cad7)}$ |
| MK1818R | $h^{90}/h^{+N}$ *leu1-32/leu1-32 ade6-210/ade6-216 ura4-d18/ura4-d18 cap::ura4*$_{(cad11)}$ |
| MK1818d | $h^{90}$ *leu1-32 ade6-210 ura4-d18 cap::ura4*$_{(cad11)}$ |
| *S. cerevisiae* | |
| SKN32 | *MATa leu2 ura3 trp1 ade8 can1 cap::HIS3* |
| SKN37 | *MATa leu2 ura3 trp1 ade8 can1 RAS2*$^{val19}$ *cap::HIS3* |
| T158-5AT | *MATa leu2 ura3 trp1 ade8 his3 cyr1::URA3* (pTPK1-TRP1) |

SP870 and SP826 were obtained from Dr. D. Beach. MK7 and MK141 were previously described (Kawamukai et al., 1991). MK141 contains an integrated copy of pALY4 (see MATERIALS AND METHODS). pALY4 encodes a fusion protein containing the peptide MYPYDVPDYASLGGPMSTLD, which is recognized by the monoclonal antibody 12CA5, fused to the amino terminus of adenylyl cyclase. The construction of MK1009, MK1818R, and MK1818d are described in text. SKN32, SKN37 (Field et al., 1990), and T158-5AT (Toda et al., 1988) were previously described. T158-5AT has pTPK1-TRP1, a plasmid expressing the *TPK1* gene that encodes one of the isoforms of the cAMP-dependent protein kinase catalytic subunit and that suppresses the disruption of CYR1.

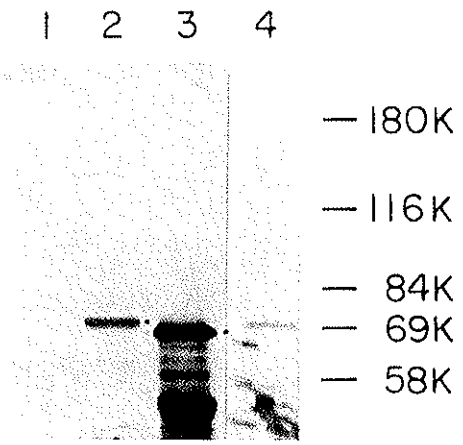


**Figure 1.** Detection of CAP-related protein in *S. pombe* by western blot analysis. Western blot analysis of protein samples (prepared as described in MATERIALS AND METHODS) was performed using a 1:300 dilution of the antisera KF191. Protein bands recognized by KF191 were detected with alkaline phosphatase-conjugated anti-rabbit IgG antibody, as described in MATERIALS AND METHODS. KF191 is a polyclonal rabbit antisera raised against the *S. cerevisiae* CAP protein purified from *E. coli* strain BL-21(DE3)pLysS that contains the CAP expression plasmid pT7.CAP, as previously described (Kawamukai *et al.*, 1991). Protein samples analyzed were as follows: lane 1, extract from *E. coli* strain XL-Blue harboring the control plasmid pBluescript SK-(5 μg protein/lane); lane 2, extract from *E. coli* strain XL-Blue harboring pCAP7, which express *S. pombe* cap protein (5 μg protein/lane); lane 3, extract from *E. coli* strain BL-21(DE3)pLysS harboring pT7.CAP, which express *S. cerevisiae* CAP protein (1 μg protein/lane); lane 4, partially purified adenylyl cyclase complex from *S. pombe* MK141 membrane extracts (~1 μg protein/lane). Dots indicate protein bands of interest.

coding *S. pombe* cap from pCAP7-3 into the *Not* I site of pAALN (Xu *et al.*, 1990). pACL7 was constructed from pACL1 by digesting *Pfl*MI and *Sna*BI, filling in the cleaved ends with Klenow enzyme, and ligating. pA70L was constructed by filling in the ends of the 1.8-kb *Bam*HI-*Hin*dIII fragment containing the *S. cerevisiae CAP* cDNA from pCAP-2 (Field *et al.*, 1990) with Klenow enzyme and inserting it into the *Sma* I site of pART1 (McLeod *et al.*, 1987). pALY12 was constructed by inserting the 6.9-kb *Sal* I-*Sna*BI fragment containing the *S. cerevisiae CYR1* gene from pYCYR into the *Sal* I-*Sma* I site of pAIC (Kawamukai *et al.*, 1991). pYALY2 was constructed by inserting the 6.2-kb *Sal* I-*Sac* I fragment containing the *S. pombe CYR1* gene from pALY1 into pAD5. pAD5 was derived from pAD1 (Toda *et al.*, 1988) by insertion of a synthetic oligonucleotide encoding the epitope recognized by the antibody 12CA5. pUDN1 was derived from pUC118 by insertion of a *Not* I linker in the *Hin*dIII site, and the multicloning sites between the *Eco*RI and *Sph* I sites were deleted by digesting with *Eco*RI and *Sph* I, filling in with Klenow enzyme, and ligating. pCAD1 was constructed by inserting the 2.5-kb *Not* I fragment containing the *S. pombe cap* cDNA from pCAP7-3 into the *Not* I site of pUDN1. pCAD3 was derived from pCAD1 by replacing the 0.5-kb *Hin*dIII fragment with the 1.8-kb *Hin*dIII fragment containing the *ura4* gene from pIRT5. pCAD5 was constructed by inserting the 1.8-kb *Hin*dIII fragment containing the *ura4* gene from pIRT5 into the *Hin*dIII site of pCAD1. pCAD11 was constructed by inserting the 1.2-kb *Xba* I fragment containing the 5' proximal region of the *cap* gene from pCGE2 into the *Xba* I site of pCAD5. pCGE2 is a genomic clone of *cap* in the pWH5 vector (see text and Figure 3).

## *Construction of S. pombe cDNA Libraries*

A cDNA library was constructed in the lambda ZAPII expression vector (Stratagene). Poly A+ mRNA was purified from a logarithmically growing culture of the *S. pombe* strain SP66 (Table 1) by a previously described procedure (Rose *et al.*, 1990). cDNA was synthesized from 10 μg of poly A+ RNA using molonez murine leukemia virus (M-MLV) reverse transcriptase (Bethesda Research Labs, Gaithersburg, MD) as follows. The first strand synthesis reaction containing 10 μg RNA, 50 μg/ml oligo dT, 10 mM each of dATP, dCTP, dGTP, and dTTP, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (CDTT), 50 mM tris(hydroxymethyl) aminomethane (Tris) (pH 8.3), and 2.5 μl (25 units) M-MLV reverse transcriptase in 50 μl was incubated for 1 h at 37°C. Second strand synthesis was achieved by adjusting the reaction to contain 100 mM KCl, 10 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 250 μM each dATP, dCTP, dGTP, and dTTP, 0.15 mM NAD, 5 mM DTT, 25 mM Tris (pH 8.3), 3.4 units RNAse H, and 100 units DNA polymerase I in 400 μl, then incubating at 16°C for 2 h. Samples were digested with RNAse A, and the cDNA was precipitated. *Not* I linkers (New England Biolabs, Beverly, MA) were ligated to the ends of the cDNAs, which were then digested with *Not* I and fractionated on a Sepharose CL-4B column. Fractions containing cDNAs with an average molecular weight of >500 bp were pooled and cloned into the *Not* I site of the lambda ZAP II vector. Over $2 \times 10^6$ independent cDNA clones were obtained.

DNA was prepared from the library and digested with *Not* I. *Not* I fragments containing the cDNAs were fractionating by agarose gel electrophoresis and purified by electroelution. They were then cloned into the *Not* I site in the *S. cerevisiae* expression vector pADANS, which contains the *ADH1* promoter and terminator sequences flanking the *Not* I site, the *LEU2* gene, and the 2-μm sequence (Colicelli *et al.*, 1989).

## *Adenylyl Cyclase Activity Measurement*

Adenylyl cyclase activity was measured in crude membrane extracts as previously described (Young *et al.*, 1989). One-liter yeast cultures

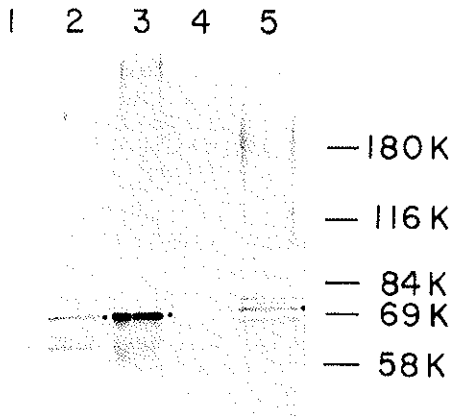


**Figure 2.** Characterization of the antisera AB321. Western blot analysis was performed, as described in MATERIALS AND METHODS, using a 1:300 dilution of the antisera AB321. Protein bands recognized by AB321 were detected with alkaline phosphatase-conjugated anti-mouse IgG antibody, as described in MATERIALS AND METHODS. AB321 is a polyclonal rabbit antisera raised against a partially purified adenylyl cyclase complex from *S. pombe*, as described in MATERIALS AND METHODS. Lane 1, extract from *E. coli* strain XL-Blue harboring the control plasmid pBluescript SK−; lane 2, extract from *E. coli* strain XL-Blue harboring pCAP2-1, which express the bip-related protein; lane 3, extract from *E. coli* XL-Blue harboring pCAP7-3, which express *S. pombe* cap; lane 4, extract from *E. coli* strain B121 (DE3) pLysS harboring pT7.CAP that expresses *S. cerevisiae* CAP; lane 5, partially purified adenylyl cyclase complex from *S. pombe* strain MK141. The amount of proteins loaded in each lanes is as indicated in Figure 1. Dots indicate protein bands of interest.

