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 over-expressing all or parts of this gene in various strains. *cyr1* mutants 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* mutants, 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,
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 ascis 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 DY14 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

![Diagram of plasmid maps](image)

**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), BamHI (B), EcoRI (R), Nco I (N), HindIII (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 carboxy-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' untranscribed 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 carboxy-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 MYPYDVPYASLGPMSTLD (Field et al., 1988), shown by the solid black boxes, fused to either the full length adenyl cyclase, the amino terminal domain, or the carboxy-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-adenyl cyclase fusion protein but contains a different auxotrophic marker. The construction of these plasmids is described in detail in Methods.
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 adenyl cyclase appear to be required to inhibit the sexual commitment of S. pombe during growth in rich medium.

Table 1. Genotypes of S. pombe strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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</thead>
<tbody>
<tr>
<td>SP826</td>
<td>h^+/h^+ leu-1-32 ade6-210 ura4-d18/ura4-d18</td>
</tr>
<tr>
<td>SP870</td>
<td>h90 leu-1-32 ade6-210 ura4-d18</td>
</tr>
<tr>
<td>MK7</td>
<td>h90 leu-1-32 ade6-210 ura4-d18 cyr1::LEU2</td>
</tr>
<tr>
<td>DY114</td>
<td>h90 leu-1-32 ade6-210 ura4-d18</td>
</tr>
<tr>
<td>DY114</td>
<td>h90 leu-1-32 ade6-210 ura4-d18 cyr1::ura4</td>
</tr>
<tr>
<td>MK141</td>
<td>h90 leu-1-32 ade6-210 ura4-d18 cyr1::pALY4 (LEU2)</td>
</tr>
<tr>
<td>MK251</td>
<td>h90 leu-1-32 ade6-210 ura4-d18 cyr1::pALY5 (ura4)</td>
</tr>
<tr>
<td>SPRU</td>
<td>h90 leu-1-32 ade6-210 ura4-d18 ras1::ura4</td>
</tr>
</tbody>
</table>

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 SP870 as described in Methods. SPRU was constructed in our lab by H. P. Xu, by disruption of the ras1 gene in SP870.

We investigated the effect of expressing high levels of adenyl cyclase in S. pombe. We built three plasmids that directed the expression of various regions of adenyl cyclase from the S. pombe alcohol dehydrogenase 1 (adh1) promoter. The plasmid pALY1 encodes the full-length 1692 amino acid residue adenyl 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 adenyl cyclase activity was measured in crude membrane preparations from Ura^+ transformants. SP870 harboring pALY1 or pALY9 have 60- to 240-fold higher levels of adenyl cyclase activity, respectively, than do normal cells (Table 2).

In S. cerevisiae, RAS proteins greatly stimulate adenyl cyclase activity as measured in vitro. We thus tested if the adenyl 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 adenyl cyclase display divergent regulation. However, we cannot exclude the possibility that overexpressed S. pombe adenyl cyclase lacks another component required for RAS responsiveness.

Despite the higher adenyl 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 adenyl cyclase does not form a produc-

Table 2. Adenylyl cyclase activity measured in various strains

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Plasmid*</th>
<th>Activity (pmol/min g^-1 mg^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP870</td>
<td>pIRT5</td>
<td>2.2</td>
</tr>
<tr>
<td>SP870</td>
<td>pALY1</td>
<td>3.2</td>
</tr>
<tr>
<td>SP870</td>
<td>pALY9</td>
<td>3.3</td>
</tr>
<tr>
<td>DY114</td>
<td>pALY1</td>
<td>Undetectable</td>
</tr>
<tr>
<td>TK-B111</td>
<td></td>
<td>112.9</td>
</tr>
</tbody>
</table>

This table shows the levels of adenyl cyclase activities measured in membranes prepared from the indicated strains harboring the indicated plasmids. Adenylyl cyclase activity was measured as described below. Adenylyl cyclase activities are expressed as the average pmol 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 adenyl cyclase, respectively, from the adh1 promoter. DY114 is isogenic to SP870, but has a disruption of the adenyl cyclase gene. TK-B111 is a S. cerevisiae strain with the genotype MATa ras1::HIS3 ras2::URA3 trp1 leu2 ade8 can1 that harbors the plasmid pADH-CYR1 and expresses the S. cerevisiae adenyl cyclase protein (Field et al., 1987).

