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Genetics

The adenylyl cyclase gene from *Schizosaccharomyces pombe*

(signal transduction/cAMP/evolution/cell regulation)

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ABSTRACT **We cloned the adenylyl cyclase gene from the fission yeast *Schizosaccharomyces pombe* using low-stringency hybridization to the *Saccharomyces cerevisiae* adenylyl cyclase gene. The *Sc. pombe* gene encodes a 1692-amino acid-residue protein. The identity of this gene was confirmed by studies of its expression in *Sa. cerevisiae*. Expression of the carboxyl-terminal region of the *Sc. pombe* adenylyl cyclase protein will suppress a temperature-sensitive mutation in the *Sa. cerevisiae* adenylyl cyclase gene. Furthermore, *Sa. cerevisiae* that lack their endogenous adenylyl cyclase gene and express the carboxyl-terminal region of the *Sc. pombe* adenylyl cyclase protein have measurable adenylyl cyclase activity. The carboxyl-terminal region of this protein has strong homology with the catalytic domain of the *Sa. cerevisiae* adenylyl cyclase. Also, *Sc. pombe* adenylyl cyclase, like *Sa. cerevisiae* adenylyl cyclase, contains a tandemly repeated motif rich in leucine. Neither yeast protein is particularly homologous to the recently cloned G_s-responsive mammalian adenylyl cyclase [Krupinski, J., Coussen, F., Bakalyar, H. A., Tang, W.-J., Feinstein, P. G., Orth, K., Slaughter, C., Reed, R. R. & Gilman, A. G. (1989) *Science* 244, 1558–1564].**

Adenylyl cyclase catalyzes the conversion of ATP into the second messenger cAMP, which plays an important role in the regulation of a variety of cellular responses in eukaryotic organisms. In the budding yeast *Saccharomyces cerevisiae*, cAMP can regulate a range of cellular events, including cell growth, cell-cycle progression, glycogen metabolism, and heat shock sensitivity (1–4). The gene encoding adenylyl cyclase from *Sa. cerevisiae*, *CYR1*, has been previously cloned in this laboratory (5) and others (6, 7). This gene encodes a 2026-amino acid-residue protein. Deletion analysis has revealed that the 418-amino acid carboxyl-terminal end of the protein is sufficient for enzymatic activity. Using the region of *CYR1* that encodes the catalytic domain as a hybridization probe, we were able to detect and clone the adenylyl cyclase gene from the distantly related fission yeast *Schizosaccharomyces pombe*.† We present here a comparison of the amino acid sequence of the adenylyl cyclase from *Sc. pombe* with that of *Sa. cerevisiae*.

MATERIALS AND METHODS

DNA Manipulation. DNA was purified from the *Sc. pombe* strain SP67 (8) by a described procedure (9). *Sc. pombe* DNA was cut with restriction enzymes (New England Biolabs), fractionated on a 1% agarose gel, and blotted onto nitrocellulose paper (10). DNA fragments that were homologous to the *Sa. cerevisiae* adenylyl cyclase gene were detected by low-stringency hybridization to a nick-translated (11), ^{32}P-labeled *Pvu* II–*Cla* I fragment of the plasmid pCYR1-2 (5). Low-stringency hybridization was performed in 6× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate), 1× Denhardt's solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.2% bovine serum albumin), and denatured calf thymus DNA (50 μg/ml) at 55°C for 24 hr followed by several washings in 2× SSC/12 mM Na_2HPO_4/8 mM NaH_2PO_4/1.35 mM $Na_4P_2O_7$/1.25% SDS at 55°C. The *Sa. cerevisiae CYR1* probe detected a 3.2-kilobase-pair (kbp) *Eco*RI–*Xba* I *Sc. pombe* DNA fragment. To clone this fragment, *Sc. pombe* DNA was cut with *Eco*RI and *Xba* I and fractionated on an agarose gel. DNA fragments 2.7 to 3.7 kbp in length were purified by electroelution and inserted into the λ ZAP vector (Stratagene). Individual plaques containing the 3.2-kbp DNA insert homologous to *CYR1* were detected by filter hybridization (12). The plasmid pPC28 was constructed by subcloning the 3.2-kbp *Eco*RI–*Xba* I DNA insert into pUC118. A library of *Sc. pombe* DNA that was cut with *Sal* I and *Xba* I was constructed in pUC118, and clones hybridizing to the 3.2-kbp fragment were detected by colony-filter hybridization (13). The DNA sequence of one such clone, pPC2, was determined by a modified procedure (14) of the dideoxynucleotide chain-termination method (15).