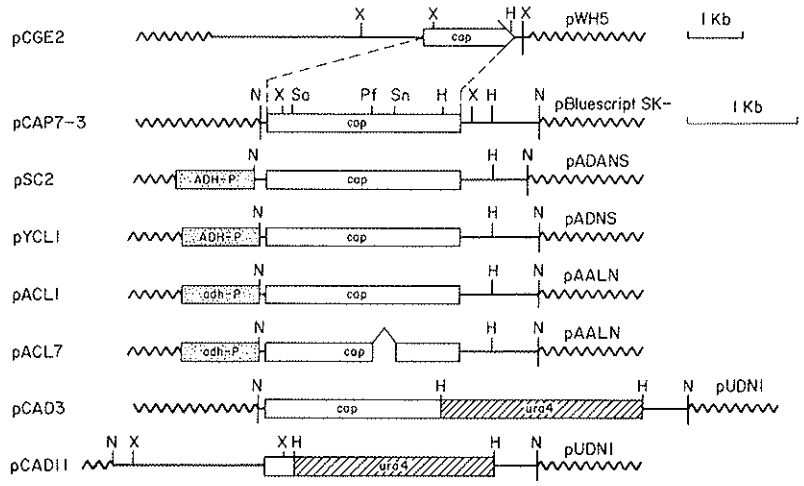


**Figure 3.** Restriction maps of plasmids. The construction of all plasmids are described in MATERIALS AND METHODS. pCAP7-3 contains a *S. pombe* cDNA encoding cap in the vector pBluescript SK−. pYCL1 contains the *cap* cDNA in the *S. cerevisiae* expression vector pADNS. pSC2 contains an overlapping *cap* cDNA in the *S. cerevisiae* expression vector pADANS. pACL1 contains the *cap* cDNA in the *S. pombe* expression vector pAALN. pCGE2 contains the *cap* gene. It was isolated from a genomic *S. pombe* library constructed in the vector pWH5 (gift of D. Beach). pACL7 contains *cap* with deletions between codons 296 and 367. pCAD3 and pCAD11 contain disruptions of the *cap* gene, in which regions of the *cap* gene have been replaced with the selectable *ura4* genes. The arrow in pCGE2 indicates the direction and location of sequences encoding cap. The positions of sites for *Hin*dIII (H), *Not* I (N), *Pfl*MI (Pf), *Sal* I (Sa), *Sna*BI (Sn), and *Xba* I (X) are indicated. Open boxes indicate *cap* coding sequences, hatched boxes the *ura4* gene, stippled boxes the *ADH1* promoter of *S. cerevisiae* and the *adh1* promoter of *S. pombe*, and the squiggly lines represent vector sequences. Noncoding sequences of the *cap* locus are indicated by the flat line. Size markers are indicated on the right and the names of vectors to the left.

were grown to a density of $1 \times 10^7$ cells/ml. Cells were washed in buffer C (20 mM Mes, pH 6.2, 0.1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride), resuspended in 35 ml of buffer C, and lysed in a French press at 20 000 psi. The lysate was centrifuged at 1000 × *g* for 10 min. The pellet was discarded, and the supernatant was centrifuged at 15 000 rpm for 90 min in a Sorvall SS34 rotor. The pellet was resuspended in 2 ml of buffer C. Adenylyl cyclase reactions containing 50 μg of membrane protein in Buffer C with 1 mM ATP, 10 μCi [$^{32}$P]ATP, 0.25 mM cAMP, 2.5 mM $MnCl_2$, 20 mM creatine phosphate, and 20 units of creatine phosphokinase (Sigma, St. Louis, MO) in 100 μl were incubated for 30 min at 30°C. Protein concentration was determined by the bicinchoninic acid method, as previously described (Pierce, Rockford, IL).

## *Purification of Adenylyl Cyclase Complex*

Adenylyl cyclase was purified from a 100-l culture of the *S. pombe* strain MK141 (Table 1) by a procedure previously developed to purify the *S. cerevisiae* adenylyl cyclase complex (Field *et al.*, 1990). MK141 expresses high levels of an adenylyl cyclase protein that is tagged at the amino terminal end with the peptide MYPYDVPDYASLGGPM-STLD (Kawamukai *et al.*, 1991). This fusion protein is expressed from a gene that has been integrated into the chromosomal DNA. Cells were grown to a density of $1 \times 10^7$ cells/ml in YEA medium, harvested, frozen in liquid nitrogen, and stored at −70°C. Later, cells were thawed and disrupted using a French Press. Unbroken cells were removed by centrifugation at 200 × *g* for 10 min. Membrane pellets were precipitated by centrifugation at 44 000 × *g* for 30 min. Membranes were solubilized in Buffer C containing 1% Lubrol PX (Pierce) and 0.5M NaCl. Insoluble material was removed by centrifugation at 44 000 × *g* for 30 minutes. Soluble membrane from 2 l of cells was passed over columns containing 2 ml of protein A-sepharose beads cross-linked to 2 mg of the monoclonal antibody 12CA5. 12CA5 was cross-linked to protein A-sepharose beads by a procedure described elsewhere (Harlow and Lane, 1988). After soluble membrane was passed through the column, the column was washed with 50 ml of Buffer C containing 1% Lubrol PX and 0.5 M NaCl and then with 50 ml Buffer C containing 0.01% Lubrol PX, 0.5M NaCl, and 20% glycerol at 4°C. Bound protein was eluted with 1 mg of the peptide YPYDVPDYA at 30°C for 15 min. Samples were collected, and the column was washed five times with 2 ml of Buffer C containing 0.01% Lubrol PX and 0.5M NaCl. The samples from a total of 10 l of cultured yeast were pooled and concentrated 100-fold by ultrafiltration.

## *Raising Antisera to S. pombe cap*

Preparations enriched for the 70-kDa *S. pombe* adenylyl cyclase-associated protein were obtained by immunoprecipitation of the adenylyl cyclase complex and purified by the procedure described above by the antisera KF191. The immunoprecipitated protein was resuspended in Freund's adjuvent and subcutaneously injected into rabbits at 2- to 3-wk intervals. The polyclonal antisera AB321 was obtained from one of the injected rabbit.

## *Immunological Methods*

Immunoprecipitations were performed typically by mixing the soluble membrane extract described above, 2 μl of antisera and 30 μl of 50% protein A-Sepharose beads (Boehringer-Mannheim, Indianapolis, IN), in a final 100-μl solution. The mixture was rotated for 1 h at 4°C and spun down. The pellets were washed three times with Buffer C containing 1% Lubrol PX and 0.5M NaCl. Adenylyl cyclase activity in these pellets was measured.

Western blot analysis was performed using the kit and the procedure recommended by the manufacturer (Bio-Rad, Richmond, CA). As control proteins, extracts of *Escherichia coli* expressing cap or CAP were prepared as follows: *E. coli* was grown on LB medium containing 100 μg/ml ampicillin. Log-phase cultures were harvested and washed once with Buffer C. Cells on ice were disrupted by sonication. Unbroken cells were removed by centrifugation (3000 rpm × 10 min in Sorvall SS34 rotor). Soluble extracts were used for Western blotting. Protein amounts were determined by the BCA method. One to five micrograms of proteins were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfered to nitrocellulose paper. Membranes were blocked with 3% nonfat dry milk in phosphate-buffered saline (PBS) for 1–2 h. After membranes were washed with PBS, they were incubated with the antisera (1:300 dilution). Alkaline phosphatase-conjugated anti-IgG antibody (Bio-Rad) was used for detection.

