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Biochemistry

Adenylate cyclases in yeast: A comparison of the genes from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*

(cyclic AMP/fission yeast/evolution)

YURIKO YAMAWAKI-KATAOKA, TATSUYA TAMAOKI, HYE-RYUN CHOE, HIDEHO TANAKA, AND TOHRU KATAOKA

Department of Cellular and Molecular Physiology, Harvard Medical School, 25 Shattuck Street, Boston, MA 02115

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ABSTRACT **A *Schizosaccharomyces pombe* gene encoding adenylate cyclase has been cloned by cross-hybridization with the *Saccharomyces cerevisiae* adenylate cyclase gene. The protein encoded consists of 1692 amino acids and has adenylate cyclase activity that cannot be activated by the *Sa. cerevisiae* RAS2 protein. *Sc. pombe* cyclase has a high degree of homology (≈60%) with the catalytic domain of *Sa. cerevisiae* cyclase precisely mapped by a gene-deletion analysis. A 25–40% identity is observed throughout the middle segments of ≈1000 residues of both cyclases, large parts of which are composed of repetitions of a 23-amino acid motif similar to those found in human glycoproteins, *Drosophila* chaoptin, and *Toll* gene product. However, a segment corresponding to the NH_2-terminal 620 residues of *Sa. cerevisiae* cyclase appears lost from *Sc. pombe* cyclase, and the COOH-terminal 140 residues are not well conserved between the two yeast species. Deletions involving the COOH-terminal residues of *Sa. cerevisiae* cyclase cause loss of activation by the RAS2 protein. These results suggest that *Sc. pombe* cyclase may have lost the ability to interact with RAS proteins by the loss of a regulatory site.**

Adenylate cyclase has been shown to play essential roles in regulation of cellular metabolism by catalyzing the synthesis of a second messenger, cAMP. A guanine nucleotide-binding (G) protein called G_s mediates hormone-dependent activation of mammalian adenylate cyclase (for review, see ref. 1). However, in the yeast *Saccharomyces cerevisiae*, adenylate cyclase is regulated by a different set of G proteins, RAS1 and RAS2, which are homologs of mammalian ras oncoproteins (2, 3). It was shown that mammalian ras proteins could activate yeast adenylate cyclase but neither mammalian ras nor yeast RAS proteins could regulate mammalian adenylate cyclase (3–5). Although the precise mode of interaction between RAS proteins and yeast adenylate cyclase is unknown, regulatory proteins of adenylate cyclase appear to have somehow switched during the course of evolution. Specifically, it was shown that the cAMP concentration of the fission yeast *Schizosaccharomyces pombe* cells was not affected by the disruption or mutational activation of its sole *ras* gene, suggesting that its adenylate cyclase is not regulated by ras proteins (6). *Sa. cerevisiae* adenylate cyclase comprises 2026 amino acids in which the COOH-terminal 417 residues, called the catalytic domain, retains a Mn^{2+}-dependent cyclase activity (7). The remaining portion of the NH_2 terminus was proposed to be essential for the RAS protein- and GTP-dependent activation in the presence of Mg^{2+} (7–9). In this report, we describe the primary structure of *Sc. pombe* adenylate cyclase.* Sequence comparison with *Sa. cerevisiae* adenylate cyclase reveals four segments displaying varying extents of homology. The functional significance of these segments is discussed.

MATERIALS AND METHODS

Cell Strains, Growth Media, and Transformation. A *Sa. cerevisiae* strain, T50-3A (*MATα, leu2, his3, trp1, ura3, cyr1-2*) was described (7). A *Sc. pombe* strain 972h^{-s} was obtained from D. Beach, Cold Spring Harbor Laboratory. Culture media for yeast cells and method of yeast transformation were as described (2–4, 10).

DNA and RNA Preparation and Hybridization Analyses. Isolation of yeast genomic DNA and Southern blot hybridization were done as described (4). The low-stringency hybridization was performed at 50°C in 1 M NaCl, and washing of the filters was done in 0.15 M NaCl at 50°C. Preparation of poly(A)$^+$ RNA from *Sc. pombe* cells and Northern blot hybridization were done as described (7, 11, 12).

