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Genetic and biochemical analysis of the adenylyl cyclase of *Schizosaccharomyces pombe*

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The adenylyl cyclase gene, *cyr1*, of *Schizosaccharomyces pombe* has been cloned. We have begun an analysis of the function and regulation of adenylyl cyclase by disrupting this gene and by overexpressing all or parts of this gene in various strains. *cyr1*$^-$ strains are viable and contain no measurable cyclic AMP. They conjugate and sporulate under conditions that normally inhibit wild-type strains. Strains containing the *cyr1* coding sequences transcribed from the strong *adh1* promoter contain greatly elevated adenylyl cyclase activity, as measured in vitro, but only modestly elevated cAMP levels. Such strains conjugate and sporulate less frequently than wild-type cells upon nutrient limitation. Strains which carry the wild-type *cyr1* gene but that also express high levels of the amino terminal domain of adenylyl cyclase behave much like *cyr1*$^-$ strains, suggesting that the amino terminal domain can bind a positive regulator. A protein that copurifies with the adenylyl cyclase of *S. pombe* cross-reacts to antiserum raised against the *S. cerevisiae* adenylyl cyclase-associated regulatory protein, CAP.

Introduction

Cyclic AMP is an important second messenger in signal transduction pathways in a variety of eucaryotic organisms, including mammals and yeast. Adenylyl cyclase, the enzyme which generates cAMP, has been studied extensively in the budding yeast *Saccharomyces cerevisiae.* Changes in the regulation or activity of *S. cerevisiae* adenylyl cyclase adversely affect cellular responses to nutrient conditions (Kataoka *et al.,* 1984; Cameron *et al.,* 1988). Mutational activation of RAS proteins, which regulate adenylyl cyclase, prevents *S. cerevisiae* from arresting in the G1 phase of the cell cycle and becoming heat-shock resistant in response to nutrient starvation. Attenuation of RAS or adenylyl cyclase results in a strong inhibition of cell growth (Matsumoto *et al.,* 1982; Kataoka *et al.,* 1984; Toda *et al.,* 1987a). Adenylyl cyclase is positively regulated by RAS proteins in *S. cerevisiae* (Toda *et al.,* 1985), but evidence suggests that RAS proteins do not modulate cAMP levels in vertebrates (Beckner *et al.,* 1985; Birchmeier *et al.,* 1985), or in the fission yeast *Schizosaccharomyces pombe* (Fukui *et al.,* 1986; Nadin-Davis *et al.,* 1986). To understand better the evolution of RAS and cAMP signalling pathways, we have begun to explore the latter pathway in the yeast *S. pombe.* Previous observations suggest that cAMP may be involved in regulating conjugation and meiosis in this yeast: agents that stimulate cAMP-dependent protein kinase activity inhibit meiosis (Calleja *et al.,* 1980; Beach *et al.,* 1985); a mutation in the gene, *cgs1,* which encodes the regulatory subunit of cAMP-dependent protein kinase, inhibits both conjugation and meiosis (M. McLeod, personal communication, 1990); and transcription of genes important for control of conjugation and meiosis, including *mei2,* is inhibited by exogenous cAMP (Watanabe *et al.,* 1988). To explore this issue further and to begin studying the regulation of the cAMP pathway, we cloned the gene, *cyr1,* encoding the *S. pombe* adenylyl cyclase (Young *et al.,* 1989). Here we report the effects of disrupting or overexpressing the adenylyl cyclase gene on cell growth, mating, and meiosis in *S. pombe* and report some observations on the biochemical properties of the *S. pombe* adenylyl cyclase complex.

Results

Disruption of the adenylyl cyclase gene in S. pombe

To investigate the phenotypes associated with loss of adenylyl cyclase activity we disrupted the adenylyl cyclase gene, *cyr1,* in both diploid and haploid *S. pombe* strains, as described in the Methods section. In brief, two plasmids,

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pPCU1 and pPCL1, were constructed that contain different disruptions of *cyr1* (Figure 1). In pPCU1, a region encoding the catalytic domain of adenylyl cyclase was replaced with the *S. pombe ura4* gene. In pPCL1, a larger region was replaced with the *S. cerevisiae LEU2* gene. One copy of the endogenous adenylyl cyclase gene of the diploid strain SP826 was replaced with the disrupted *cyr1* gene of pPCU1 (see Methods and Table 1 for strain descriptions). Proper integration of the disrupted *cyr1* gene in diploid transformants was confirmed by southern blot analysis (data not shown).

Tetrad analysis was performed on several independently derived diploid strains containing the *cyr1* gene disruption. In many cases asci contained either three or four viable spores, and two of the viable spores were Ura^+, indicating that they contained the disrupted *cyr1* allele. Adenylyl cyclase activity and cAMP were undetectable in the Ura^+ haploid strain DY114 that was derived from these experiments (Tables 2 and 3), confirming that the *cyr1* gene disruption resulted in complete loss of adenylyl cyclase activity and proving that such loss is not lethal in *S. pombe.* The Ura^+ haploid strains grew with a barely longer doubling time (1.9 h) than their sibling Ura^- strains (1.7 h). Thus, loss of adenylyl cyclase in *S. pombe* does not produce the dramatic inhibition of growth seen upon the loss of adenylyl cyclase in *S. cerevisiae.*

The phenotypes of the haploid strains lacking adenylyl cyclase were investigated further. The strain MK7 was derived from the haploid *S. pombe* strain SP870 by replacing the endogenous adenylyl cyclase gene with the disrupted