† Adenylyl cyclase activities were determined, as previously described (Young et al., 1989), by measuring the production of [32P]cAMP in a 1:10 reaction mixture containing 50 μg membrane protein in 20 mM MES, 0.1 mM MgCl2, 0.1 mM EGTA, 1 mM β-mercaptoethanol, 1 mM ATP + 10 μCi [α-32P]ATP, 0.25 mM cAMP in the presence of either 2.5 mM MnCl2, 2.5 mM MgCl2, or 2.5 mM MgCl2 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

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Plasmid*</th>
<th>PMA (pmol/mg)†</th>
<th>PMA stationary</th>
<th>YEA stationary</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP870</td>
<td>pRT5</td>
<td>1.15</td>
<td>0.58</td>
<td>1.12</td>
</tr>
<tr>
<td>SP870</td>
<td>pALY1</td>
<td>1.64</td>
<td>1.27</td>
<td></td>
</tr>
<tr>
<td>SP870</td>
<td>pALY9</td>
<td>1.82</td>
<td>1.84</td>
<td></td>
</tr>
<tr>
<td>DY114</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MK7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 i-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.

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 cyclase-associated protein, 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-
adenylyl 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 \( \sim 70 \) kDa, and in preliminary experiments can be detected by western blot analysis using antisera raised against \( S. \text{cerevisiae} \) 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 \( E. \text{coli} \) strain that expresses \( S. \text{cerevisiae} \) CAP protein. Thus, it appears that a CAP-related protein is complexed with adenylyl cyclase in \( S. \text{pombe} \).

**Discussion**

The budding yeast \( S. \text{cerevisiae} \) and the fission yeast \( S. \text{pombe} \) 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 \( S. \text{cerevisiae} \) 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 \( \sim 60 \) kDa that is also required for RAS responsiveness (Colicelli et al., 1990). Over-expression 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). \( S. \text{pombe} \) contains an adenylyl cyclase that resembles the \( S. \text{cerevisiae} \) protein (Ya-
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. Antiserum 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.
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.

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

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Experiment I</th>
<th>Experiment II</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>21/1287</td>
<td>16/2008</td>
</tr>
<tr>
<td>12CA5</td>
<td>807/981</td>
<td>425/1853</td>
</tr>
<tr>
<td>KF191</td>
<td>534/984</td>
<td>251/1593</td>
</tr>
</tbody>
</table>

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 MnCl2, as described in Table 2 and in Methods. Values represent pmol of cAMP produced in 30 min.

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 Ncol fragment with a 1.8-kb fragment encoding the S. pombe ura4 gene. The 1.8-kb fragment has Ncol 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’ HpaI site and the 3’ Ncol site with a 2.2-kb HindIII fragment encoding the S. cerevisiae LEU2 gene contained on pART1 (McLeod et al., 1987). pALY1 was constructed by the following steps. First, a SaI 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 SalI-KpnI fragment containing the adenylyl cyclase coding sequence of the resulting plasmid was then cloned into the SaI-KpnI sites of the vector pAIL. pAIL contains the previously described Ndcl-SalI oligonucleotides encoding a peptide derived from the hemagglutinin antigen of influenza virus (Field et al., 1988) cloned in the Ndcl-SalI sites of the plasmid pPART9. pART9 is the same as pART1 (McLeod et al., 1987) except that the 2.2-kb HindIII fragment containing the S. cerevisiae LEU2 gene has been replaced with a 1.8-kb HindIII fragment containing the S. pombe ura4 gene and the Ndcl site has been removed from the ars1 element. pALY8 was constructed from pALY1 by deleting the 3.0-kb EcoRI fragment containing the 3’ end of the cyr1 gene. pALY9 was constructed by replacing the 6.8-kb SalI-BamHI 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 Sphi-SalI fragment of pALY1, containing the coding and 3’ untranslated sequences of the cyr1 gene, to the 5.4-kb Sphi-SalI fragment of pART5. pART5 was derived from pPART1 by deletion of the 1.2-kb EcoRI fragment containing the ars1 element. pALY5 is identical to pALY4 except for its selectable marker. It was constructed from the 6.8-kb Sphi-SalI fragment of pALY1 and the 5.1-kb Sphi-SalI fragment of pART8. pART8 is identical to pART5 except that the 2.2-kb HindIII fragment containing the S. cerevisiae LEU2 gene has been replaced with the 1.8-kb HindIII fragment containing the ura4 gene. pIRT5 was derived from pIRT2 (Hindley et al., 1987) by replacing the 2.2-kb HindIII fragment containing the S. cerevisiae LEU2 gene with the 1.8-kb HindIII 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 BamHI fragment of pPCU1 containing the ura4 gene, and Ura− transformants were selected on minimal defined media (PMA) lacking ura. 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 adenyllyl cyclase genes (data not shown). hmo/ hmo revertants of these strains, which occur at a frequency of \(10^{-4}\), 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 cyrl gene in the haploid strain SP870 with the 8.0-kb Sphl-Sacl fragment of pPCL1, which contains the disrupted cyrl 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 adenyllyl 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 (Olempaska-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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**References**


Matsumoto, K., Uno, I., Oshima, Y., and Ishikawa, T. (1982). Isolation and characterization of yeast mutants deficient in...