Cloning, Sequencing, and Deletion-Mutagenesis. Construction of a cDNA library from *Sc. pombe* poly(A)$^+$ mRNA was done (13) by using pUC18 as a vector. Fragments of the *Sc. pombe* gene were cloned into M13 vectors and subjected to DNA sequencing (14). Protein sequences were compared by the DNA/PROTEIN sequence analysis program (International Biotechnologies). Expression of *Sc. pombe* cyclase in *Sa. cerevisiae* was done using the vector pAD-1 (obtained from J. Nikawa, Gunma University, Gunma, Japan) (see Fig. 4*A*). *YRp7-ADC1-CYR1* (15), which over-expressed the complete

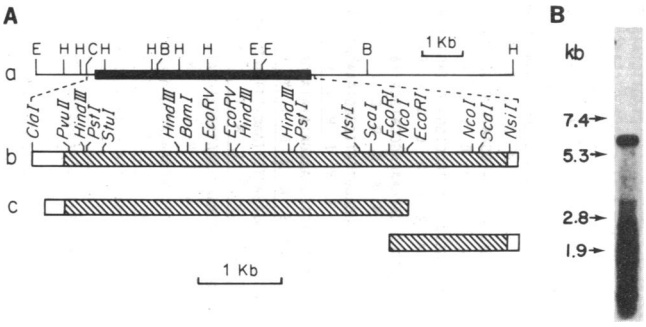

FIG. 1. Structure and Northern (RNA) blot analysis of the *spCYR1* gene. (*A*) Restriction maps of the genomic *spCYR1* gene (*a*), of the sequenced region (*b*), and of the cDNA clones (*c*). The hatched regions represent the protein-coding sequence. Abbreviations used are as follows: B, *Bam*HI; C, *Cla* I; E, *Eco*RI; and H, *Hin*dIII. (*B*) Five micrograms of poly(A)$^+$ RNA from *Sc. pombe* 972h^{-s} was fractionated in 1% agarose gel containing formaldehyde. A Northern blot of the gel was hybridized with the ^{32}P-labeled *Eco*RI–*Hin*cII fragment of the *spCYR1* (corresponding to the amino acid positions 1341–1502) and autoradiographed. RNA size markers were obtained from Boehringer Mannheim.

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Abbreviation: p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate.

*The sequence reported in this paper has been deposited in the EMBL/GenBank data base (accession no. M24942).