Yeast Expression Plasmids. The plasmid pAD4 contains the yeast *LEU2* gene from YEp213 (9), the yeast 2-μm origin of replication, the ampicillin resistance gene, and the bacterial origin of replication from pUC18, as well as the yeast *ADH1* (alcohol dehydrogenase) promoter and terminator sequences (16). pAD4 is identical to the plasmid pADNS, which was described (17), except that the promoter and terminator sequences are separated by the pUC18 polylinker sequence. The plasmid pADPC was constructed by inserting the 4.0-kbp *Hin*dIII–*Sac* I fragment of pPC2 into the vector pAD4 at the *Hin*dIII–*Sac* I sites. The plasmid pYCYR contains the 8.2-kbp region of pEF-CYR1 (18), containing the alcohol dehydrogenase promoter linked to the entire coding sequence of *Sa. cerevisiae* adenylyl cyclase, cloned into the vector YEp13 (9).

Yeast Strains and Genetics. Yeast were grown, transformed, and analyzed as described. The genotype of the *Sa. cerevisiae* strain T50-3A is *MATα his3 leu2 trp1 ura3 cyr1-2*, and its construction has been described (5). The *cyr1-2* allele encodes a temperature-sensitive adenylyl cyclase (19). The genotype of the *Sa. cerevisiae* strain T158-5AT is *MATa his3 leu2 trp1 ura3 ade8 cyr1::URA3*. In T158-5AT the entire adenylyl cyclase gene is deleted and replaced with the *Sa. cerevisiae URA3* gene. This strain has been described (20).

Adenylyl Cyclase Assays. Adenylyl cyclase assays were performed using crude yeast membrane extracts. Yeast membrane extracts were prepared from 1-liter yeast cultures that were grown to a density of 1×10^7 cells per ml. Cells were washed in buffer C {200 mM Mes [2-(*N*-morpholino)ethanesulfonic acid], 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

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M26699).