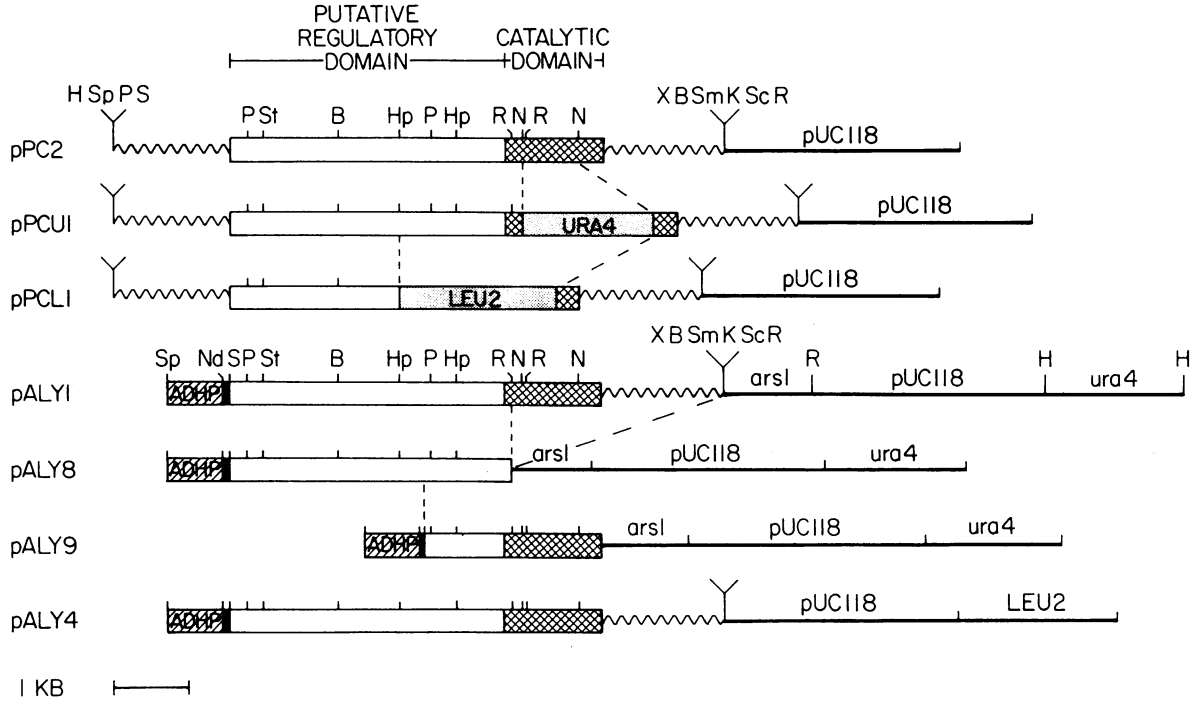

Figure 1. **Plasmid maps.** pPC2 contains the *S. pombe* adenylyl cyclase gene in the pUC118 vector and has been previously described (Young *et al.,* 1989). The positions of sites for *Stu* I (St), *Pst* I (P), *Hpa* I (Hp), *Bam*HI (B), *Eco*RI (R), *Nco* I (N), *Hin*dIII (H), *Sph* I (Sp), *Sal* I (S), *Xba* I (X), *Sma* I (Sm), *Kpn* I (K), and *Sac* I (Sc) are indicated. The sequence encoding the carboxyl-terminal catalytic region of the protein is represented by the cross-hatched region, whereas the remainder of the coding sequence is represented by the open box. 5′ and 3′ untranslated sequences of the *cyr1* gene are represented by squiggly lines. Thin lines represent the indicated sequences. pPCU1 contains a disruption of the adenylyl cyclase gene. It was derived from pPC2 by replacing a region encoding a portion of the catalytic domain with the *S. pombe ura4* gene, which is indicated by the shaded box. Similarly, pPCL1 was derived from pPC2 by replacing the carboxyl-terminal domain with the *S. cerevisiae LEU2* gene, also indicated with a shaded box. The plasmids pALY1, pALY8, and pALY9 encode fusion proteins consisting of the 20 amino acid residue epitope MYPYDVPDYASLGGPMSTLD (Field *et al.,* 1988), shown by the solid black boxes, fused to either the full length adenylyl cyclase, the amino terminal domain, or the carboxyl-terminal catalytic domain, respectively. The sequences encoding these fusion proteins are linked to the *adh1* promoter (ADHP), represented by the slashed boxes. pALY4 was derived from pALY1 and encodes an identical epitope-adenylyl cyclase fusion protein but contains a different auxotrophic marker. The construction of these plasmids is described in detail in Methods.

Table 1. Genotypes of *S. pombe* strains

SP826	*h⁺ⁿ/h⁺ⁿ leu-1-32/leu1-32 ade6-210/ade6-210 ura4-d18/ura4-d18*
SP870	*h90 leu-1-32 ade6-210 ura4-d18*
MK7	*h90 leu1-32 ade6-210 ura4-d18 cyr1::LEU2*
DY112	*h90 leu1-32 ade6-210 ura4-d18*
DY114	*h90 leu1-32 ade6-210 ura4-d18 cyr1::ura4*
MK141	*h90 leu1-32 ade6-210 ura4-d18 cyr1::pALY4 (LEU2)*
MK251	*h90 leu1-32 ade6-210 ura4-d18 cyr1::pALY5 (ura4)*
SPRU	*h90 leu1-32 ade6-210 ura4-d18 ras1::ura4*

SP826 and SP870 were obtained from Dr. David Beach.

MK7 and MK141 were derived from SP870 as described in Methods.

DY112 and DY114 were derived from SP826 as described in Methods.