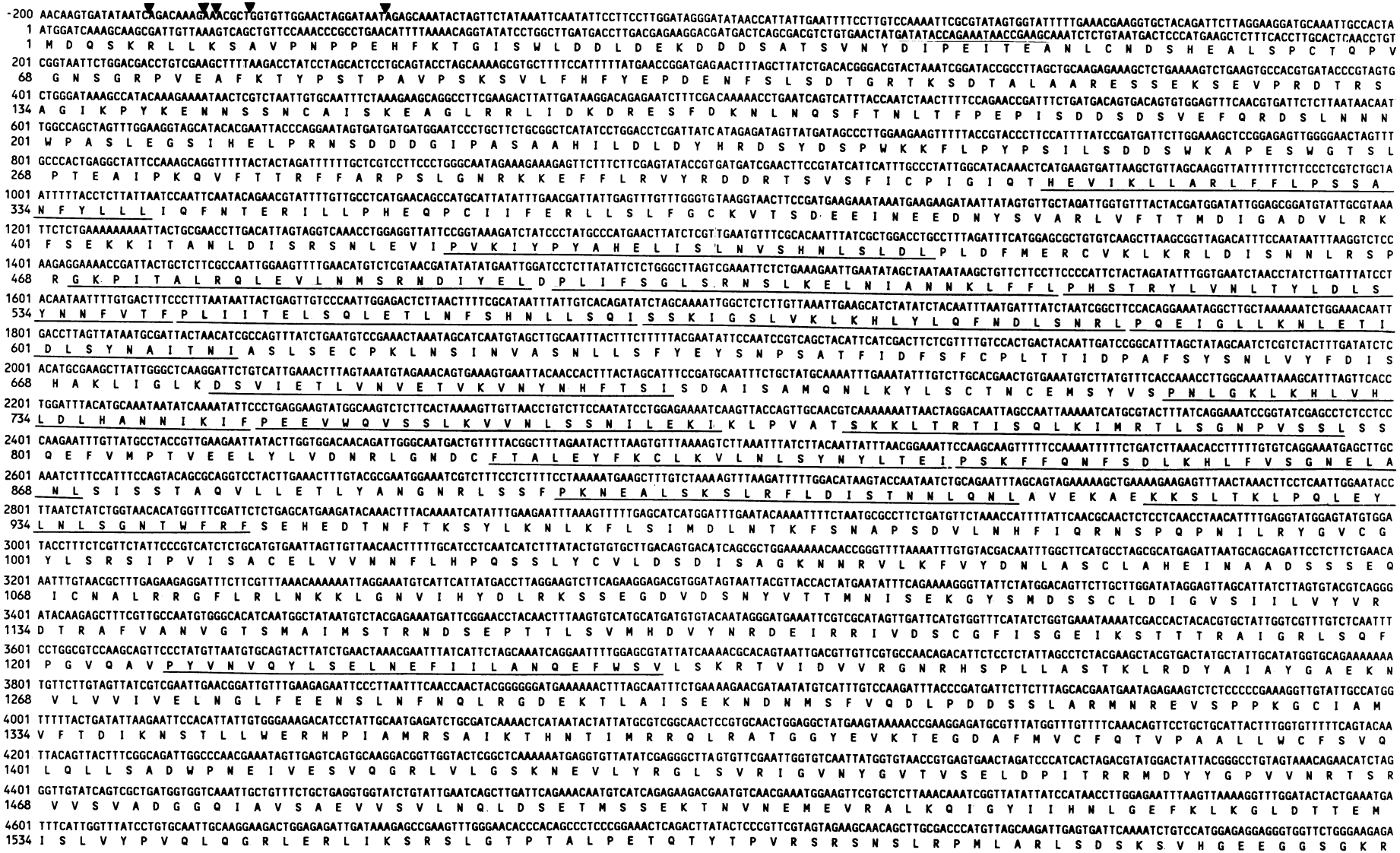

FIG. 2. DNA sequence and deduced amino acid sequence of the *spCYR1* gene. The DNA sequence of the genomic *spCYR1* gene is shown. Deduced amino acid sequence is shown below the nucleotide sequence using the one-letter code. Filled triangles and an open triangle indicate the 5′-ends of the five different upper cDNA clones and the 3′-end of the one lower cDNA clone shown in Fig. 1*A*, respectively. Underlined are repetitive units of 23-amino acid residues that are homologous to the consensus sequence.

Sa. cerevisiae adenylate cyclase gene (*CYR1*) from the yeast alcohol dehydrogenase I (*ADC1*) promoter, was cleaved with *Cla* I and then digested with the BAL-31 exonuclease. DNA fragments carrying deletions were used to reconstruct the *YRp7-ADC1-CYR1* plasmids having deletions. The plasmids were designated as *YRp7-ADC1-CYR1*(x–x), in which x's represented the ranges of deletions in amino acid positions.

Antibody Preparation and Western Blot Analysis. A polypeptide of *Sa. cerevisiae* adenylate cyclase corresponding to the amino acid positions 335–596 was expressed as a fusion protein with *Escherichia coli* β-galactosidase in *E. coli* JM101 by using pUR292 (16). The fusion protein was purified by absorption and elution from Sepharose 4B coupled with anti-β-galactosidase antibody (Promega) and used to immunize rabbits. For Western (immunologic) blotting 50 μg of membrane proteins was electrophoresed on 5% SDS/polyacrylamide gels and transferred to nitrocellulose filters (17, 18). Detection of *Sa. cerevisiae* adenylate cyclase protein was done by using the anticyclase antibody according to the manufacturer's (Promega) instructions.