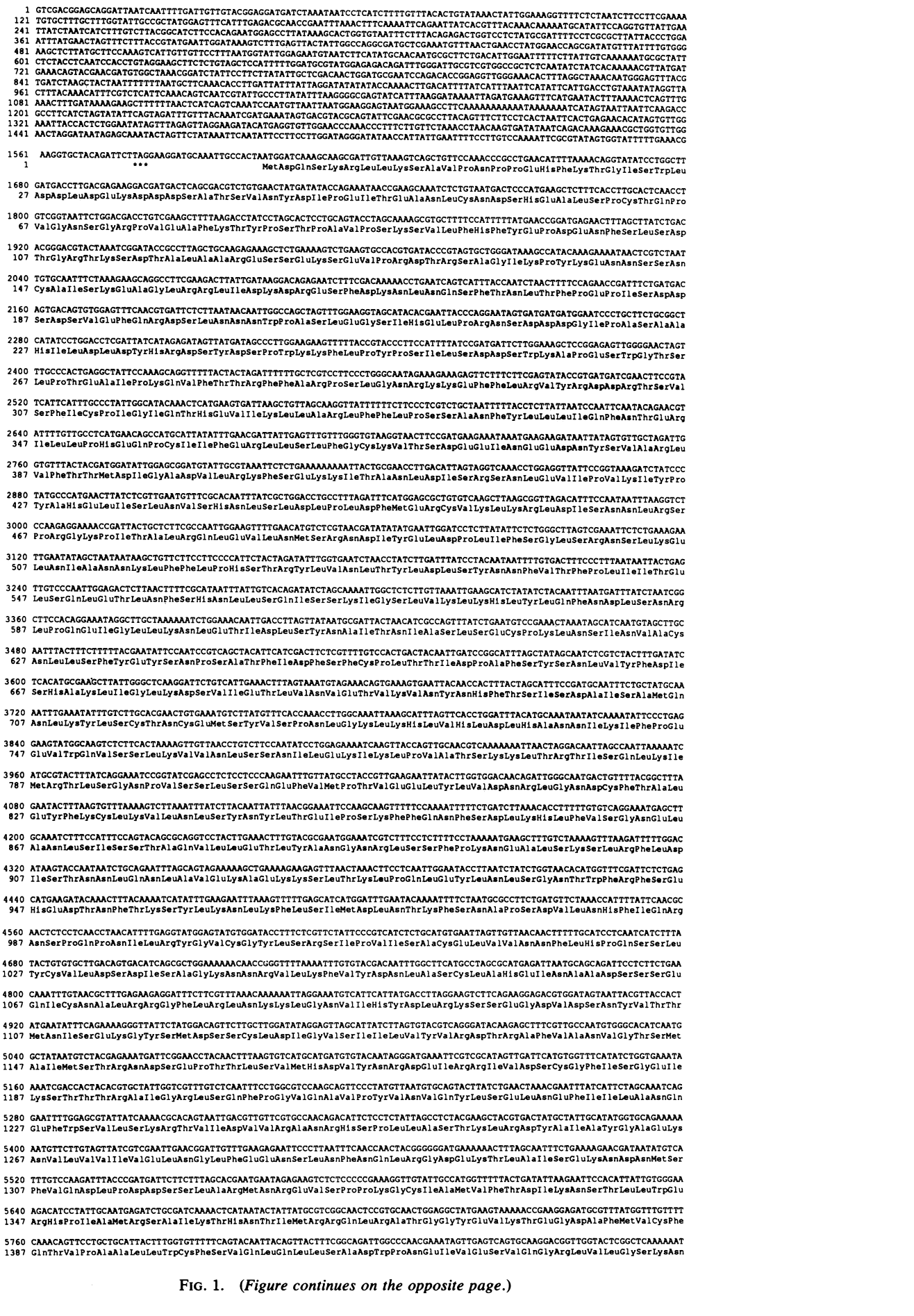

FIG. 1. (*Figure continues on the opposite page.*)

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5880 GAGGTGTTATATCGAGGGCTTAGTGTTCGAATTGGTGTCAATTATGGTGTAACCGTGAGTGAACTAGATCCCATCACTAGACGTATGGACTATTACGGGCCTGTAGTAAACAGAACATCT
1427 GluValLeuTyrArgGlyLeuSerValArgIleGlyValAsnTyrGlyValThrValSerGluLeuAspProIleThrArgArgMetAspTyrTyrGlyProValValAsnArgThrSer

6000 AGGGTTGTATCAGTCGCTGATGGTGGTCAAATTGCTGTTTCTGCTGAGGTGGTATCTGTATTGAATCAGCTTGATTCAGAAACAATGTCATCAGAGAAGACGAATGTCAACGAAATGGAA
1467 ArgValValSerValAlaAspGlyGlyGlnIleAlaValSerAlaGluValValSerValLeuAsnGlnLeuAspSerGluThrMetSerSerGluLysThrAsnValAsnGluMetGlu

6120 GTTCGTGCTCTTAAACAAATCGGTTATATTATCCATAACCTTGGAGAATTTAAGTTAAAAGGTTTGGATACTACTGAAATGATTTCATTGGTTTATCCTGTGCAATTGCAAGGAAGACTG
1507 ValArgAlaLeuLysGlnIleGlyTyrIleIleHisAsnLeuGlyGluPheLysLeuLysGlyLeuAspThrThrGluMetIleSerLeuValTyrProValGlnLeuGlnGlyArgLeu

6240 GAGAGATTGATAAAGAGCCGAAGTTTGGGAACACCCACAGCCCTCCCGGAAACTCAGACTTATACTCCCGTTCGTAGTAGAAGCAACAGCTTGCGACCCATGTTAGCAAGATTGAGTGAT
1547 GluArgLeuIleLysSerArgSerLeuGlyThrProThrAlaLeuProGluThrGlnThrTyrThrProValArgSerArgSerAsnSerLeuArgProMetLeuAlaArgLeuSerAsp

6360 TCAAAATCTGTCCATGGAGAGGAGGGTGGTTCTGGGAAGAGATCGGTTTCATCCTTGCGCAACGTATCACCATCAGAGAGTACTGGTGGATATGAAGGTTGTATTTTTGATGACCAACAG
1587 SerLysSerValHisGlyGluGluGlyGlySerGlyLysArgSerValSerSerLeuArgAsnValSerProSerGluSerThrGlyGlyTyrGluGlyCysIlePheAspAspGlnGln

6480 TATCAATTACTTTATGAACTTTGTGAGCGTCTTGAAGACCATGCCGCTATACTGCATGGGTTTCCTGAACCACCGCCTTGCGATACCGGTCTGGCAGCTCCCGTAAACCAGGCCGAGGAG
1627 TyrGlnLeuLeuTyrGluLeuCysGluArgLeuGluAspHisAlaAlaIleLeuHisGlyPheProGluProProProCysAspThrGlyLeuAlaAlaProValAsnGlnAlaGluGlu