SPRU was constructed in our lab by H.-P. Xu, by disruption of the *ras1* gene in SP870.

cyr1 gene from pPCL1. cAMP levels in MK7 were also undetectable (Table 3). Unlike normal wild-type strains, which mate and sporulate only upon starvation in minimal defined medium, both DY114 and MK7 conjugate and sporulate readily in rich (YEA) medium (Figure 2). Premature sexual activity in these strains is inhibited by the addition of exogenous cAMP. Thus, wild-type levels of adenylyl cyclase appear to be required to inhibit the sexual commitment of *S. pombe* during growth in rich medium.

Overexpression of adenylyl cyclase in S. pombe

We investigated the effect of expressing high levels of adenylyl cyclase in *S. pombe.* We built three plasmids that directed the expression of various regions of adenylyl cyclase from the *S. pombe* alcohol dehydrogenase I (*adh1*) promoter. The plasmid pALY1 encodes the full-length 1692 amino acid residue adenylyl cyclase protein; pALY8 encodes only the amino-terminal 1281 amino acid residues, and pALY9 encodes only the carboxyl-terminal 792 amino acid residues, which includes the catalytic domain (Figure 1). All of these plasmids also contain the selectable *S. pombe ura4* gene and *ars1* sequence. pALY1 and pALY9 were used to transform the haploid strain SP870 and adenylyl cyclase activity was measured in crude membrane preparations from Ura⁺ transformants. SP870 harboring pALY1 or pALY9 have 60- to 240-fold higher levels of adenylyl cyclase activity, respectively, than do normal cells (Table 2).

In *S. cerevisiae,* RAS proteins greatly stimulate adenylyl cyclase activity as measured in vitro. We thus tested if the adenylyl cyclase activity in *S. pombe* strains was stimulated by purified *S. cerevisiae* RAS2 proteins. It was not (Table 2). This result is consistent with a previous report (Yamawaki-Kataoka *et al.,* 1989) and strengthens the notion that *S. cerevisiae* and *S. pombe* adenylyl cyclase display divergent regulation. However, we cannot exclude the possibility that overexpressed *S. pombe* adenylyl cyclase lacks another component required for RAS responsiveness.

Despite the higher adenylyl cyclase activity measured in SP870 harboring pALY1 or pALY9, cAMP levels were not significantly more than two-fold higher relative to normal cells in logarithmically growing or stationary cultures (Table 3). This result suggests either that excess adenylyl cyclase does not form a produc-

Table 2. Adenylyl cyclase activity measured in various strains

		Activity (pmol · min^{-1} · mg^{-1})†		
Strain*	Plasmid*	Mn^{2+}	Mg^{2+}	Mg^{2+} + RAS2
SP870	pIRT5	2.2	2.2	2.4
SP870	pALY1	141.1	3.2	3.5
SP870	pALY9	524.4	3.4	3.3
DY114		Undetectable		
TK-B111		112.9	7.5	67.8

This table shows the levels of adenylyl cyclase activities measured in membranes prepared from the indicated strains harboring the indicated plasmids. Adenylyl cyclase activity was measured as described below. Adenylyl cylcase activities are expressed as the average pmoles of cAMP produced per minute per microgram of total membrane protein from two independent experiments.

* The genotypes of *S. pombe* strains used are given in Table 1 and plasmids are described in Methods. SP870 is a wild-type haploid strain. The plasmids pALY1 and pALY9 direct the expression of the full length or the catalytic region of *S. pombe* adenylyl cyclase, respectively, from the *adh1* promoter. DY114 is isogenic to SP870, but has a disruption of the adenylyl cyclase gene. TK-B111 is a *S. cerevisiae* strain with the genotype *MATα ras1::HIS3 ras2::URA3 trp1 leu2 ade8 can1* that harbors the plasmid pADH-CYR1 and expresses the *S. cerevisiae* adenylyl cyclase protein (Field *et al.,* 1987).

† Adenylyl cyclase activities were determined, as previously described (Young *et al.,* 1989), by measuring the production of [^{32}P]cAMP in a 100 μl reaction mixture containing 50 μg membrane protein in 20 mM MES, 0.1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM β-mercaptoethanol, 1 mM ATP + 10 μCi [α-^{32}P]ATP, 0.25 mM cAMP in the presence of either 2.5 mM $MnCl_2$, 2.5 mM $MgCl_2$, or 2.5 mM $MgCl_2$ and *S. cerevisiae* RAS2 protein (8.5 μg/100 μl). RAS2 protein was purified from *E. coli* and bound to GTP as previously described (Broek *et al.,* 1985). Measurements of adenylyl cyclase activity in strain DY114 were equivalent to background levels, and at least 10-fold lower than wild type.

Table 3. cAMP levels measured in various strains

Strain*	Plasmid*	cAMP (pmol/mg)† PMA log	PMA stationary	YEA stationary
SP870	pIRT5	1.15	0.58	1.12
SP870	pALY1	1.64	1.27	
SP870	pALY9	1.82	1.84	
DY114				Undetectable
MK7				Undetectable

This table shows the levels of cAMP measured from the indicated strains harboring the indicated plasmids. cAMP levels were measured as described below. The given values are the picomoles of cAMP per milligram of total cellular protein, averaged from two independent experiments.

* The strains and plasmids used are the same as in Table 2, except for MK7 which was derived from SP870 by disrupting the adenylyl cyclase gene.