Assay of Adenylate Cyclase Activity. Yeast cells were grown to the density of 6–10 × 10^6 cells per ml, harvested, and lysed by shaking with glass beads using a Mini Beads-Beater (Biospec Products, Bartlesville, OK). Crude membrane fractions were prepared, and 30 μg of proteins was assayed for adenylate cyclase activity essentially as described (2, 3, 7). Where indicated, 5 μg of purified RAS2 protein (3) was preincubated with 5 μl of 1 mM guanosine 5'-[β,γ-imido]-triphosphate (p[NH]ppG) and added to the reaction mixture as described (3, 7, 15).

RESULTS

Cloning of *Sc. pombe* Adenylate Cyclase Gene. A Southern blot of *Sc. pombe* DNA digested with *Eco*RI was hybridized with a probe covering the catalytic domain of *Sa. cerevisiae* cyclase (a *Pvu* II–*Cla* I fragment) (7) at reduced stringency (see *Materials and Methods*). Two bands, 14 and 4 kilobases (kb) in size, were detected (data not shown). These *Eco*RI fragments were cloned by hybridization screening from a DNA library in which *Eco*RI partial digests of *Sc. pombe* DNA were cloned into λ phage vector EMBL3. The 14-kb *Eco*RI fragment was obtained in a clone containing three *Eco*RI fragments of 5.6, 0.18, and 14 kb and analyzed further. By sequencing 5.3 kb surrounding the hybridizing sequence (see Fig. 1*A*), we found a single long open reading frame designated *spCYR1* that could encode 1692-amino acid residues as shown in Fig. 2. The 4-kb *Eco*RI fragment was also cloned and found by DNA sequencing not to have significant homology with the *Sa. cerevisiae CYR1* gene. Because *Sc. pombe* genomic genes often contain introns, we isolated cDNA clones from a *Sc. pombe* cDNA library by hybridization screening with an internal 5-kb *Bam*HI fragment of the *spCYR1*. The structure of the cDNA clones depicted in Fig. 1*A* was identical with that of the corresponding part of the *spCYR1*, although the cDNAs obtained were split at internal *Eco*RI sites, presumably due to incomplete treatment with