For screening the *S. pombe* lambda ZAP II cDNA library, *E. coli* strain BB4 was infected and incubated for 6 h. Then, plaques were incubated for 4 h with nitrocellulose filters soaked in 10 mM Isopropyl-

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-88 GCGGCCGCAACACACTTACTATATTTGTTGCGTTGCATTATAAAAATCACTTAACCTCCGCTAAACACATTTTCTTTTCGTCTAACCATGTCGGATATGATCAATATA
  1                                                                                                 METSerAspMetIleAsnIle

 22 CGTGAAACAGGCTACAATTTTACCACGATTCTCAAACGACTGGAAGCGGCAACTTCTCGTTTGGAGGATCTTGTGGAAAGTGGTCATAAGCCTCTTCCCAACATGCAT
  8 ArgGluThrGlyTyrAsnPheThrThrIleLeuLysArgLeuGluAlaAlaThrSerArgLeuGluAspLeuValGluSerGlyHisLysProLeuProAsnMetHis

130 CGTCCTTCTAGAGATTCAAACAGTCAAACCCACAACATATCCTTCAACATTGGTACTCCAACGGCCCCAACCGTGTCGACTGGTAGTCCTGCTGTCGCATCATTACAT
 44 ArgProSerArgAspSerAsnSerGlnThrHisAsnIleSerPheAsnIleGlyThrProThrAlaProThrValSerThrGlySerProAlaValAlaSerLeuHis

238 GATCAAGTTGCGGCTGCTATCAGTCCTCGGAACAGAAGTCTTACATCCACCTCTGCCGTTGAAGCGGTTCCTGCTTCCATTTCCGCCTACGATGAATTTTGTTCCAAG
 80 AspGlnValAlaAlaAlaIleSerProArgAsnArgSerLeuThrSerThrSerAlaValGluAlaValProAlaSerIleSerAlaTyrAspGluPheCysSerLys

346 TATTTATCTAAATACATGGAATTGAGTAAAAAAATTGGTGGTTTGATTGCCGAACAAAGTGAACACGTCGAGAAGGCTTTTAACCTTTTACGTCAGGTTTTGAGCGTC
116 TyrLeuSerLysTyrMetGluLeuSerLysLysIleGlyGlyLeuIleAlaGluGlnSerGluHisValGluLysAlaPheAsnLeuLeuArgGlnValLeuSerVal

454 GCTTTGAAAGCCCAGAAACCCGACATGGATTCCCCGGAACTCTTGGAATTCCTCAAGCCTATCCAATCTGAACTGCTCACTATTACCAATATTCGAGATGAACACCGC
152 AlaLeuLysAlaGlnLysProAspMetAspSerProGluLeuLeuGluPheLeuLysProIleGlnSerGluLeuLeuThrIleThrAsnIleArgAspGluHisArg

562 ACCGCCCCCGAGTTCAATCAACTTTCTACCGTCATGAGTGGAATTAGTATTTTGGGCTGGGTCACAGTTGAGCCCACTCCATTATCCTTTATGTCTGAAATGAAAGAT
188 ThrAlaProGluPheAsnGlnLeuSerThrValMetSerGlyIleSerIleLeuGlyTrpValThrValGluProThrProLeuSerPheMetSerGluMetLysAsp

670 TCGTCCCAATTCTACGCCAACCGTGTAATGAAAGAGTTTAAAGGTAAAGATGACTTGCAAATCGAATGGGTTCGTTCTTACCTTACTCTGTTGACTGAATTGATTACA
224 SerSerGlnPheTyrAlaAsnArgValMetLysGluPheLysGlyLysAspAspLeuGlnIleGluTrpValArgSerTyrLeuThrLeuLeuThrGluLeuIleThr

778 TACGTCAAGACCCACTTCAAAACCGGTCTCACCTGGTCTACTAAACAGGATGCAGTACCCTTGAAAACGGCTTTGGCAAACTTGTCTGCTTCGAAAACTCAGGCACCA
260 TyrValLysThrHisPheLysThrGlyLeuThrTrpSerThrLysGlnAspAlaValProLeuLysThrAlaLeuAlaAsnLeuSerAlaSerLysThrGlnAlaPro

886 TCTTCTGGCGATTCTGCAAACGGCGGCTTGCCTCCACCACCACCACCACCTCCTCCATCTAATGATTTTTGGAAGGATAGCAACGAGCCCGCACCTGCTGATAATAAA
296 SerSerGlyAspSerAlaAsnGlyGlyLeuProProProProProProProProSerAsnAspPheTrpLysAspSerAsnGluProAlaProAlaAspAsnLys

994 GGTGATATGGGAGCGGTGTTTGCCGAAATTAATAAAGGCGAGGGAATTACATCCGGACTTCGTAAGGTGGATAAGAGTGAAATGACCCACAAAAATCCGAATTTACGT
332 GlyAspMetGlyAlaValPheAlaGluIleAsnLysGlyGluGlyIleThrSerGlyLeuArgLysValAspLysSerGluMetThrHisLysAsnProAsnLeuArg

1102 AAAACGGGACCCACTCCTGGTCCTAAGCCTAAAATCAAAAGCTCTGCTCCTTCTAAGCCAGCGGAAACTGCTCCCGTTAAACCACCTCGCATTGAGTTGGAAAATACT
 368 LysThrGlyProThrProGlyProLysProLysIleLysSerSerAlaProSerLysProAlaGluThrAlaProValLysProProArgIleGluLeuGluAsnThr

1210 AAGTGGTTTGTTGAAAACCAAGTCGACAATCACAGTATAGTCTTGGATTCTGTGGAGCTCAACCACTCAGTGCAAATTTTCGGTTGCTCCAATTGCACAATTATAATT
 404 LysTrpPheValGluAsnGlnValAspAsnHisSerIleValLeuAspSerValGluLeuAsnHisSerValGlnIlePheGlyCysSerAsnCysThrIleIleIle

1318 AAAGGAAAGTTAAACACCGTTTCCATGAGCAACTGTAAACGTACTAGCGTTGTAGTAGATACTTTAGTGGCCGCTTTTGATATTGCTAAGTGTTCCAATTTTGGCTGC
 440 LysGlyLysLeuAsnThrValSerMetSerAsnCysLysArgThrSerValValValAspThrLeuValAlaAlaPheAspIleAlaLysCysSerAsnPheGlyCys

1426 CAAGTTATGAATCACGTTCCAATGATTGTTATCGACCAATGCGATGGAGGTTCCATCTATTTGAGCAAATCTTCGTTGTCGTCCGAAGTTGTCACTAGTAAATCAACA
 476 GlnValMetAsnHisValProMetIleValIleAspGlnCysAspGlyGlySerIleTyrLeuSerLysSerSerLeuSerSerGluValValThrSerLysSerThr

1534 AGCTTGAACATCAACGTCCCCAACGAAGAGGGAGATTATGCTGAACGTGCTGTTCCCGAGCAGATCAAGCACAAGGTGAATGAAAAGGGAGAGCTTGTCTCTGAGATT
 512 SerLeuAsnIleAsnValProAsnGluGluGlyAspTyrAlaGluArgAlaValProGluGlnIleLysHisLysValAsnGluLysGlyGluLeuValSerGluIle

1642 GTACGTCACGAATAAGTTTTTCTCAGAGAGCTTTTTCAAGCTATATTCCTAACGGTTTCATAGTAAAGATTTAAGTTGATTTGTCTAGAGCTTTTTATTCATCTTCAT
 548 ValArgHisGlu  .

1750 CTTTTTCTTTTACTGCTACTAACGACACACTTTGCTCGATGTTTTGTAAAATTGATCAATCAAATCTTCGGCTTAATTTCAGAGATACAAATATTTGTTTTCCCTTCG

1858 CTTTCGACTTTCCGATGCGATTTACCTTTTGCCCAAATATCCCTTGGACTCAATGTGTACTTCCCCACATACATCTTTATATTGAATGGGATCGGATAATGCGCGGGA

1966 AAAGTTGTTGTCCGTACCACGAATTGCTACGTTGACTTTATGATATATGCGTGCATGCATACATACGAATAGTGGTTATTTTACGAGTTAAACGATATAGAAGCTTAA

2074 ATTTAGTGATTGTTTGTGTGCAAATAGAATTTATGGATGACAATGATAAGGCTGTTGTATATGACGCTTCCTCATTTCTTTTTCCCTGTTGATTTTAACTCGAAAGGA

2182 TTTTAAGTGTCTTCCGTTCGCCAATTGAATTCATCATCATCTTCTTTTTGTTTTTGTTTTTCAGTCTAAACAAATCCTCTAGCTAAGGTATCATTTTCTCGATTGCTG

2290 TTTTGCTCATGTTATGTTTTTATGAAAAAAAGCCATGCTGTTCAATAAAAGAGGAAAGGAAGATTTTGGAAGTTTTATAGTTGAATGAGTTCATCTATAAATATATAC

2398 ATATATACATATATATAATATACAAAATATTTTTTCTTTGAGGCTTGAACTATAAAAAAAAAAAATTGCGGCCGC
```

**Figure 4.** Sequence of *S. pombe cap* cDNA and encoded protein. The DNA sequence shown is a composite derived from the two overlapping cDNA inserts in pCAP7-3 and pSC2 (see Figure 3). The 5′ and 3′ end *Not* I sites were derived from linkers used in cloning the cDNAs. The amino acid sequence of the encoded *S. pombe* cap protein is also shown. The inframe stop codons, located at the 3′ end of the coding sequence and 36 bp 5′ from the start codon, are indicated in bold. The cDNA encodes a 551 amino acid residue protein. Numbers on the left-hand side indicate the number of base pairs from the *Not* I site and the number of amino acid residues from the beginning of the encoded protein.

