Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7814–7818, October 1990
Biochemistry

Adenylyl cyclase is dispensable for vegetative cell growth in the fission yeast *Schizosaccharomyces pombe*

(adenylate cyclase/gene disruption/enzyme activity/cAMP depletion/sexual development)

TATSUYA MAEDA, NOBUYOSHI MOCHIZUKI, AND MASAYUKI YAMAMOTO

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, P.O. Hongo, Tokyo 113, Japan

Communicated by Masayasu Nomura, July 19, 1990 (received for review May 4, 1990)

ABSTRACT **Disruption of the *cyr1* gene of *Schizosaccharomyces pombe*, which encodes adenylyl cyclase, did not confer lethality to fission yeast cells, although they grew 40% slower than wild-type strains in complete medium. These cells contained no measurable amount of cAMP and no adenylyl cyclase activity. When h^+ and h^- *cyr1* disruptants were mixed, they underwent mating even in rich medium. Propagation of homothallic *cyr1* disruptants was difficult, probably because such cells readily mate and produce asci and thus stop growing. A >10-fold increase in the amount of *cyr1* mRNA was observed when cloned *cyr1*$^+$ was introduced into *Sch. pombe* cells on a multicopy plasmid. The total adenylyl cyclase activity was similarly high in these transformants. However, the level of intracellular cAMP was hardly affected. Evidence suggests that this was not due to increased phosphodiesterase activity. Thus, cAMP level in growing fission yeast cells appears to be regulated not by the amount of adenylyl cyclase protein but by a feedback mechanism at the enzyme level. The cAMP level fell by ≈50% under nitrogen starvation, which triggers sexual development in *Sch. pombe*. We suggest that fission yeast controls the level of intracellular cAMP primarily to regulate sexual development rather than to drive or arrest the cell cycle.**

Adenylyl cyclase is the key enzyme for biosynthesis of cAMP, which regulates various cellular processes as a second messenger. In the fission yeast *Schizosaccharomyces pombe*, addition of cAMP has been shown to inhibit mating and meiosis (1, 2), primarily by repressing expression of genes required for sexual development (3). The intracellular cAMP level is lowered when *Sch. pombe* is subjected to nitrogen starvation (ref. 4; see *Results*), which is a physiological prerequisite for mating and meiosis in fission yeast (5). Regulation of adenylyl cyclase activity thus appears to be highly relevant to the initiation of sexual development in this organism.

It is, however, unclear whether cAMP plays a role in cell cycle progression in *Sch. pombe*. In the prokaryotic microbe *Escherichia coli*, cAMP regulates catabolite repression but is not essential for cell growth (6). Some prokaryotes have very low cAMP levels, and cAMP appears to stimulate synthesis of proteins that are not always required for cell growth in prokaryotes (7). However, in the simple eukaryote *Saccharomyces cerevisiae*, *cyr1* mutants defective in adenylyl cyclase activity can hardly grow (8), although they may not be completely inviable (9), suggesting that cAMP is important for cell growth in this microbe. Growth of mammalian cells is in general inhibited by a high concentration of cAMP, although conflicting results have been reported (reviewed in refs. 10 and 11). No conclusions can easily be drawn regarding how cAMP participates in control of cell growth in mammals. One reason for this is that experimental conditions have not yet been established where cAMP is completely depleted from mammalian cells. These observations make it worth examining whether adenylyl cyclase is essential for cell growth in another simple eukaryote, *Sch. pombe*, an organism to which various molecular genetic manipulations can be applied. This question may be particularly interesting because conservation of control mechanisms of the cell cycle has been demonstrated between this microbe and mammalian cells (reviewed in ref. 12).

Analysis of *Sch. pombe* adenylyl cyclase is also interesting from another viewpoint. This enzyme is regulated by Ras proteins in *S. cerevisiae* (13). However, mutational analysis of the *Sch. pombe ras1* gene suggested that the Ras protein does not regulate adenylyl cyclase in *Sch. pombe* (4), which also appears to be the case with adenylyl cyclase of higher organisms (14–16). Sequence analyses of the adenylyl cyclase gene from *S. cerevisiae* (17), *Sch. pombe* (18, 19), and human (20) have shown that the two yeast enzymes share some structural features whereas the human one is quite different from them. Thus, analysis of *Sch. pombe* adenylyl cyclase may provide useful information on how Ras proteins and their target molecules came to interact during evolution.

Cloning and sequencing of the gene encoding *Sch. pombe* adenylyl cyclase (*cyr1*) have been reported by two groups (18, 19). We also cloned the same gene and determined its entire sequence (unpublished data). On this basis we set out to disrupt the *cyr1* gene. It turned out that, in contrast to *S. cerevisiae*, loss of adenylyl cyclase activity does not cause severe growth arrest in *Sch. pombe*, although the disruptants grow slower than the wild type. The *cyr1* mutants showed a tendency to enter the sexual reproduction pathway under rich nutritional conditions. It is suggested that the activity of adenylyl cyclase is strictly regulated at the enzyme level in *Sch. pombe* cells.

We were informed during preparation of this manuscript that M. Kawamukai and M. Wigler also reached the conclusion that disruption of *Sch. pombe cyr1* is not lethal (personal communication).