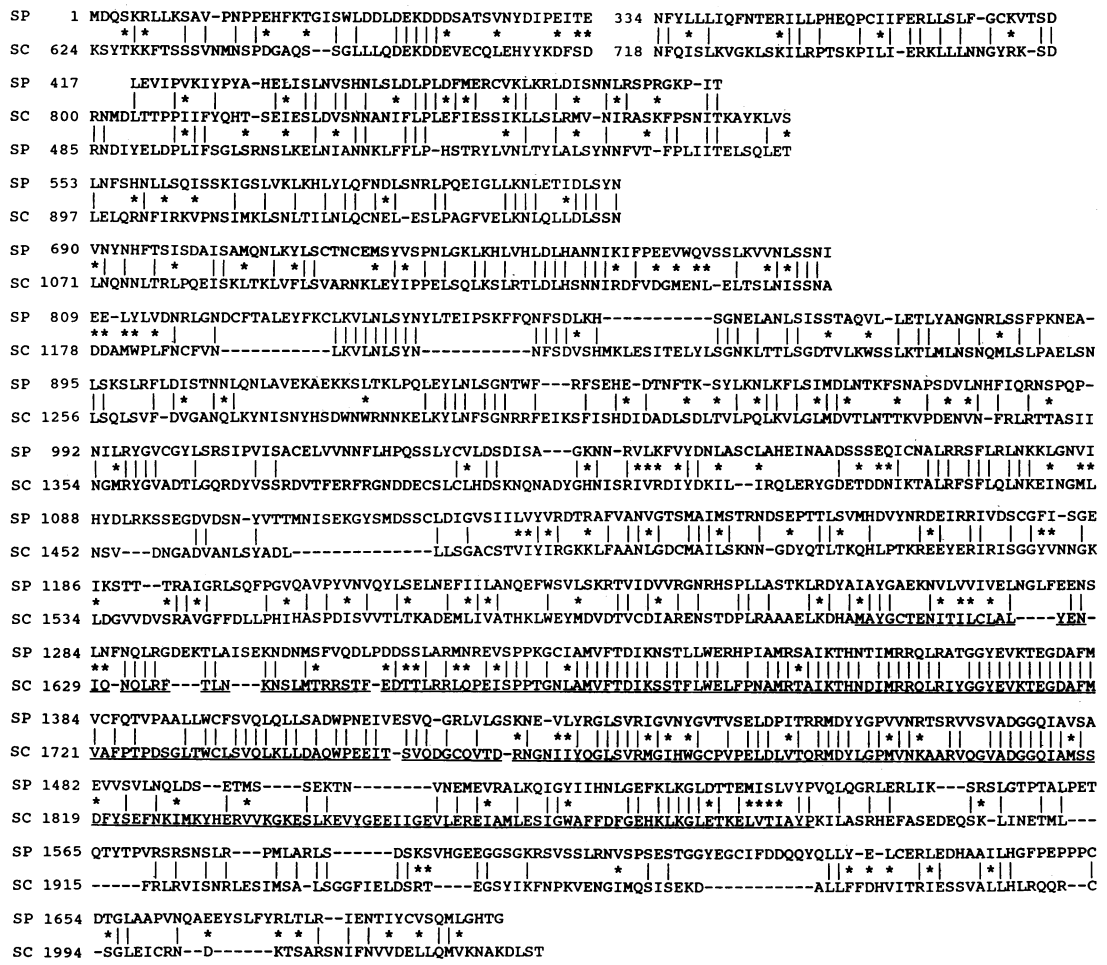

FIG. 3. Protein sequence comparison between *Sc. pombe* (SP) and *Sa. cerevisiae* (SC) adenylate cyclases. The amino acid sequences of regions of *Sc. pombe* and *Sa. cerevisiae* cyclases found to have homology are shown. Vertical lines represent identities, and asterisks represent conservative amino acid substitutions. Dashes indicate insertions introduced for the best alignment. Underlined is the range of the catalytic domain of *Sa. cerevisiae* cyclase described in text.

*Eco*RI methylase upon cloning. Therefore the *spCYR1* gene had no intron in its coding sequence. The molecular size of mRNA derived from the *spCYR1* gene was determined as ≈6 kb (see Fig. 1*B*), which approximately corresponded with the length (5.5 kb) of sequence represented in the cDNA clones.

Structure of the *spCYR1* Gene Product and Its Homology with *Sa. cerevisiae* Adenylate Cyclase. The nucleotide sequence of the *spCYR1* gene and the deduced amino acid sequence are shown in Fig. 2. The protein sequence was compared with that of *Sa. cerevisiae* adenylate cyclase (7), and an alignment that showed the best match is shown in Fig. 3. The result indicated that the *spCYR1* product consisted of four segments having different degrees of homology with *Sa. cerevisiae* cyclase. The highest homology (≈60%) was seen between amino acid positions 1313 and 1540 of the *spCYR1* product and 1650 and 1890 of *Sa. cerevisiae* cyclase. The middle part of the *spCYR1* product (positions 334–1313) was ≈25–40% homologous with the corresponding part of *Sa. cerevisiae* cyclase, although short stretches of nonconserved regions were interspersed. A large portion of this segment was composed of repetitions of a 23-amino acid motif very similar to that seen in *Sa. cerevisiae* cyclase (7) (see Fig. 2). The motif was PXXαXXLXXLXX-LXLXXNXαXXα (P, proline; L, leucine; N, asparagine; α, aliphatic amino acids; and X, any amino acids). As reported, this motif has striking similarity to repetitive motifs identified in human glycoproteins and *Drosophila* proteins (7, 19–23). A segment corresponding to the NH_2-terminal 620 residues of *Sa. cerevisiae* cyclase appeared lost from the *spCYR1* product, whereas ≈280 residues (positions 50–334) of the *spCYR1* product did not have a corresponding sequence in *Sa. cerevisiae* cyclase. The COOH-terminal segments of *Sa. cerevisiae* cyclase (positions 1891–2026) and of the *spCYR1* product (1540–1692) had <25% identity with each other, and introduction of many insertions were required for their alignment. As seen in *Sa. cerevisiae* cyclase, the *spCYR1* product had no typical nucleotide-binding consensus sequences (24) and no particularly hydrophobic segment.