† Strains were grown either in minimal defined medium (PMA + leucine) or in rich medium (YEA) to a density of approximately $O.D._{600}$ = 1.0 (log) or $O.D._{600}$ = 2.0 (stationary). Cells were harvested and nucleotides were extracted with 1 M formic acid saturated with 1-butanol, as previously described (Olempska-Beere and Freese, 1984). cAMP levels were measured by radioimmunoassay with antibody raised against acetylated cAMP, as previously described (Harper and Brooker, 1975). cAMP levels in DY114 and MK7 were at least .01 lower than wild type.

tive complex in *S. pombe,* due for example to limiting quantities of a positive regulatory component, or that cAMP levels are regulated by a feedback mechanism. Powerful feedback mechanisms for regulating cAMP levels are found in the yeast *S. cerevisiae* (Nikawa, *et al.,* 1987).

Cells expressing high levels of adenylyl cyclase appear to grow normally in rich media, but they do not respond like wild-type cells upon starvation. After reaching stationary phase, the wild-type strain SP870 sporulates, whereas strains harboring either pALY1 or pALY9 are relatively sterile and have an elongated morphology (Figures 3 and 4). These phenotypes are similar to that of *S. pombe* strains expressing the *S. cerevisiae* adenylyl cyclase (Beach *et al.,* 1985; Nadin-Davis *et al.,* 1986) or cAMP, dependent protein kinase catalytic subunit (D. Beach, personal communication). Similar phenotypes are observed in a *S. pombe* strain containing a mutation, *cgs1,* in the regulatory subunit of cAMP-dependent protein kinase (M. McLeod, personal communication). The phenotypes associated with high levels of adenylyl cyclase activity can be suppressed by expression of either the *S. cerevisiae* high-affinity cAMP phosphodiesterase or the regulatory subunit of cAMP-dependent protein kinase (Figure 3B). Thus these phenotypes are almost surely the consequence of the hyperactivity of the cyclic AMP-signalling pathway during starvation.

Interference with adenylyl cyclase function

In *S. cerevisiae,* expression of the regulatory amino terminal domain of adenylyl cyclase dominantly interferes with normal adenylyl cyclase activity, presumably by binding to regulatory proteins (Field *et al.,* 1990b). To test if expression of the amino terminal domain of adenylyl cyclase could similarly interfere with adenylyl cyclase function in *S. pombe,* we examined the phenotype of SP870 cells harboring the plasmid pALY8 that expresses only the N-terminal domain of the adenylyl cyclase protein. Such cells behave very similarly to haploid strains containing the *cyr1* disruption (Figure 5). They conjugate and sporulate at a high frequency before nutrient starvation, suggesting that expression of the amino-terminal domain interferes with adenylyl cyclase function. Such interference may reflect the existence of a positive regulator of adenylyl cyclase that binds to its amino-terminal domain.

Adenylyl cyclase-associated protein

The *S. cerevisiae* adenylyl cyclase complex contains a tightly associated protein which appears to be required for the proper regulation of adenylyl cyclase (Field *et al.,* 1990a). To demonstrate this, we developed a procedure to purify adenylyl cyclase from *S. cerevisiae* by immunoaffinity chromatography using a monoclonal antibody raised against a specific peptide epitope that was genetically engineered into the amino-terminal domain of the enzyme (Field *et al.,* 1988). A protein with an apparent mobility of 70 kDa co-purifies with adenylyl cyclase purified from *S. cerevisiae* by this procedure. We have recently cloned the gene, *CAP,* encoding the adenylyl *c*yclase-*a*ssociated *p*rotein, and have raised antisera to the CAP protein purified from an *Escherichia coli* expression system (Field *et al.,* 1990a).

To explore whether *S. pombe* adenylyl cyclase associates with a protein similar to CAP, we constructed the pALY4 plasmid vector that expresses an epitope-fusion adenylyl cyclase protein. The strain MK141 contains an integrated copy of this plasmid and expresses high levels of the epitope fusion protein (see Table 4). Ad-