6600 TATTCATTGTTCTACCGTCTGACTTTGCGTATCGAGAATACTATTTATTGTGTCAGTCAAATGCTTGGACACACTGGCTAAATGAATGCATATAATGACTGCTATATTTAAATAATGTTT
1667 TyrSerLeuPheTyrArgLeuThrLeuArgIleGluAsnThrIleTyrCysValSerGlnMetLeuGlyHisThrGly***                                    ***

6720 AACCCCATAAATAGTTTATATATATATATTTATTTTTTGTATATATAATTTGCATATTTTTAATAAAATTTTTTTTTTTTTTGCTATACCCTAAAGTTGGAATTATGGAATACCAAATTA

6840 ACGTACGTGTTTCGAGGCAAATCAACTTTTATTTACTATTAGTAAA
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FIG. 1. The DNA sequence and predicted amino acid sequence of the *Sc. pombe* adenylyl cyclase gene. The DNA sequence of a 10-kbp *Sal* I–*Xba* I fragment from the plasmid pPC2, beginning from the *Sal* I site and extending 6885 base pairs (bp), is shown. pPC2 was isolated from a genomic *Sc. pombe* DNA library. Both strands of the entire DNA sequence presented were determined. Numbers at left indicate nucleotide and amino acid positions. An open reading frame encoding a 1692-amino acid protein begins at nucleotide 1602 and is bracketed by stop codons at nucleotide 1578, 24 bp upstream from the start codon, and position 6677. ***, Stop codons.

at 20,000 psi (1 psi = 6.9 kPa). 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. The procedures described above were done at 4°C. Protein concentrations were measured following a described procedure (21). Adenylyl cyclase reactions containing 15–60 μg of membrane extract protein, 1 mM [α-^{32}P]ATP (126 cpm/pmol), 2.5 mM $MnCl_2$, 20 mM creatine phosphate, 20 units of creatine phosphokinase (Sigma), and 0.25 mM cAMP in 100 μl were incubated for 30 min at 30°C. cAMP produced was measured by a published procedure (22).

RESULTS

Cloning and Sequencing a Gene from *Sc. pombe* Homologous to the Adenylyl Cyclase Gene of *Sa. cerevisiae*. We first detected a DNA sequence in *Sc. pombe* that is homologous to the *Sa. cerevisiae* gene encoding adenylyl cyclase by Southern blot-hybridization. The plasmid pPC2, which contains a 10-kbp DNA sequence derived from genomic *Sc. pombe* DNA, was isolated as described, and the nucleotide sequence was determined (Fig. 1). The sequence contains an open reading frame that is 5097 bp long and encodes a protein 1692-amino acid residues in length. The protein encoded by this sequence has significant homology with *Sa. cerevisiae* adenylyl cyclase (Fig. 2)—particularly in the carboxyl-terminal catalytic domains.

Expression of the Carboxyl-Terminal Region of the Protein Encoded by the *Sc. pombe* Gene in *Sa. cerevisiae* Containing a Temperature-Sensitive Adenylyl Cyclase. To determine the identity of the *Sc. pombe* gene, we first conducted a genetic test. We built a plasmid, pADPC, that contains the *Hin*dIII–*Sac* I fragment of pPC2, encoding the 727-amino acid carboxyl-terminal region of the *Sc. pombe* protein, linked to the yeast *ADH1* (alcohol dehydrogenase) promoter in the yeast expression vector pAD4. This plasmid contains the *Sa. cerevisiae LEU2* gene (see *Materials and Methods* for details). pADPC was used to transform the *Sa. cerevisiae* strain T50-3A, which contains the *cyr1-2* allele and is temperature sensitive for growth due to a thermolabile adenylyl cyclase (5, 19). Four independent Leu$^+$ clones of T50-3A that were transformed with pADPC were able to grow at the restrictive temperature of 36°C, whereas four independent Leu$^+$ clones transformed with the vector pAD4 were unable to grow at the restrictive temperature (data not shown).