The *spCYR1* Gene Encoded Adenylate Cyclase. The striking homology with the catalytic domain of *Sa. cerevisiae* cyclase strongly suggested that the *spCYR1* gene encoded adenylate cyclase. To prove that, we constructed two plasmids, pAD-*spCYR1*-1 and pAD-*spCYR1*-2, which could express the *spCYR1* product starting from the amino acid position 85 or 1, respectively, under the *ADC1* promoter (see Fig. 4*A*). The plasmids were transformed into the *Sa. cerevisiae* strain, T50-3A (7), which has a temperature-sensitive adenylate cyclase, the *cyr1-2* mutation (25). Leu^+ transformants were obtained at the nonpermissive temperature 34°C on selection plates for leucine auxotrophy supplemented with 1 mM cAMP, which had been shown to rescue the *cyr1-2* mutant at the nonpermissive temperature (25). The transformants were streaked on the cAMP-containing plates, replica-plated on plates with and without cAMP, and incubated at 34°C. As shown in Fig. 4*B*, the *spCYR1* plasmids could suppress temperature sensitivity of T50-3A without cAMP. The colonies were grown at 34°C in the presence of cAMP, and membrane fractions were isolated and assayed for adenylate cyclase activity. The result shown in Table 1 indicated that T50-3A cells having the *spCYR1* expression plasmids gained a low but significant adenylate cyclase activity in the presence of Mn^{2+} or Mg^{2+}. These data lead us to conclude that the *spCYR1* gene encoded adenylate cyclase. However, we could not detect any activation of the *Sc. pombe* adenylate cyclase activity by adding the purified RAS2 protein and p[NH]ppG at the concentrations sufficient to activate *Sa. cerevisiae* cyclase by 50-fold (see Table 1). No activity was detected in soluble fractions. Because the relatively low activity and loss of activation by the RAS2 protein might be due to degradation or improper modification of the *Sc. pombe* cyclase protein in *Sa. cerevisiae* cells, crude membrane was