```
Sp  001   MSDMINIRETGYNFTTILKRLEAATSRLEDLVESGHKPLPNMHRPSRDSNSQTHNISFNIGTPTAPTVSTGSPAVASLHDQVAAAISPRNRSLTSTSAVE
Sc  001    MPDSKYTMQGYNLVKLLKRLEEATARLEDVTIYQEGYIQNKLEASKNNKPS----------------------------DSGADANTTNEPSAENAPEVE

Sp  101   AVPASISAYDEFCSKYLSKYMELSKKIGGLIAEQSEHVEKAFNLLRQVLSVALKAQKPDMDSPELLEFLKPIQSELLTITNIRDEHRTAPEFNQLSTVMS
Sc  073   QDPKCITAFQSYIGENIDPLVELSGKIDTVVLDALQLLKGGFQSQLTFLRAAVRSRKPDYSSQTFADSLRPINENIIKLGQLKESNRQSKYFAYLSALSE

Sp  201   GISILGWVTVEPTPLSFMSEMKDSSQFYANRVMKEFKGKDDLQIEWVRSYLTLLTELITYVKTHFKTGLTWSTKQDAVPLKTALANLSASKTQAPSSGDS
Sc  173   GAPLFSWVAVD-TPVSMVTDFKDAAQFWTNRILKEYRESDPNAVEWVKKFLASFDNLKAYIKEYHTTGVSWKKDGMDFADAMAQSTKNTGATSSPSPASA

Sp  301   ANGGLPPPPPPPPP------SNDFWKDSNEPAPADNKGDMGAVFAEINKGEGITSGLRKVDKSEMTHKNPNLRKTGPTPGPKPKIKSSA-PSKPAETAPV
Sc  272   TAAPAPPPPPPAPPASVFEISNDTPATSSDA----NKGGIGAVFAELNQGENITKGLKKVDKSQQTHKNPELRQSSTVSSTGSKSGPPPRPKKPSTLKTK

Sp  394   KPPRIELENTKWFVEN-QVDNHSIVLDSVELNHSVQIFGCSNCTIIIKGKLNTVSMSNCKRTSVVVDTLVAAFDIAKCSNFGCQVMNHVPMIVIDQCDGG
Sc  368   RPPRKELVGNKWFIENYENETESLVIDANKDE-SIFIGKCSQVLVQIKGKVNAISLSETESCSVVLDSSISGMDVIKSNKFGIQVNHSLPQISIDKSDGG