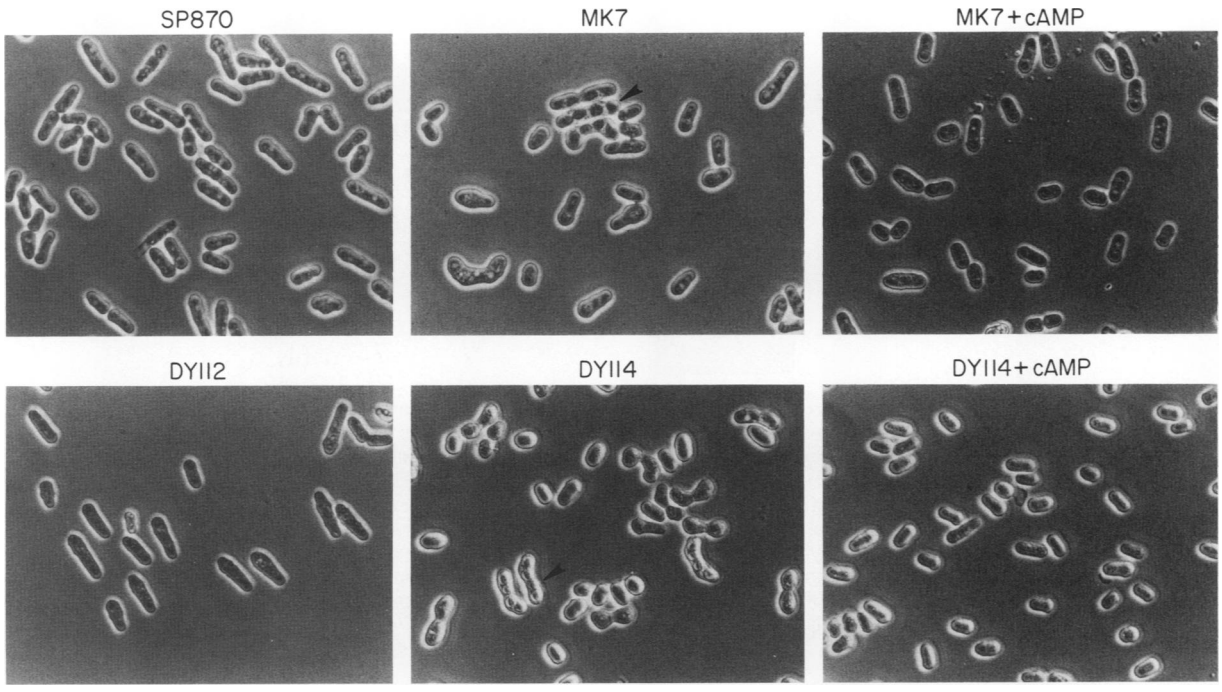

***Figure 2. S. pombe* strains containing a disruption of the adenylyl cyclase gene conjugate and sporulate in rich media.** The designated strains were grown on rich medium (YEA) agar plates with or without 5 mM cAMP for 2 d. Cells were dispersed in liquid medium (YEA) and photographs were taken. On this rich medium, conjugation and meiosis are inhibited in SP870 and DY112, which have normal adenylyl cyclase activity, whereas strains MK7 and DY114, which lack adenylyl cyclase activity, conjugate and undergo meiosis. The presence of 5 mM cAMP inhibits sporulation in MK7 and DY114. Arrows point to asci that result from conjugation and sporulation of cells.

enylyl cyclase partially purified from this strain by the immunoaffinity method contains several proteins. One of these proteins migrates by polyacrylamide gel electrophoresis with an apparent molecular weight of ~70 kDa, and in preliminary experiments can be detected by western blot analysis using antisera raised against *S. cerevisiae* CAP (data not shown). Most importantly, the CAP antisera is capable of immunoprecipitating adenylyl cyclase from cell extracts from MK141 (Table 4). This immunoprecipitation can be specifically blocked by the addition of extract from an *E. coli* strain that expresses *S. cerevisiae* CAP protein. Thus, it appears that a CAP-related protein is complexed with adenylyl cyclase in *S. pombe.*

Discussion

The budding yeast *S. cerevisiae* and the fission yeast *S. pombe* express structurally related adenylyl cyclases (Yamawaki-Kataoka *et al.,* 1989; Young *et al.,* 1989). Adenylyl cyclase is the major target for RAS protein action in the budding yeast (Toda *et al.,* 1985), but not in the fission yeast (Fukui *et al.,* 1986; Nadin-Davis *et al.,* 1986). We are trying to understand the basis for this difference by comparing the function of adenylyl cyclases in these yeasts.

The *S. cerevisieae* adenylyl cyclase is a large protein of 200 kDa. This protein has been divided into several functional domains, based both on biochemical and genetic evidence. The carboxyl terminal 40 kDa contains the entire catalytic domain (Kataoka *et al.,* 1985), and the most C-terminal portion of this domain is required for RAS responsiveness (Yamawaki-Kataoka *et al.,* 1989). Another 30 kDa separate this domain from a large leucine-rich repeat region of ~60 kDa that is also required for RAS responsiveness (Colicelli *et al.,* 1990). Overexpression of the leucine-rich repeat itself appears to interfere with RAS protein/adenylyl cyclase interaction (Field *et al.,* 1990b). The function of the 70-kDa N-terminal domain is unknown, although a 100-amino acid domain just N-terminal to the leucine-rich repeat is required for optimal RAS responsiveness (Colicelli *et al.,* 1990). *S. pombe* contains an adenylyl cyclase that resembles the *S. cerevisiae* protein (Ya-

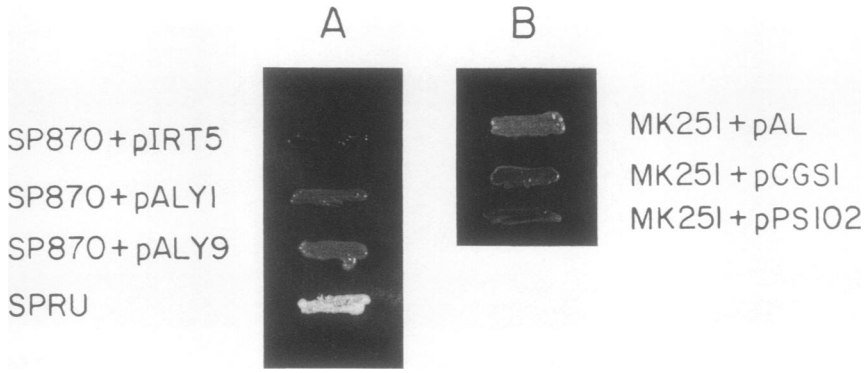

Figure 3. **Sporulation in strains that express high levels of adenylyl cyclase.** Patches of the designated strains containing the designated plasmids were grown on minimal medium (PMA + leucine) plates for 2 d and stained with iodine vapor for 1 min. Strains that have sporulated appear dark upon iodine staining. (A) SP870 cells, harboring the control vector pIRT5, appear dark indicating that this strain has sporulated. In contrast, the sterile strain, SPRU, in which the *ras1* gene has been deleted, does not sporulate and does not stain. SP870 cells harboring either pALY1 or pALY9, plasmids that direct expression of high levels of adenylyl cyclase activity, are partially sterile as indicated by the intermediate color upon staining. (B) MK251 cells, which express high levels of adenylyl cyclase, are partially sterile. This phenotype is suppressed in cells that contain either the plasmid pCGS1, which encodes the regulatory subunit of the cAMP-dependent protein kinase, or the plasmid pPS102, which expresses the *S. cerevisiae PDE2* gene encoding a high-affinity cAMP phosphodiesterase, but is not suppressed in cells containing the control plasmid pAL.

mawaki-Kataoka *et al.,* 1989; Young *et al.,* 1989). The carboxyl terminal catalytic domain shows the greatest similarity, but there are scattered similarities in other domains as well. In particular, both enzymes contain a large leucine-rich repeat region.