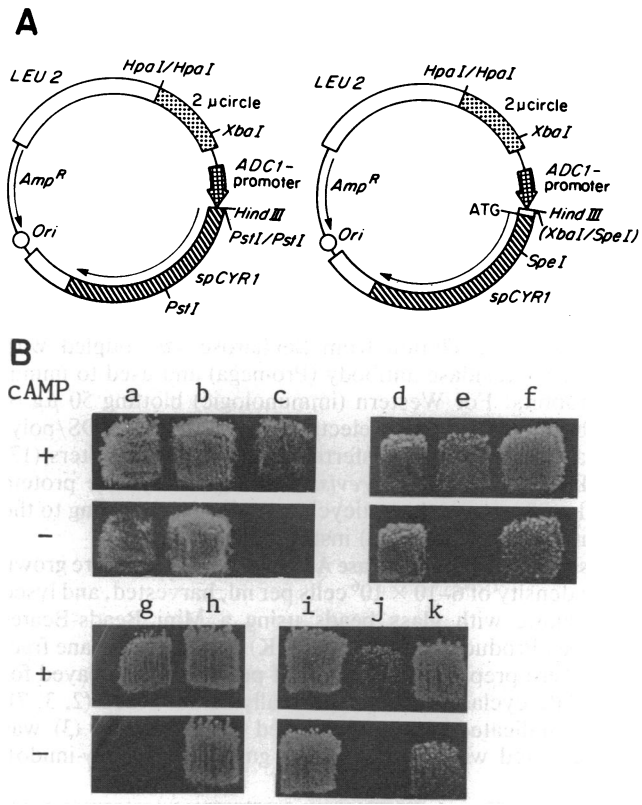

FIG. 4. Complementation of *cyr1-2* mutation by various plasmids. (*A*) Structures of plasmids pAD-*spCYR1*-1 (*Left*) and -2 (*Right*), which express the *spCYR1* gene under the *ADC1* promoter, are shown. Restriction sites in parentheses mean the sites are lost after ligation. (*B*) T50-3A cells transformed with the pAD-*spCYR1*-1 (a), pAD-*spCYR1*-2 (b), pAD-1 (c), *YRp7-ADC1-CYR1*(1916–2026) (d), *YRp7-ADC1-CYR1*(1881–2026) (e), *YRp7-ADC1-CYR1*(1900–2026) (f), *YRp7* (g), *YRp7-ADC1-CYR1*(1932–2026) (h), *YRp7-ADC1-CYR1*(1906–2026) (i), *YRp7-ADC1-CYR1*(1882–2026) (j), and *YRp7-ADC1-CYR1*(1890–2026) (k) were grown on suitable auxotrophic selection plates with and without 1 mM cAMP for 2 days and photographed.

prepared from *Sc. pombe* $972h^{-s}$ cells and assayed for adenylate cyclase activity as shown in Table 1. The adenylate cyclase activity of the *Sc. pombe* cells was low compared with that of *Sa. cerevisiae* and unable to be activated by RAS2 protein plus p[NH]ppG or p[NH]ppG only.

Mapping of Functional Domains of *Sa. cerevisiae* Adenylate Cyclase. The NH_2-terminal border of the catalytic domain

Table 1. Adenylate cyclase activity of various cell membranes

Plasmid	Host cell	Adenylate cyclase activity			
		Mn^{2+}	Mg^{2+}	Mg^{2+} + p[NH]-ppG	Mg^{2+} + p[NH]-ppG + RAS2
pAD-*spCYR1*-1	T50-3A	1.74	0.14	0.12	0.12
pAD-*spCYR1*-2	T50-3A	0.72	0.10	0.11	0.10
pAD-1	T50-3A	0.02	0.00	0.00	0.00
None	$972h^{-s}$	1.21	0.59	0.46	0.37
None	SP-1	9.8	0.19	1.32	4.3
YRp7-ADC1-CYR1	T50-3A	106.1	2.4	NT	117.5
CYR1(1906–2026)*	T50-3A	428.9	9.3	NT	5.2
CYR1(1932–2026)*	T50-3A	178.8	8.8	NT	11.7

All values presented are units of activity where one unit is defined as the production of 1 pmol of cAMP per min per mg of protein. NT, not tested.

*Abbreviation of the corresponding *YRp7-ADC1-CYR1* (deletion) plasmids.

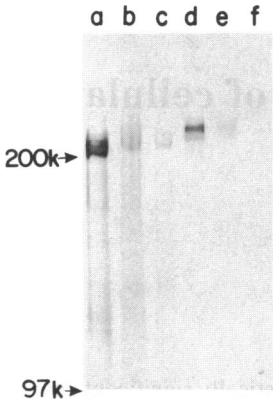

FIG. 5. Detection of adenylate cyclase proteins from deletion mutant plasmids. Membrane fractions isolated from T50-3A cells harboring *YRp7-ADC1-CYR1*(1906–2026) (lane a), *YRp7-ADC1-CYR1*(1932–2026) (lanes b and c), *YRp7-ADC1-CYR1* (lane d), and *YRp7* (lane f), and from a wild-type *Sa. cerevisiae* strain SP1 (7) (lane e) were fractionated in 5% polyacrylamide gel. The Western blot of the gel was subjected to detection of the *Sa. cerevisiae* adenylate cyclase protein as described. The molecular mass markers were myosin heavy chain and phosphorylase b. k, kDa.