Sp  493   SIYLSKSSLSSEVVTSKSTSLNINVP-NEEGDYAERAVPEQIKHKVNEKGELVSEIVRHE
Sc  467   NIYLSKESLNTEIYTSCSTAINVNLPIGEDDDYVEFPIPEQMKHSFAD-GKFKSAVFEHA
```

**Figure 5.** Comparison of *S. pombe* and *S. cerevisiae* CAP proteins. The *S. cerevisiae* (Sc) CAP and *S. pombe* (Sp) cap protein sequences were compared by the FASTDB program (Brutlag *et al.*, 1990; IntelliGenetics Inc., Mountain View, CA), using a PAM-250 similarity matrix, as previously described (Young *et al.*, 1991). Horizontal dashes represent gaps that were inserted during the alignment process. Numbers on the left indicate the amino acid residue position. Vertical lines are between identical residues and semicolons are between conserved residues.

β-D-thiogalactopyranoside to induce protein synthesis. Filters were blocked by PBS containing the 3% nonfat dry milk for 2 h and incubated with PBS containing the antisera AB321 (1:300 dilution) and 3% bovine serum albumin. Membranes were further incubated with the alkaline phosphatase-conjugated anti-IgG antibody for 2 h and colorized.

## RESULTS

### *Detection of S. pombe CAP-Related Protein*

We previously reported that a polyclonal antisera, KF191, raised against *S. cerevisiae* CAP protein immunoprecipitates an adenylyl cyclase complex from a solubilized membrane preparation from the *S. pombe* strain, MK141 (Kawamukai *et al.*, 1991). This strain (Table 1) contains high levels of an adenylyl cyclase tagged at its amino-terminal end with a short peptide epitope, expressed under the control of the *adh1* promoter (Kawamukai *et al.*, 1991). To further examine proteins associated with adenylyl cyclase, we partially purified the adenylyl cyclase complex from membrane extracts of MK141 cells using a column containing protein A-sepharose beads linked to the monoclonal antibody 12CA5 directed against the peptide epitope, as described in MATERIALS AND METHODS (Field *et al.*, 1988). Adenylyl cyclase specific activity was 600-fold higher in preparations purified by this method than in unpurified membrane extracts. We performed a Western blot analysis on the partially purified adenylyl cyclase complex to determine the molecular weights of proteins recognized by the KF191 antisera (Figure 1). KF191 recognized a single protein band with an apparent molecular mass of 70 kDa, similar in size to that of *S. cerevisiae* CAP. These results strongly suggest the existence of an adenylyl cyclase binding protein in *S. pombe* that is related to *S. cerevisiae* CAP.

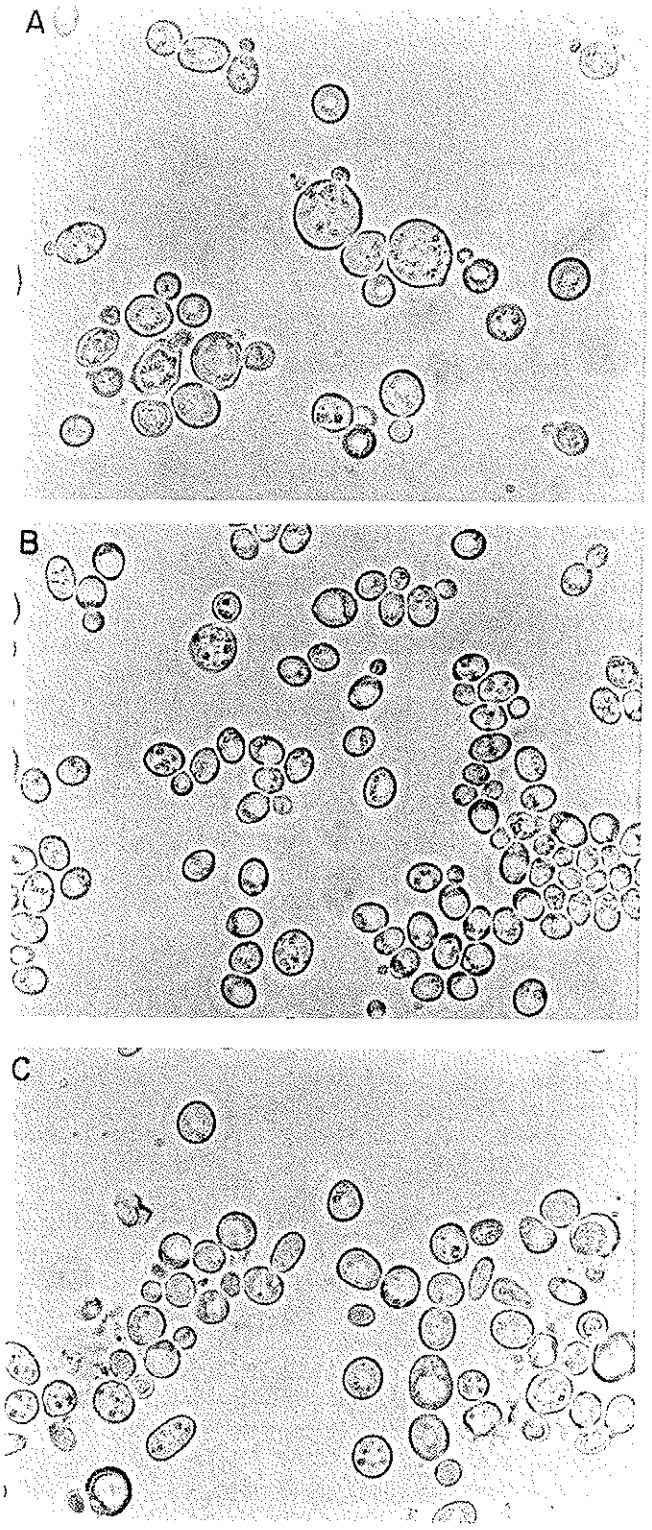
Because KF191 immunoprecipitated adenylyl cyclase activity inefficiently compared with 12CA5 antibody, we postulated that KF191 may react only weakly with the putative *S. pombe* CAP-related protein. To facilitate the characterization of the 70-kDa *S. pombe* protein, we raised antisera against preparations enriched for this protein. To increase the purity of the putative CAP-related protein before immunization of rabbits, we immunoprecipitated the *S. pombe* adenylyl cyclase complex with KF191 from preparations purified by the scheme described above. The polyclonal antisera AB321 was derived from a rabbit that had been serially injected with the immunoprecipitates (see MATERIALS AND METHODS). AB321 could immunoprecipitate about 30% of the adenylyl cyclase activity from membrane extracts from MK141 cells. Several proteins were detected by Western blot analysis of the partially purified *S. pombe* adenylyl cyclase complex using AB321 (Figure 2), but AB321 hardly recognized *S. cerevisiae* CAP in Western blots of extracts from the *E. coli* strain BL21(DE3)pLysS harboring the plasmid pT7.CAP that expresses this protein (Figure 2).

### *Cloning S. pombe cDNAs Encoding a CAP-Related Protein*

We used two different approaches to clone *S. pombe* cDNAs encoding a CAP-related protein. First, we screened a *S. pombe* cDNA library, constructed in the lambda ZAPII expression vector (Stratagene), with AB321 antisera (see MATERIALS AND METHODS). Ten positive clones were obtained after screening ~2 × $10^5$ plaques. Plasmids containing the cDNAs were recovered from lambda ZAPII clones using the in vivo excision procedure (see MATERIALS AND METHODS). Two distinct types of clones, represented by pCAP2-1 and pCAP7-3, were obtained and characterized. The *E.*

*coli* strain XL-Blue, containing either pCAP2-1 or pCAP7-3, expressed proteins with the expected mobility detectable by Western blot analysis using AB321 antisera (Figure 2). However, only the product of pCAP7-3 cross-reacted with KF191 antisera by Western blot analysis (Figure 1). Furthermore, only extracts from *E. coli* strain XL-Blue containing pCAP7-3 specifically blocked the immunoprecipitation of adenylyl cyclase from MK141 extracts by AB321 antisera. Thus, pCAP7-3 appeared to encode an *S. pombe* protein related to *S. cerevisiae* CAP.

We isolated *S. pombe* homologs of CAP using a second approach by identifying *S. pombe* cDNAs that could functionally substitute for CAP in the *S. cerevisiae* strain SKN32 (Table 1), in which the *CAP* gene had been deleted. SKN32 is unable to grow on rich media (YPD) due to the lack of CAP function (Field *et al.*, 1990). A *S. pombe* cDNA library, cloned in the *S. cerevisiae* expression vector pADANS (see MATERIALS AND METHODS), was transformed into SKN32. Several clones that suppressed the inability of SKN32 to grow on YPD medium were identified. One of the plasmids recovered, pSC2, had the same restriction endonuclease map as pCAP7-3, except that the 5′ end of the pSC2 cDNA insert was longer and the 3′ end was shorter (see Figure 3).




### *Sequence of S. pombe cap and Comparison with S. cerevisiae CAP*

The DNA sequences of the cDNAs contained on pCAP2-1, pCAP7-3, and pSC2 were determined (see MATERIALS AND METHODS). pCAP2-1 encodes a heat shock-related protein (data not shown) that is highly homologous (70%) with *S. cerevisiae* KAR2 and other BIP-type heat shock proteins involved in protein folding in the endoplasmic reticulum (Pelham, 1989). Its sequence has been submitted to Genebank. pCAP7-3 and pSC2 encode a protein that we have named cap that has significant homology to *S. cerevisiae* CAP (Figures 4 and 5). There is a single open reading frame encoding a 551 amino acid residue protein. This is slightly larger than the 526 amino acid residue *S. cerevisiae* CAP (Field *et al.*, 1990). An inframe stop codon, which is included in pSC2 but not in pCAP7-3, is just 36 bases 5′ upstream of the ATG start codon.

*S. cerevisiae* CAP and *S. pombe* cap share 34% identity (Figure 5). In *S. cerevisiae* CAP, a stretch of proline residues separates the two functional domains (Gerst *et al.*, 1991). *S. pombe* cap also contains a stretch of proline residues: nine in a row (residues 306–314). Homology between the two yeast proteins is stronger between the C-terminal regions (40% identity for residues 315–551

**Figure 6.** Suppression of morphological phenotypes due to deletion of CAP gene in *S. cerevisiae* by expression of *S. pombe* cap protein. Photomicrographs of cells were taken after cells were grown on SC synthetic media for 2 d. (A) *S. cerevisiae* strain SKN32, in which the *CAP* gene has been replaced with the *LEU2* gene, harboring YEp13 M4 (control plasmid); (B) SKN32 harboring pADH-CAP, a plasmid expressing high levels of CAP; (C) SKN32 harboring pYCL1, a plasmid expressing high levels of *S. pombe* cap.

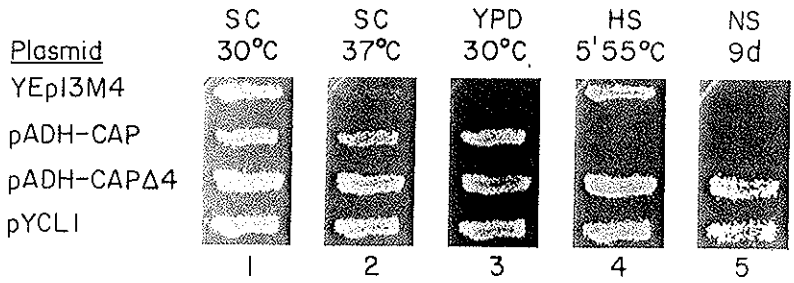