Several lines of evidence indicate that the proper function of adenylyl cyclase in *S. pombe* requires other protein components. First, overexpression of the leucine-rich repeat results in a phenotype resembling that of adenylyl cyclase-deficient cells, and thus this region appears to interfere with adenylyl cyclase function. As mentioned above, we have made similar observations upon expressing the leucine-rich repeat domain in *S. cerevisiae.* These observations suggest that the leucine-rich repeat domains have a conserved interaction with a regulatory protein. Second, we have found evidence that *S. pombe* contains a homolog of the *S. cerevisiae* CAP protein. In the budding yeast, CAP protein is found in the adenylyl cyclase complex and appears to be required for the proper functioning of the RAS/adenylyl cyclase pathway. Antisera to CAP protein can be used to immunoprecipitate adenylyl cyclase from both *S. cerevisiae* and *S. pombe.* At this time we feel it is unlikely that the CAP protein interacts with the leucine-rich repeat. In *S. cerevisiae,* overexpression of CAP does not overcome the overexpression of the leucine-rich repeat domain. Moreover, this domain does not appear to be required for CAP protein to bind to adenylyl cyclase in *S. cerevisiae* (Jeffrey Field and Roymarie Ballester, unpublished observations). Third, there is a large difference between *S. pombe* adenylyl cyclase activity measured with Mn^{2+} and activity measured with Mg^{2+}. This is seen too for the *S. cerevisiae* adenylyl cyclase, and in the latter case, the difference is narrowed when enzymatic activity is measured in the presence of RAS proteins (Toda *et al.,* 1985; Broek *et al.,* 1985). We suspect that an unknown protein component regulates activity of *S. pombe* adenylyl cyclase in the presence of Mg^{2+}. Fourth, and finally, we have noted that *S. pombe* strains that overexpress adenylyl cyclase have greatly elevated levels of activity measured in vitro, but have only mildly elevated levels of cyclic AMP. This observation is consistent with either of two hypotheses: *S. pombe* cells contain limiting amounts of a positive regulatory component or a powerful feedback mechanism regulates cAMP levels. Such mechanisms have been observed in *S. cerevisiae* (Nikawa *et al.,* 1987).

Studies based on the genetic perturbation of the adenylyl cyclase pathway in *S. cerevisiae* strongly suggest a role for cAMP in growth control and the sensing of nutrient conditions (Matsumoto *et al.,* 1982, 1983a,b; Kataoka *et al.,* 1985; Cannon and Tatchell, 1987; Toda *et al.,* 1987a,b). The only known physiological event that clearly affects cAMP levels in that organism

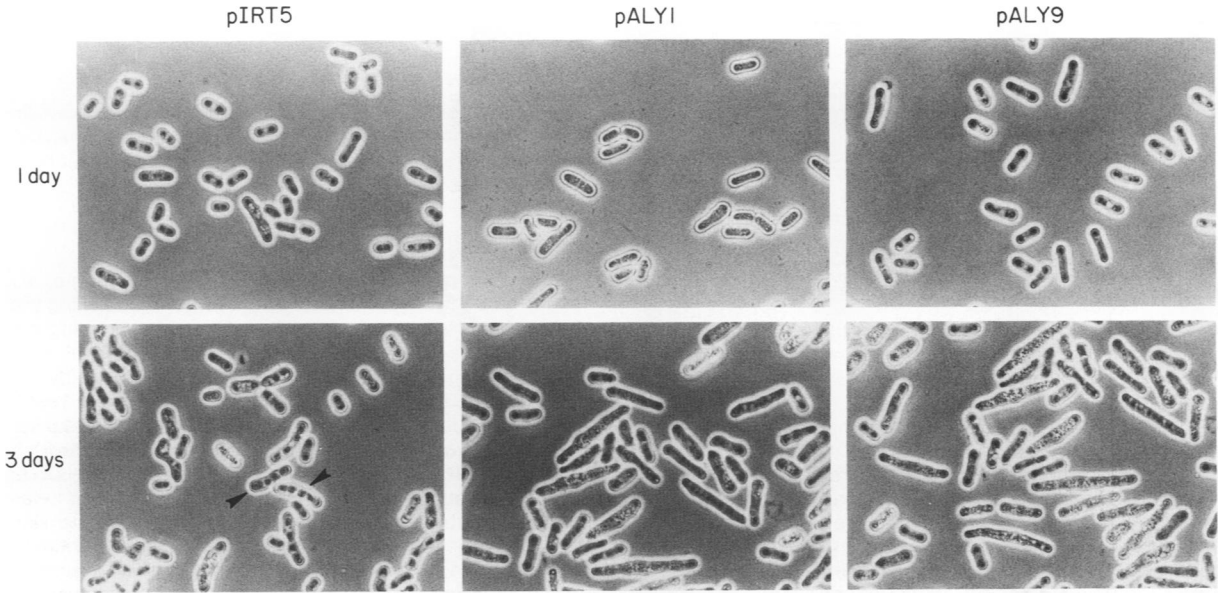

Figure 4. **Morphology of *S. pombe* strains that express high levels of adenylyl cyclase.** SP870 cells harboring the plasmids pIRT5 (control plasmid), pALY1 or pALY9 (which direct high levels of expression of adenylyl cyclase) were grown on minimal selective medium (PMA + leucine) agar plates for either 1 or 3 d. All strains appear essentially the same after 1 d of growth. After 3 d of growth, cells harboring the control vector pIRT5, like wild-type *S. pombe,* have sporulated. Cells harboring pALY1 or pALY9, which express high levels of adenylyl cyclase, fail to sporulate and have an elongated morphology. Arrows point to asci that result from the conjugation and sporulation of cells.