Adenylyl Cyclase Activity in Yeast Expressing the Carboxyl-Terminal Region of the Protein Encoded by the *Sc. pombe* Gene. We next tested whether the *Sc. pombe* gene encodes an adenylyl cyclase by measuring adenylyl cyclase activity in a *Sa. cerevisiae* strain that lacks its own adenylyl cyclase but expresses the carboxyl-terminal region of the *Sc. pombe* protein. The strain T158-5AT, in which the endogenous adenylyl cyclase gene has been completely replaced with the yeast *URA3* gene, contains a high-copy plasmid that encodes the yeast *SCH9* gene (20). Overexpression of *SCH9*, which encodes a protein homologous to the cAMP-dependent protein kinase catalytic subunits, permits the normal growth of strains lacking adenylyl cyclase (20). We transformed T158-5AT with the plasmids pAD4, pADPC, or pYCYR. pYCYR is a plasmid that contains the *Sa. cerevisiae* adenylyl cyclase coding sequence linked to the *ADH1* promoter. *Sa. cerevisiae* containing pYCYR have high levels of adenylyl cyclase activity relative to wild-type yeast strains. We measured adenylyl cyclase activity in transformed T158-5AT cells containing these plasmids (Table 1). We found that the levels of adenylyl cyclase activity in cells expressing the region of the *Sc. pombe* gene contained on the plasmid pADPC were at least 30-fold higher than levels found in cells harboring the vector pAD4. These results provide conclusive evidence that the *Sc. pombe* gene encodes adenylyl cyclase. We have named this *Sc. pombe* gene *cyr1*.

DISCUSSION

In the yeast *Sa. cerevisiae*, adenylyl cyclase is regulated by RAS proteins (24). There is no evidence that RAS proteins regulate adenylyl cyclase in either *Sc. pombe* or in mammalian cells (25–28). In mammals, one major form of adenylyl cyclase is regulated by G_s protein (29). Although both G_s and RAS proteins bind guanine nucleotides, they belong to very distinct families of proteins. Recently, a mammalian gene encoding a G_s-responsive adenylyl cyclase was cloned and sequenced (30). It contains two large multi-membrane-spanning domains and two 40-kDa domains that are proposed to be catalytic. There is very little homology between these putative catalytic domains and the catalytic domain of the *Sa. cerevisiae* adenylyl cyclase. Moreover, the *Sa. cerevisiae* enzyme contains no transmembrane domains. Thus, the two adenylyl cyclases are very different.

The yeast *Sc. pombe* is quite diverged from *Sa. cerevisiae*. In fact, when proteins conserved between *Sa. cerevisiae*, *Sc. pombe*, and mammals have been compared, they have generally been observed as equally diverged (31–35). It is, therefore, of some interest to compare *Sc. pombe* adenylyl cyclase with those of *Sa. cerevisiae* and mammals. The *Sa. cerevisiae* and *Sc. pombe* proteins show striking homology within their respective catalytic domains (63% identity in a 158-amino acid region). In contrast, the *Sc. pombe* adenylyl cyclase, like the *Sa. cerevisiae* enzyme, shows little homol-

FIG. 2. Alignment of the amino acid sequences (in one-letter code) of the adenylyl cyclases of *Sc. pombe* and *Sa. cerevisiae*. The *Sc. pombe* protein is aligned above the *Sa. cerevisiae* protein. This alignment was done by the method of Dayhoff (23) using a logarithm of odds matrix for 250 accepted point mutations per 100 amino acids. Numbers at left indicate amino acid positions from the beginning of the proteins; the first 370 amino acids of the *Sa. cerevisiae* protein are not shown. Amino acids that are identical or highly conserved at corresponding positions are indicated by | or :, respectively. Amino acid residues are indicated as highly conserved when both residues fall within one of the following amino acid groups: (N,D), (D,E,Q), (Q,H), (H,N), (H,R), (R,K), (R,W), (M,I,L,V), (F,L), (F,Y).

ogy to the mammalian adenylyl cyclase. A weak consensus sequence derived from aligning the putative mammalian catalytic domains with four guanylyl cyclases and the *Sa. cerevisiae* adenylyl cyclase also weakly fits the *Sc. pombe* adenylyl cyclase, but not much better than one would expect given the homology between the *Sc. pombe* and *Sa. cerevisiae* proteins in their catalytic domains. Thus, at least two quite distinct branches exist in the evolution of adenylyl cyclase in eukaryotes: the one represented by the recently cloned gene from mammalian cells and the other represented by the genes conserved between two divergent yeasts. The cloned mammalian gene encodes a G_s-responsive adenylyl cyclase. What regulates the *Sc. pombe* enzyme (25, 26) is not clear; nor is it clear how many distinct forms of adenylyl cyclase are present in mammalian cells (36–39).