had been mapped approximately to the amino acid position 1609 (7). Comparison between the *Sa. cerevisiae* and *Sc. pombe* cyclase protein sequences suggested that the unconserved COOH-terminal 140 residues might not be included in the catalytic domains. Therefore, we introduced step-wise deletions into the COOH-terminal part of *Sa. cerevisiae* cyclase by using exonuclease BAL-31. The deletions started from a *Cla* I cleavage site at amino acid position 1959 and extended to the upstream sequence, and the resultant cyclase genes bearing deletions were expressed under the *ADC1* promoter. The plasmids were transformed into T50-3A strain, and complementation of the *cyr1-2* mutation was examined as described above for the *spCYR1* gene. As shown in Fig. 4*B*, the deletions that extended further upstream than amino acid position 1890 abolished the complementation ability. Therefore, we concluded that the catalytic activity resided in the 280 residues (positions 1609–1890) of *Sa. cerevisiae* cyclase. Some of the *cyr1-2* strains having deletion cyclase plasmids were grown at 34°C, and their membranes were assayed for adenylate cyclase activity. As shown in Table 1, the deletion cyclases failed to be activated by the RAS2 protein and p[NH]ppG even though they retained the Mn^{2+}-dependent activity. Loss of the activation did not appear to be ascribed to degradation of the mutant cyclase protein as shown in Fig. 5, where the mutant cyclase proteins were detected by Western blot analysis using an antibody against *Sa. cerevisiae* cyclase. The T50-3A strain having *YRp7-ADC1-CYR1* overproduced the complete cyclase protein of the estimated M_r of 220,000, which coincided with the predicted value. The cyclase proteins detected in the cells having the mutant plasmids were found to be proportionally smaller depending on the extent of the deletions.

DISCUSSION

The gene encoding adenylate cyclase was cloned from the fission yeast *Sc. pombe* to trace the evolutionary path of adenylate cyclase from *Sa. cerevisiae* to *Sc. pombe*. In contrast to *Sa. cerevisiae* cyclase, *Sc. pombe* cyclase is not likely to be regulated by RAS proteins as suggested by Fukui *et al.* (6) and confirmed in this study. Therefore we think the structural comparison of the two cyclases might give insight into a possible switch in regulatory proteins of adenylate cyclase during evolution. To further the understanding of adenylate cyclase regulation, we have been dissecting the *Sa. cerevisiae* cyclase protein into separate functional domains. The catalytic domain is mapped to 280 residues near the COOH terminus and corresponds exactly to the only region highly conserved between cyclases of the two yeast species. However, much less homology is seen in the remaining parts. Only a moderate degree of conservation exists in the middle segments of ≈1000 residues between *Sa. cerevisiae* and *Sc. pombe* adenylate cyclases. About 600 residues of these segments mainly consist of repetitions of a 23-amino acid motif, which has a homology to 24-amino acid repetitive motifs of human leucine-rich α_2 glycoprotein (19), platelet glycoprotein Ib (20, 21), *Drosophila* chaoptin (22), and the *Toll* gene product (23). Previously we suggested that the repetitive sequence in *Sa. cerevisiae* cyclase might be involved in membrane anchoring (7). However, the cyclase mutants that have lost the whole repetitive region are still recovered in membrane fraction upon expression in yeast (our unpublished data). Therefore, the function of the repetitive sequence is still unknown.

We have been trying to map a domain of *Sa. cerevisiae* adenylate cyclase that is responsible for interaction with RAS proteins. Preliminary mapping showed that a deletion of the NH_2-terminal 1317 residues abolished the activation by RAS2 protein (7). At this point we still do not know whether the NH_2-terminal 620 residues, found to be deleted from *Sc. pombe* cyclase, are indispensible for interaction with RAS proteins. In this paper we found another segment at the COOH-terminal region of *Sa. cerevisiae* cyclase that appears indispensible for interaction with RAS2 protein. Which of the two segments (or both) is directly responsible for interaction with RAS proteins remains uncertain. In any case the two proteins, especially the COOH-terminal one, are much less conserved in *Sc. pombe* cyclase than the catalytic domain. Therefore, in *Sc. pombe* adenylate cyclase the loss of conservation of the regulatory regions might correlate with the loss of interaction with RAS proteins. So far nothing is known about the regulation of *Sc. pombe* adenylate cyclase, especially regarding its regulatory G protein.

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