is glucose feeding (Mazon *et al.,* 1982; Francois *et al.,* 1988; Mbonyi *et al.,* 1988). There is no evidence that cAMP plays a role in the conjugal functions of *S. cerevisiae.* In contrast, previous studies have indicated that perturbation of the cAMP signalling pathway has profound effects on the conjugation of *S. pombe* and does not affect growth per se (Calleja *et al.,* 1980; Beach *et al.,* 1985). Our studies confirm this. *S. pombe* strains lacking the *cyr1* gene have no measur-

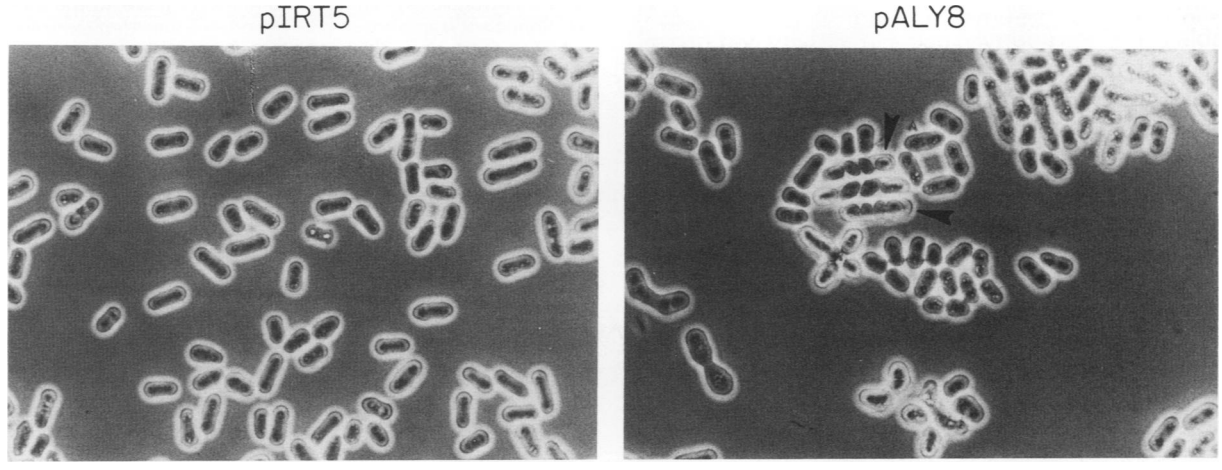

Figure 5. **Cells expressing truncated adenylyl cyclase protein, like strains lacking adenylyl cyclase, conjugate and undergo meiosis before nutrient starvation.** SP870 cells containing either pIRT5 or pALY8 during log phase growth in liquid minimal medium (PMA + leucine) were photographed after 1 d of growth. Although cells harboring the control plasmid pIRT5 appear normal, cells containing pALY8, which directs the expression of an adenylyl cyclase lacking the catalytic domain, have undergone conjugation and meiosis. This phenotype is similar to that of strains in which the adenylyl cyclase gene has been deleted (see Figure 2). Arrows point to asci that result from sporulation of cells.

Table 4. Immunoprecipitation of adenylyl cyclase from high-level expression strain MK141

		Immunoprecipitable/soluble adenylyl cyclase activity	
Antibody		Experiment I	Experiment II
None		21/1287	16/2008
12CA5		807/981	425/1853
KF191		534/984	251/1593
Antibody	Extract	Experiment III	Experiment IV
None		11/437	16/497
KF191		131/268	198/282
KF191	Control	128/263	161/254
KF191	CAP	22/362	30/458

MK141 cells overexpress a full-length adenylyl cyclase-epitope fusion protein. Solubilized lysates from MK141 were incubated with antibody to CAP (KF191), to the epitope (12CA5), or with buffer (none). In some incubations, extracts from *E. coli* were added to the antisera and incubated for 1 h before incubating with yeast lysates. "Control" extracts were from the strain BL-21(DE3)pLyS, and "CAP" extracts were from this strain containing the vector pT7.CAP, which expresses the *S. cerevisiae* CAP protein when induced by isopropyl-β-D-thiogalactopyranoside (IPTG) (Field *et al.*, 1990a). *E. coli* extracts were from IPTG-induced strains. KF191 is a rabbit polyclonal antisera raised to CAP protein purified from this *E. coli* expression system. 12CA5 is a mouse monoclonal antibody that has been previously described (Field *et al.*, 1988). Protein A sepharose beads were added to incubations, and after 1 h the beads were precipitated by centrifugation. The amount of adenylyl cyclase that remained soluble or immunoprecipitable was determined by assaying activity in the presence of 2.5 mM $MnCl_2$, as described in Table 2 and in Methods. Values represent pmol of cAMP produced in 30 min.

able cAMP or adenylyl cyclase activity, yet grow at near normal rates. Such strains conjugate readily, even in a rich medium that inhibits conjugation in wild-type strains. In addition, strains that overexpress adenylyl cyclase are relatively sterile. Since sexual commitment in *S. pombe* is normally a response to nutrient limitation, the cAMP pathway may be involved in the recognition of the nutrient status in that organism, as we believe it to be in *S. cerevisiae.*

The adenylyl cyclases found in yeasts do not resemble the adenylyl cyclase found in mammals (Krupinski *et al.*, 1989). Since we have shown that *cyr1*$^-$ *S. cerevisiae* strains (Kataoka *et al.*, 1985) and *cyr1*$^-$ S. pombe strains (this study) contain no measurable cAMP or adenylyl cyclase activity, it is unlikely that either yeast contains a homolog to the adenylyl cyclase gene that has been identified in mammals. It is not yet clear if mammals contain a homolog of the adenylyl cyclase gene found in yeasts.