Homology between the yeast enzymes is not as striking outside the catalytic domains (29% identity over 1274 amino acids), perhaps reflecting divergent regulation. However, both enzymes share a common motif outside their catalytic domains. The *Sa. cerevisiae* enzyme contains a 23-amino acid leucine-rich consensus sequence that is tandemly repeated ≈22 times. A similarly tandemly repeated sequence is found in the *Sc. pombe* adenylyl cyclase (Fig. 3). Very similar motifs are also found tandemly repeated in a variety of eukaryotic proteins (40). Results from our laboratory suggest that this region is important for activation of the *Sa. cerevisiae* adenylyl cyclase by RAS protein. Although the regulation of *Sc. pombe* and *Sa. cerevisiae* adenylyl cyclase may

Table 1. Adenylyl cyclase activity

Plasmid*	Adenylyl cyclase activity, pmol/min per μg	
	Experiment I	Experiment II
pAD4	<1.0	<1.0
pADPC	35.8	35.6
pYCYR	881.1	479.2

The *Sa. cerevisiae* strain T158-5AT harboring the designated plasmids was tested for adenylyl cyclase activity as described. Values are expressed as the average pmol of cAMP produced per min per μg of total membrane protein for three separate assays.

*Plasmids pADPC and pYCYR direct the expression of the catalytic region of *Sc. pombe* adenylyl cyclase and the entire *Sa. cerevisiae* adenylyl cyclase, respectively.

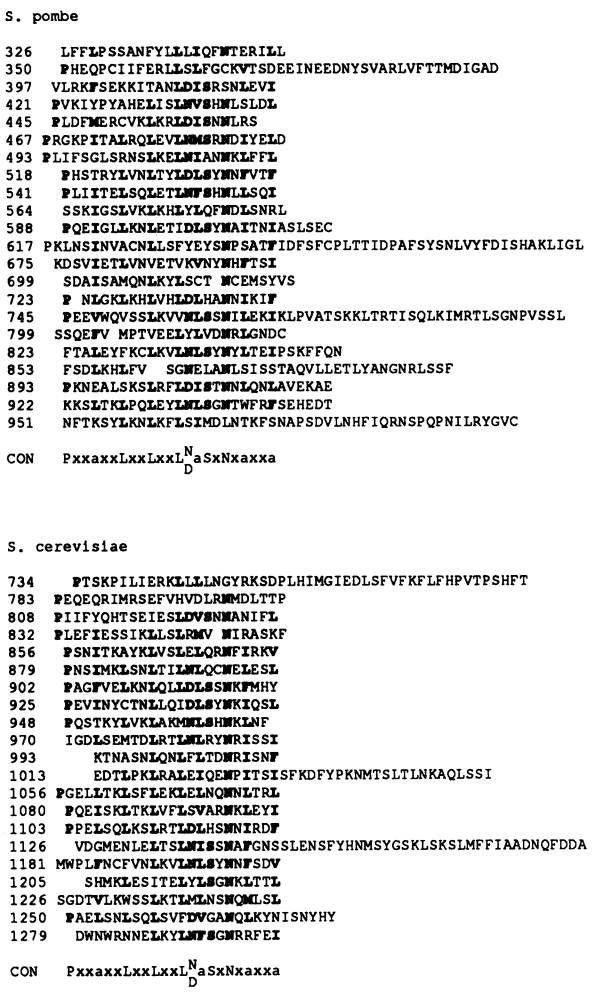

FIG. 3. Alignments of the leucine-rich repeats of *Sc. pombe* and *Sa. cerevisiae* adenylyl cyclases. A region of the protein sequence of the *Sc. pombe* adenylyl cyclase, from amino acid-residue positions 326 to 999, is shown in segments aligned to give the best fit to the consensus sequence shown below. Residues that match the consensus sequence are in boldface. Numbers at left indicate the amino acid position of the first amino acid residue of each segment. A similar alignment of the region of the *Sa. cerevisiae* adenylyl cyclase, from amino acid-residue positions 734 to 1300, is also shown. In the consensus sequence, x indicates any amino acid residue, and a indicates any aliphatic amino acid residue included in the amino acid group M, I, L, and V (one-letter code). Consensus sequences derived from each protein are identical.

differ in essential respects, this regulation will probably share common features. The isolation of the gene encoding *Sc. pombe* adenylyl cyclase should facilitate the characterization of the regulation of this enzyme.

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