**Figure 7.** Expression of *S. pombe cap* in an *S. cerevisiae cap* disruption strain. An *S. cerevisiae* strain disrupted at the *CAP* locus (see MATERIALS AND METHODS), SKN37, was transformed with plasmids expressing full-length *S. cerevisiae* CAP (pADH-CAP), the C-terminal domain of *S. cerevisiae* CAP (pADH-CAPΔ4), full-length *S. pombe* cap (pYCL1), or a control vector (YEp13M4). Transformed yeast were grown on SC-leu plates for 2 d at 30°C to maintain selection of the plasmids. At this time, patches were replica plated onto SC-leu plates and were incubated at 30°C (lane 1) or, to test for temperature sensitive growth, at 37°C (lane 2). To assay growth on rich medium, transformed cells were replica plated onto YPD plates (lane 3). To assay for heat shock (HS) sensitivity, transformed cells were replica plated onto preheated (55°C) SC-leu plates and were incubated at 55°C for another 5 min (lane 4). To assay for sensitivity to nitrogen starvation (NS), transformed cells were plated onto YNB-N plates, lacking nitrogen, grown for 9 d at 30°C, and replica plated back onto SC-leu plates (lane 5). After replica plating, all transformed yeast strains were incubated for 2 d at 30°C, with the exception of lane 2, which was incubated at 37°C.

of s.p. cap) than between the N-terminal regions (24% identity for residues 1–305). The most conserved region (residues 306–367) is 66% identical. Ironically, this region of strong homology between the *S. cerevisiae* CAP and *S. pombe* cap proteins is functionally silent for the phenotypic assays we have performed in *S. cerevisiae* (Gerst *et al.*, 1991) and in *S. pombe* (see below).

### *Complementation of CAP Minus Phenotypes in S. cerevisiae by Expression of S. pombe cap*

Disruption of the N-terminal functional domain of CAP suppresses the heat-shock sensitive phenotype associated with the RAS2$^{val19}$ mutation in *S. cerevisiae*, suggesting that the N-terminal domain is required for the proper regulation of adenylyl cyclase by RAS (Field *et al.*, 1990; Gerst *et al.*, 1991). Loss of the C-terminal functional domain results in abnormal cellular morphology (round and enlarged cells), growth inhibition at 37°C, growth inhibition on rich medium, and failure to survive starvation. The functions of the two domains are separable and appear, at least superficially, to be unrelated (Gerst *et al.*, 1991; Vojtek *et al.*, 1991).

To test whether the two yeast CAP proteins are functionally conserved in either or both domains, we expressed the *S. pombe cap* cDNA in different *S. cerevisiae* strains. To perform these experiments, we constructed the plasmid pYCL1, which contains the *S. pombe cap* cDNA under the control of the *S. cerevisiae ADH1* promoter (see MATERIALS AND METHODS). pYCL1 suppressed abnormal growth phenotypes associated with the loss of C-terminal CAP function in *S. cerevisiae* strain SKN37. SKN37 cells containing the plasmid pYCL1 grew normally at 37°C and on rich medium, were resistant to nitrogen starvation (Figure 6), and appeared to have normal morphology (Figure 6). However, the heat shock-resistant phenotype, caused by the loss of the N-terminal portion of CAP, in the *S. cerevisiae RAS2*$^{val19}$ strain SKN37 (Table 1) was not suppressed by expression of *S. pombe* cap (Figure 7). SKN37 cells transformed with pYCL1 remained heat-shock resistant, whereas SKN37 cells transformed with pADH-CAP, which expresses full-length *S. cerevisiae* CAP protein, became heat-shock sensitive. Thus, expression of *S. pombe* cap appears insufficient to restore RAS responses in *S. cerevisiae*.

### *Phenotypes Caused by Deletion of S. pombe cap*

We sought to examine further the function of *S. pombe* cap and to determine whether it is also a bifunctional protein by deleting and expressing different regions of the *cap* gene in *S. pombe*. We made two types of deletions in the *S. pombe cap* gene; we replaced either nearly the entire coding sequence or only the C-terminal domain coding sequences with the *S. pombe ura4* selectable marker. To disrupt the entire *cap* coding sequence, we first cloned the genomic locus of *cap*. We obtained the plasmid pCGE2 (Figure 3), which contains the *cap* gene, from a *S. pombe* genomic library by colony hybridization with pCAP7-3 (see MATERIALS AND METHODS). We then engineered the plasmid pCAD11, in which the coding sequence (codons 69–551) between the *Sal* I and *Hin*dIII sites has been replaced with the *ura4* gene (Figure 3). This plasmid was digested by *Not* I, and the 3.8-kb *Not* I fragment was purified by gel electrophoresis. This fragment was used to transform the *S. pombe* diploid SP826 ($h^{+N}/h^{+N}$, see Table 1). Several strains were subjected to Southern blot analysis. One diploid strain (MK1818) was confirmed to have the *ura4* gene correctly integrated within the *cap* locus. A spontaneous mating type revertant, MK1818R, was obtained in which one

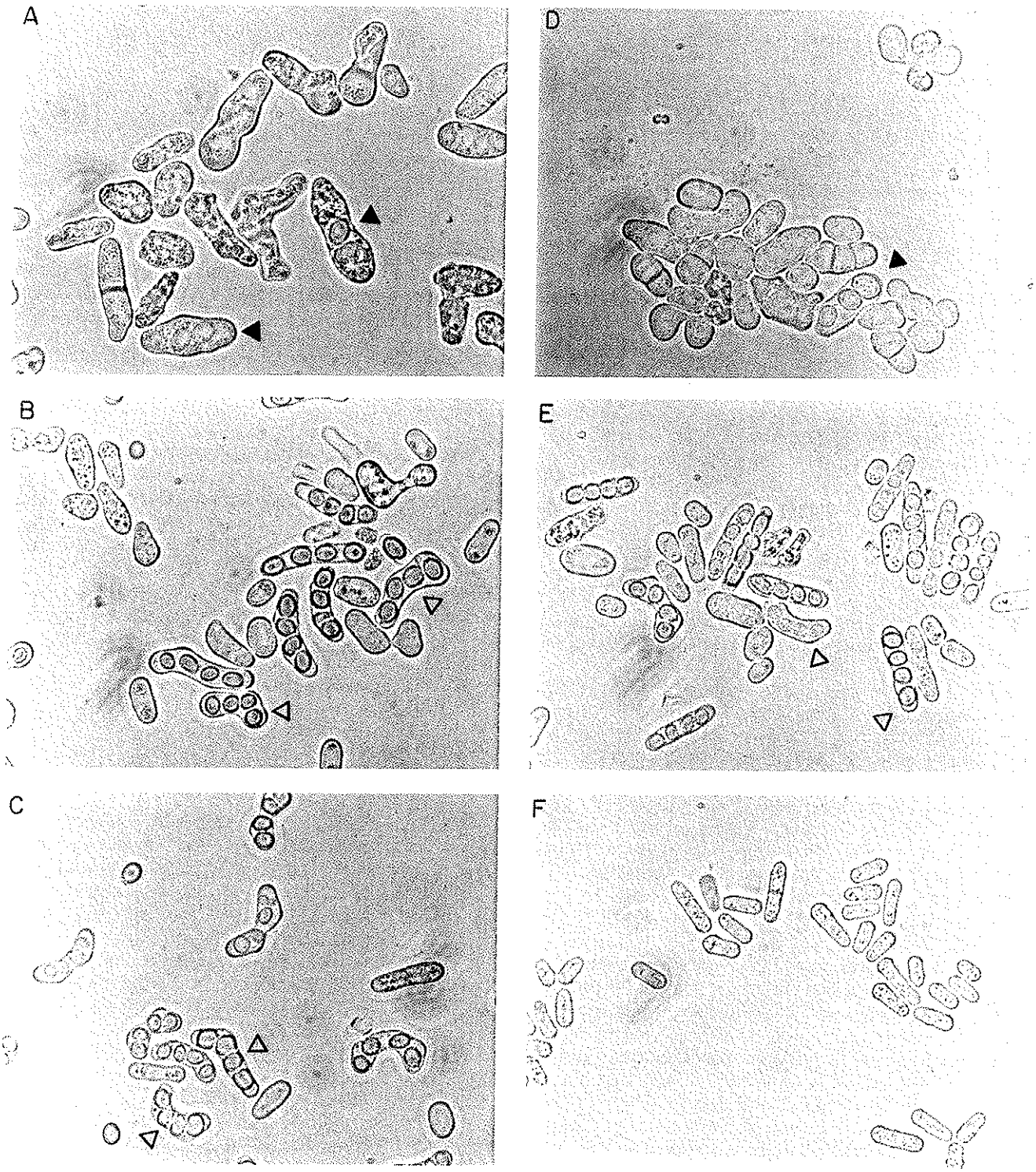



**Figure 8.** Cellular morphology of cap disruptant. Photomicrographs of homothallic cells grown on PMA synthetic medium on plates (A, B, and C) or in PMA liquid medium (D, E, and F). Cells were grown for 1 wk (A and D) or 2 d (B, C, E, and F). Homothallic wild-type cells form spores on the solid medium (starvation conditions) but not in the liquid culture. (A and D) *S. pombe* strain MK1818d, in which the *cap* gene has been replaced with *ura4* gene (see Table 1), transformed with the control plasmid pAL; (B and E) MK1818d cells transformed with pA70L, which expresses high levels of *S. cerevisiae* CAP; (C and F) MK1818d cells transformed with pCGE2, which expresses the *S. pombe cap* gene. Normal four-spore asci (arrow heads) are abundant in B, C, and E, and abnormal asci (filled arrow heads) are observed in A and D. The cells of F, where wild-type cap function has been restored, do not show evidence of conjugation or sporulation. For photomicroscopy, cells grown on solid medium were dispersed in liquid culture medium and photographed.

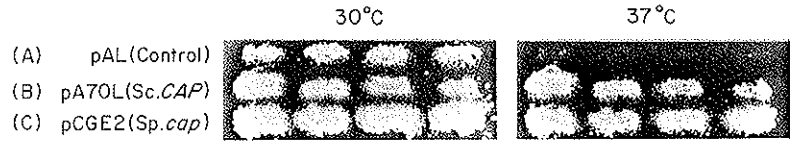



**Figure 9.** Suppression of *S. pombe cap* deficient strain by the *S. cerevisiae CAP* gene. Patches of cells were grown on PMA synthetic plates, replica plated on PMA, and grown at 30 and 37°C. (A) *S. pombe* strain MK1818d transformed with pAL (control plasmid); (B) MK1818d transformed with pA70L, which expresses high levels of the *S. cerevisiae CAP* gene; (C) MK1818d transformed with pCGE2, which expresses the *S. pombe cap* gene. For each plasmid, four independent transformants are shown.

tained in which one allele of the mating locus was presumed to be changed to $h^{90}$. Tetrad analysis of the MK1818R strain was performed. Of 10 tetrads analysed, 8 displayed a 2:2 segregation pattern for the $Ura^-$ phenotype. The $Ura^-$ ($cap^{wt}$) cells were otherwise wild-type, whereas the $Ura^+$ cells (cap::*ura4*) grew slowly. Thus, *cap* disruption does not result in lethality.

Several of the *cap* disruptants were examined further. Such cells showed an abnormal cellular morphology, especially under starvation conditions; the cells were larger and rounder than wild-type and some had branched protrusions (Figure 8). They formed abnormal asci (Figure 8) and were also temperature sensitive for growth at 37°C (Figure 9). Such cells grew poorly in synthetic medium (PMA) but well in rich medium (YEA).

To see the effect of disruption of only the C-terminal domain coding sequences of *cap*, we constructed plasmid pCAD3, in which the region between the two *Hin*dIII sites, encoding amino acid residues 513–551, was replaced with the *ura4* gene (Figure 3). This plasmid was digested with *Not* I and then used to transform the homothallic *S. pombe* haploid strain SP870 (Table 1). Proper integration of the plasmid sequences within the *cap* genomic locus was confirmed by Southern blot analysis. Cells lacking the C-terminal region of cap showed a very similar phenotype to those with *cap* entirely disrupted. Such cells had an abnormal cellular morphology, abnormal asci, grew more slowly in synthetic medium, and were temperature sensitive for growth at 37°C.