Methods

Yeast growth and genetics

The genotypes of all *S. pombe* strains used are shown in Table 1. Methods for growth, transformation, tetrad analysis, and iodine staining of *S. pombe* are described by Moreno *et al.*, (1990).

Plasmids

The plasmid pPC2 was previously described (Young *et al.*, 1989). pPCU1 was constructed from pPC2 by replacing the 0.8-kb *Nco*I fragment with a 1.8-kb fragment encoding the *S. pombe ura4* gene. The 1.8-kb fragment has *Nco*I sites that were engineered at both ends of the *ura4* gene by the polymerase chain reaction (PCR) method (Scharf, 1990). pPCL1 was derived from pPC2 by replacing the 2.5-kb sequence between the 5′ *Hpa*I site and the 3′ *Nco*I site with a 2.2-kb *Hin*dIII fragment encoding the *S. cerevisiae LEU2* gene contained on pART1 (McLeod *et al.*, 1987). pALY1 was constructed by the following steps. First, a *Sal*I site was introduced 5′ to the start codon of the *S. pombe* adenylyl cyclase coding sequence in pPC2 using the PCR method. The 6.8-kb *Sal*I-*Kpn*I fragment containing the adenylyl cyclase coding sequence of the resulting plasmid was then cloned into the *Sal*I-*Kpn*I sites of the vector pAIL. pAIL contains the previously described *Nde*I-*Sal*I oligonucleotide encoding a peptide derived from the hemagglutinin antigen of influenza virus (Field *et al.*, 1988) cloned in the *Nde*I-*Sal*I sites of the plasmid pART9. pART9 is the same as pART1 (McLeod *et al.*, 1987) except that the 2.2-kb *Hin*dIII fragment containing the *S. cerevisiae LEU2* gene has been replaced with a 1.8-kb *Hin*dIII fragment containing the *S. pombe ura4* gene and the *Nde*I site has been removed from the *ars1* element. pALY8 was constructed from pALY1 by deleting the 3.0-kb *Eco*RI fragment containing the 3′ end of the *cyr1* gene. pALY9 was constructed by replacing the 6.8-kb *Sal*I-*Bam*HI fragment of pALY1 with a 2.6-kb fragment encoding amino acid residues 901–1692 of adenylyl cyclase-derived by PCR from pPC2. pALY4 was constructed by ligating the 6.8-kb *Sph*I-*Sac*I fragment of pALY1, containing the coding and 3′ untranslated sequences of the *cyr1* gene, to the 5.4-kb *Sph*I-*Sac*I fragment of pART5. pART5 was derived from pART1 by deletion of the 1.2-kb *Eco*RI fragment containing the *ars1* element. pALY5 is identical to pALY4 except for its selectable marker. It was constructed from the 6.8-kb *Sph*I-*Sac*I fragment of pALY1 and the 5.1-kb *Sph*I-*Sac*I fragment of pART8. pART8 is identical to pART5 except that the 2.2-kb *Hin*dIII fragment containing the *S. cerevisiae LEU2* gene has been replaced with the 1.8-kb *Hin*dIII fragment containing the *ura4* gene. pIRT5 was derived from pIRT2 (Hindley *et al.*, 1987) by replacing the 2.2-kb *Hin*dIII fragment containing the *S. cerevisiae LEU2* gene with the 1.8-kb *Hin*dIII fragment containing the *ura4* gene. pAL is the same as pIRT2.

The plasmid pT7.CAP, described previously (Field *et al.*, 1990a), expresses the *S. cerevisiae* CAP protein in *E. coli.*

Gene disruption and integration in S. pombe

The adenylyl cyclase gene was disrupted in the diploid strain SP826 as previously described (Russell and Nurse, 1986). SP826 was transformed with the 5-kb *Bam*HI fragment of pPCU1 containing the *ura4* gene, and Ura$^+$ transformants were selected on minimal defined media (PMA) lacking uracil. Several independent transformants were tested for stability of the Ura$^+$ phenotype by segregation analysis. Stable transformants were analyzed by Southern blots to confirm

that they contained the proper disruption in one copy of the endogenous adenylyl cyclase genes (data not shown). h^{90}/h^{+n} revertants of these strains, which occur at a frequency of $\sim 10^{-3}$, were detected by the iodine vapor staining test. The haploid strains DY112 and DY114 were derived from spores of a single ascus from one such revertant. The strain MK7 was constructed by replacing the normal *cyr1* gene in the haploid strain SP870 with the 8.0-kb *Sph*I-*Sac*I fragment of pPCL1, which contains the disrupted *cyr1* gene, and selecting for stable Leu$^+$ transformants. MK141 was derived by insertion of pALY4 into the genome of SP870. MK251 was derived by insertion of pALY5 into the genome of SP870.

Measurements of cAMP and adenylyl cyclase activity

cAMP levels were determined by radioimmune-assay (Harper and Brooker, 1975) of cAMP from nucleotides extracted from cells with 1 M formic acid saturated with 1-butanol (Olempska-Beer and Freese, 1984). Adenylyl cyclase activity was measured in crude cell membrane preparations as previously described (Young *et al.*, 1989). See Tables for further details.

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