A difference between the entire *cap* disruptant and the C-terminal disruptant was found in sexual functions. The strain with the entire *cap* deletion agglutinated, conjugated, and sporulated when cultured in liquid PMA medium (see Figure 8), conditions under which wild-type homothallic strains and the C-terminal disruptant do not (see Figure 8 and data not shown). This phenotype is similar to that of *cyr1* deficient strains, suggesting a functional interaction between cap and adenylyl cyclase. Further evidence for functional interaction between cap and cyr1 is presented in the next section.

We next expressed *S. cerevisiae* CAP in *S. pombe cap* disruption strains to study further the functional conservation of the proteins. The phenotype of abnormal morphology and temperature sensitivity of *cap* deletion strains was suppressed by expression of *S. cerevisiae* CAP (Figures 8 and 9). However, the phenotype of agglutination, conjugation, and sporulation exhibited by the fully deleted *cap* strain in liquid medium was not suppressed by the expression of *S. cerevisiae* CAP (Figure 8). Thus, there has been functional conservation of the C-terminal region of CAP, but the N-terminal domains do not appear to function properly in the heterologous species.

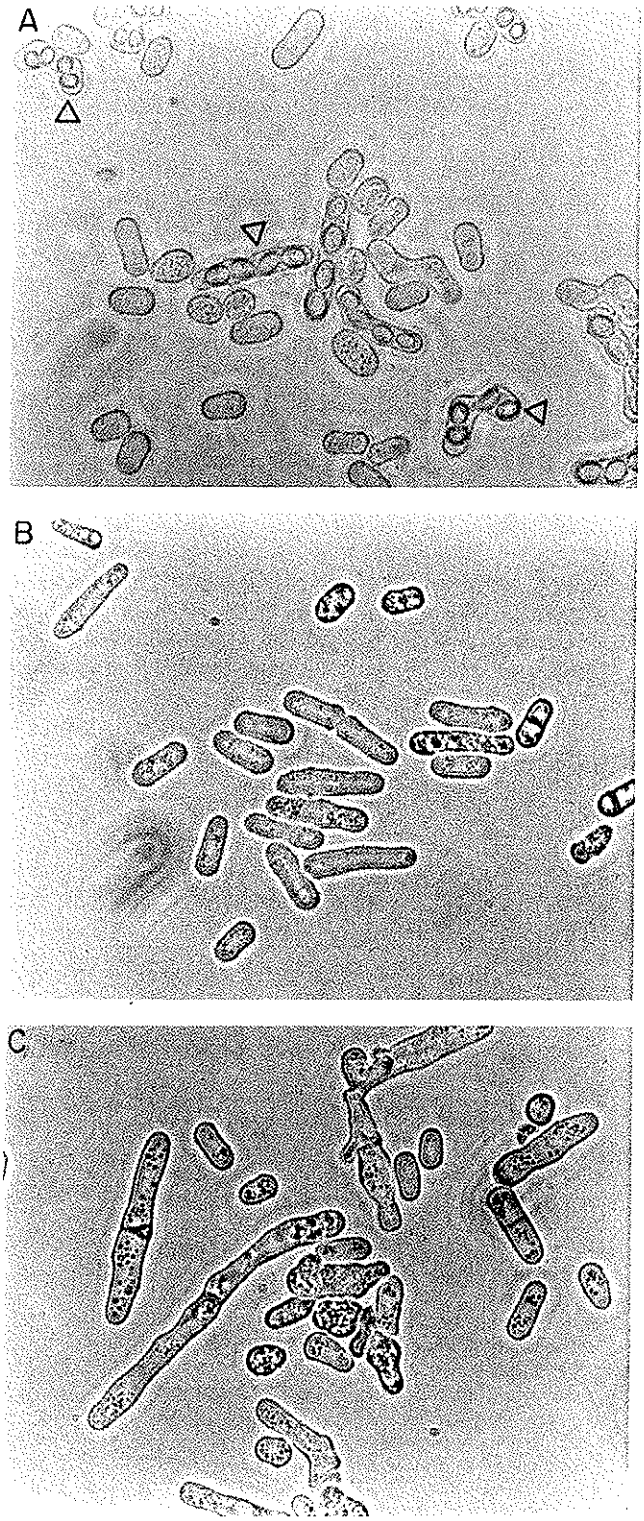
To test the significance of the highly conserved region in the center of cap (amino acids 306–367), the plasmid pACL7, which expresses *cap* having a deletion in codons 296–367, was constructed. No significant difference between pACL7 and pACL1, which expresses full-length cap, was observed when these plasmids were transformed into strains fully disrupted at the *cap* locus. Both plasmids restored morphology, temperature sensitivity, and sexual function (data not shown). This observation is similar to our failure to find a function for the homologous central domain of CAP in *S. cerevisiae* (Gerst *et al.*, 1991).

### *Overproduction of Adenylyl Cyclase and cap in S. pombe*

We previously observed that overproduction of adenylyl cyclase in the *S. pombe* strain SP870 results in partial sterility and an elongated cellular shape (Kawamukai *et al.*, 1991). The effects resulting from overproduction of both cap and adenylyl cyclase were examined in this study. We constructed the plasmid pACL1, which contains the *S. pombe cap* cDNA under the control of the *S. pombe adh1* promoter (Figure 3). When both adenylyl cyclase and cap were overexpressed, the morphology of cells dramatically changed into highly elongated and disordered shapes (Figure 10) and such cells became very sterile. These drastic changes were not seen in cells transformed with the pACL1 clone alone.

We measured adenylyl cyclase activities in membrane extracts in *S. pombe* cells overexpressing either adenylyl cyclase alone or both adenylyl cyclase and cap together (Table 2). Although overexpression of adenylyl cyclase alone resulted in an 8- to 20-fold higher level of enzymatic activity, overproduction of both *S. pombe* cap and adenylyl cyclase yielded 32- to 68-fold higher levels than in wild-type cell extracts. These results suggest that *S. pombe* cap, like *S. cerevisiae* CAP, may be involved

in regulating adenylyl cyclase activity. Expression of CAP alone had no effect on measurable adenylyl cyclase activity.




### *Physical Interaction of Heterologous Yeast CAP and Adenylyl Cyclase Proteins*

We tested the specificity of *S. pombe* cap and *S. cerevisiae* CAP interaction with homologous and heterologous yeast adenylyl cyclases expressed in the *S. pombe* strain MK7 and the *S. cerevisiae* strain T158-5AT (Table 3). The endogenous chromosomal adenylyl cyclase genes have been deleted in both of these strains (Table 1). The antisera KF211, which recognizes only *S. cerevisiae* CAP, and the antisera AB321, which recognizes only *S. pombe* cap, were used for the experiments. About 7.6% (10-fold above background) of the total adenylyl cyclase activity in membrane extracts from *S. pombe* MK7 cells harboring pALY12, which expresses the *S. cerevisiae* adenylyl cyclase, were immunoprecipitated by AB321. In contrast, ~30% of the total activity in extracts from MK7 cells harboring pALY1, which expresses the *S. pombe* adenylyl cyclase, were immunoprecipitated by AB321. Likewise, 13.8% (3-fold above background) of the activity in extracts from *S. cerevisiae* T158-5AT cells harboring pYALY2, which expresses the *S. pombe* adenylyl cyclase, were immunoprecipitated by KF211. In contrast, 58% of the activity in extracts from T158-5AT harboring pYCYR, which expresses the *S. cerevisiae* adenylyl cyclase, were immunoprecipitated by KF211. Thus, *S. cerevisiae* CAP and *S. pombe* cap each may be able to interact with adenylyl cyclase from the heterologous yeast, but apparently not as strongly as with adenylyl cyclase from the homologous source.

## DISCUSSION

CAP, the adenylyl cyclase-associated protein of *S. cerevisiae*, is a bifunctional protein. Its N-terminal domain is required for full cellular responsiveness to the activated RAS2$^{val19}$ allele (Field *et al.*, 1990; Gerst *et al.*, 1991). Ordinarily, RAS2$^{val19}$ cells are heat-shock sensitive, fail to survive nitrogen starvation, and do not accumulate glycogen. RAS2$^{val19}$ cells that, in addition, lack just the N-terminal domain of CAP behave again like wild-type cells: they are resistant to heat shock and nitrogen starvation and accumulate glycogen. These effects are probably mediated through the interaction of the N-terminal domain of CAP with adenylyl cyclase. *S. cerevisiae* cells lacking the C-terminal domain of CAP have abnormal responses to nutrient deprivation and excess, are abnormally round and heterogeneously large, bud randomly, and are temperature sensitive (Field

**Figure 10.** Morphological change induced by overexpression of cap and adenylyl cyclase. Photomicrographs of cells grown on PMA agar plates for 3 d. (A) *S. pombe* strain SP870 harboring pIRT5 (control plasmid containing *ura4* gene) and pAL (control plasmid containing *LEU2* gene); (B) SP870 harboring pAL and pALY1, which expresses high levels of *S. pombe* adenylyl cyclase; (C) SP870 harboring pALY1 and pACL1, which expresses high levels of both *S. pombe* cap and *S. pombe* adenylyl cyclase. Abundant four-spore asci (arrow heads) are seen in A.

**Table 2.** Adenylyl cyclase activity in *S. pombe* strain SP870 harboring various plasmids

| Plasmids | Exp. I | Exp. II |
|---|---|---|
| pAL and pIRT5 | 5 (±2) | 4 (±0.3) |
| pAL and pALY1 | 101 (±10) | 32 (±1) |
| pACL1 and pALY1 | 342 (±84) | 129 (±20) |

The adenylyl cyclase activities in membrane extracts from SP870 cells harboring the designated plasmids were measured as described in MATERIALS AND METHODS. Adenylyl cyclase activities are expressed as the average pmol of cAMP produced per minute per milligram of total membrane protein from two duplicate assays. The variation in these assays are in parentheses. pAL and pIRT5 are control plasmids. SP870 harboring pALY1 overexpresses adenylyl cyclase. SP870 harboring pACL1 and pALY1 overexpresses both cap and adenylyl cyclase. Lysates from independent transformants were prepared for the two experiments.

*et al.*, 1990; Vojtek *et al.*, 1991). These defects are correctable by the overexpression of profilin, a low molecular weight protein that binds actin and phosphoinositides (Vojtek *et al.*, 1991). The C-terminal function of CAP does not appear to be related to the cyclic AMP signaling pathway. Moreover, the N-terminal and C-terminal domains of CAP are capable of independent function. The two functional domains of CAP are connected by a middle domain that has as yet undetected function.

The fission yeast *S. pombe* is "wired" differently than the budding yeast *S. cerevisiae*. Both organisms have ras and adenylyl cyclase proteins (Young *et al.*, 1989; Yamawaki-Kataoka *et al.*, 1989), but, in *S. pombe*, ras does not regulate adenylyl cyclase (Fukui *et al.*, 1986; Kawamukai *et al.*, 1991). Indeed, ras and adenylyl cyclase (cyr1) have opposing influences on sexual functions: $ras^{null}$ cells are sterile whereas $cyr1^{null}$ cells conjugate and sporulate under conditions where wild-type cells do not (Kawamukai *et al.*, 1991). Cells that overexpress adenylyl cyclase are sterile whereas cells with the activated $ras1^{val17}$ allele are hyperresponsive to mating factors. It was therefore of interest to us to determine if *S. pombe* contained a homolog of the *S. cerevisiae* CAP protein and into which systems it was wired. By the criteria of immunological reactivity, biochemical properties, genetic complementation, and primary sequence, *S. pombe* does contain a homologous protein. All three domains of the *S. cerevisiae* and *S. pombe* cap proteins have primary sequence homology: the homology is highest in the middle domain (66% identity), followed by the C-terminal (40%) and N-terminal domains (24%).

Phenotypic analysis suggests that the *S. pombe* cap protein is also bifunctional. *S. pombe* cells that express C-terminal deletion mutants of *cap* are temperature sensitive, grow slowly in synthetic medium, and have an abnormal morphology. On the other hand, cells that have an extensive deletion of cap display all these phenotypes and, additionally, conjugate and sporulate under conditions in which wild-type cells do not. The most stark distinction between N-terminal and C-terminal function can be seen in *S. pombe* cells lacking endoge-

**Table 3.** Immunoprecipitable adenylyl cyclase activities in *S. pombe* and *S. cerevisiae* strains harboring various plasmids

| Antisera: strain/plasmid | Total Activity | Immunoprecipitable activity | | |
|---|---|---|---|---|
| | | None | KF211 | AB321 |
| *S. pombe* (*cyr1::LEU2*) | | | | |
| MK7/pAL(control) | <1 | ND | ND | ND |
| MK7/pALY1(*adh*-Sp.*cyr1*) | 633 (±21) | 5 (±1) | 6 (±2) | 190 (±37) |
| MK7/pALY12(*adh*-Sc.*CYR1*) | 302 (±7) | 2 (±.04) | 2 (±.02) | 23 (±1.4) |
| *S. cerevisiae* (*cyr1::URA3*) | | | | |
| T158-5AT/YEp13M4(control) | <1 | ND | ND | ND |
| T158-5AT/pYCYR(*ADH*-Sc.*CYR1*) | 2353 (±16) | 131 (±31) | 1369 (±172) | 106 (±26) |
| T158-5AT/pYALY2(*ADH*-Sp.*cyr1*) | 1290 (±68) | 55 (±10) | 178 (±5) | 63 (±8) |

Immunoprecipitations of soluble membrane extracts from the designated strains harboring the designated plasmids were performed using the indicated antisera. Adenylyl cyclase activities were measured in duplicate and presented as described in Table 2. ND, not determined. Total activity, the total adenylyl cyclase activities in the membrane extracts before immunoprecipitation. These data are representative of two independent experiments. KF211 is a rabbit polyclonal antisera raised against *E. coli* extract expressing *S. cerevisiae* CAP. KF211 was obtained by the same way, and from the same *E. coli* extract, as KF191 was. AB321 is a polyclonal antisera raised against the *S. pombe* cap-adenylyl cyclase complex, as described in MATERIALS AND METHODS. MK7 is a *S. pombe* strain in which the endogenous adenylyl cyclase gene has been replaced (Table 1). T158-5AT is a *S. cerevisiae* strain in which the endogenous adenylyl cyclase gene has been replaced and harbors the plasmid encoding TPK1 as a suppressor (Table 1). All plasmids used are described in Materials and Methods. pAL and YEp13M4 are control plasmids. pALY12 and pYCYR encode *S. cerevisiae* adenylyl cyclase. pALY1 and pYALY2 encode *S. pombe* adenylyl cyclase.

nous cap but expressing *S. cerevisiae* CAP. These cells display none of the defects seen when just the C-terminal domain of cap is disrupted but still display abnormal sexual responses.

The morphological and growth defects of *S. pombe* cells lacking the C-terminal domain of cap resemble in kind, if not in exact detail, the defects seen in *S. cerevisiae* cells that lack C-terminal domain of CAP. Both types of cells are temperature sensitive. Both are greatly inhibited for growth in certain media relative to wild-type cells. Both are morphologically abnormal, although in different ways. *S. pombe* cells lacking C-terminal cap function are large but often have branched protrusions and abnormal asci. All these defects are corrected by expressing the *S. cerevisiae* CAP. Conversely, the growth and morphological defects in *S. cerevisiae* cells lacking the C-terminal function of CAP are corrected by expression of the *S. pombe* cap. Hence, we conclude that the C-terminal function of *S. cerevisiae* and *S. pombe* cap have been highly conserved.

The N-terminal function of cap has also been conserved, although perhaps not as strongly. In *S. cerevisiae*, biochemical and genetic evidence suggests that the N-terminus of CAP is required for proper adenylyl cyclase function (Field *et al.*, 1990; Gerst *et al.*, 1991; Field, unpublished data). The same appears to be true in *S. pombe*. First, *S. pombe* cap binds to *S. pombe* adenylyl cyclase, and both *S. pombe* cap and *S. cerevisiae* CAP appear to bind to some extent the adenylyl cyclase of the heterologous species. Second, *S. pombe* cells, which lack full-length cap, conjugate and sporulate prematurely. Premature sexual activity is also seen in cells that lack adenylyl cyclase. Third, coexpression of adenylyl cyclase and cap in *S. pombe* results in higher levels of measurable adenylyl cyclase activity in cellular extracts and to a highly aggravated phenotype of elongated and sterile cells. A similar phenotype results in *S. pombe* cells from lack of the regulatory subunit of the cyclic AMP-dependent protein kinase (McLeod, personal communication).

The *S. pombe* adenylyl cyclase/cap complex and the homologous complex in *S. cerevisiae* have diverged considerably in their regulation. In *S. cerevisiae*, the complex is regulated by RAS1 and RAS2 (Toda *et al.*, 1985), proteins highly homologous to the mammalian oncogenic RAS proteins. This does not currently appear to be the case in *S. pombe*. Only one close homolog of oncogenic mammalian RAS has been described in *S. pombe*, ras1, and it does not regulate adenylyl cyclase (Fukui *et al.*, 1986). Moreover, expression of *S. pombe* cap does not complement the loss of the N-terminal domain of *S. cerevisiae* CAP nor does expression of *S. cerevisiae* CAP affect the phenotypes associated with the loss of the function of the N-terminal domain of *S. pombe* cap. This divergence in function is perhaps reflected in the divergence of the primary sequence between the N-terminal domains of the *S. pombe* and *S. cerevisiae* proteins. However, as this study and our previous studies have shown, there are considerable parallels in the structure and regulation of the adenylyl cyclase complexes from the two organisms. Therefore, it should not surprise us if an as yet undiscovered RAS-related protein is shown to modulate adenylyl cyclase in *S. pombe*. Recently, a number of loci in *S. pombe* have been identified that potentially encode regulators of adenylyl cyclase (Hoffman and Winston, 1991).

The function of the middle domain of yeast CAPs remains a puzzle. In both *S. cerevisiae* and *S. pombe*, this region can be deleted without apparent phenotypic consequences. In terms of primary sequence, however, the middle domain is the region most highly conserved between the two proteins. In this region, both proteins contain a stretch of consecutive prolines, six in *S. cerevisiae* and nine in *S. pombe*, and there is extensive homology neighboring these prolines. In our view, it is not an accident that such a highly conserved structure joins two domains with conserved function in two highly divergent yeasts. The conservation of this structural and functional arrangement suggests that the functions of the N-terminal and C-terminal domains are in someway coordinated.

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