MATERIALS AND METHODS

Strains, Media, and Genetic Methods. Table 1 summarizes the *Sch. pombe* strains used in this study. JZ222 carries the sequence encoding the catalytic region of *S. cerevisiae* adenylyl cyclase (amino acids 1609–2026) under control of the *Sch. pombe adh* promoter inserted at the *leu1* locus. Details of the construction of this strain will be published elsewhere. Complete medium YPD (21) and minimal medium PM (2, 3) were used. PM−N medium lacks a nitrogen source (NH_4Cl). General genetics methods were as described by Gutz *et al.* (22). Transformation and gene replacement of *Sch. pombe* were done as described (4, 23).

Assay of cAMP. Essentially the same procedure was followed to prepare the sample for assay as described before (4). To minimize experimental fluctuations, all samples to be compared were kept frozen in 5% (wt/vol) trichloroacetic

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Table 1. *Sch. pombe* strains used in this study

Strain	Genotype
L975	h^+ wild type
JY333	h^- *ade6-M216 leu1*
JY334	h^+ *ade6-M216 leu1*
JY362	h^+/h^- *ade6-M210/ade6-M216 leu1/leu1*
JY450	h^{90} *ade6-M216 leu1*
JY765	h^+/h^- *ade6-M210/ade6-M216 leu1/leu1 ura4-D18/ura4-D18*
JY919	h^+/h^- *ade6-M210/ade6-M216*
JZ222	h^{90} *ade6-M210 ura4-D18 leu1::{ScCYR1(1609–2026)+ura4+}*
JZ296	h^+/h^- *ade6-M210/ade6-M216 leu1/leu1 ura4-D18/ura4-D18 +/cyr1::ura4+*$_{DAC5}$
JZ298	h^+ *ade6-M216 leu1 ura4-D18 cyr1::ura4+*$_{DAC5}$
JZ300	h^- *ade6-M216 leu1 ura4-D18 cyr1::ura4+*$_{DAC5}$

acid and assayed simultaneously by using the same batch of an assay kit (Amersham). The assay protocol provided by the supplier was followed. The amount of protein was determined according to Lowry *et al.* (24).

Assay of Adenylyl Cyclase Activity. The method developed by Becker *et al.* (25) for *S. cerevisiae* was followed with minor modifications. *Sch. pombe* cells were cultured in PM to a concentration of 0.5–2 × 10^7 cells per ml. After harvest, cells at a concentration of 5 × 10^8 per ml were treated with 30% (vol/vol) dimethyl sulfoxide for 30 min. Adenylyl cyclase activity was measured using 10^7 permeabilized cells per assay. Each assay mixture (100 μl) contained 5 mM $MnCl_2$, 2 units of creatinine kinase, 1 mM [α-^{32}P]ATP, and 10 mM theophylline. The mixtures were incubated at 30°C for 15 min. The assay conditions were otherwise the same as in the original report (25).

DNA and RNA Blotting Analysis. DNA blotting analysis was performed according to Southern (26). RNA blotting analysis was essentially according to Thomas (27), with modifications as described (3).

RESULTS

Cloning of the *Sch. pombe* Gene for Adenylyl Cyclase. Cloning and sequencing of the *Sch. pombe* gene (*cyr1*) encoding adenylyl cyclase have been reported (18, 19). We cloned the same gene independently by the same strategy, that is, by using the *S. cerevisiae CYR1* gene as a probe. The nucleotide sequence of the *cyr1* gene as determined by us completely matches those of the other workers over 6.8 kilobases (data not shown). The original *cyr1* DNA we cloned was a *Bam*HI fragment that covered only the 3′ two-thirds of the gene, and an *Eco*RI fragment was subsequently cloned to cover the 5′ region. A composite segment that encompasses the entire *cyr1* gene was constructed and cloned into the *Sch. pombe–E. coli* shuttle vector pDB248′ (23). The resultant plasmid, pEAC1 (Fig. 1), could complement *cyr1* defects (see below).

Disruption of the *cyr1* Gene *in Vivo*. Disruption of the *cyr1* gene was performed essentially as described (4). Two types of disrupted alleles of *cyr1* were constructed (Fig. 1). The allele in pDAC3 has a central region of *cyr1* replaced by a *Sch. pombe ura4+* cassette (28). The C-terminal 339 amino acids specified by the *cyr1* sequence remaining in pDAC3 had no adenylyl cyclase activity (data not shown), although the catalytic domain of *Sch. pombe* adenylyl cyclase is located in the C-terminal region (refs. 18 and 19; unpublished data). Deletion of *cyr1* is more extensive in pDAC5. The whole coding region except for the N-terminal 10 amino acids has been deleted and *ura4+* inserted instead. The 3′ terminus of the deleted area is only 4 base pairs beyond the termination

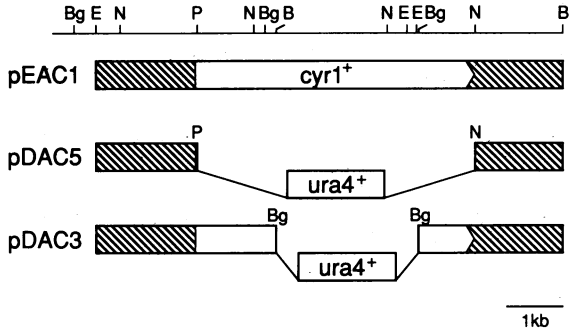

FIG. 1. Illustration of the *cyr1* locus carried on three plasmids. pEAC1 carries the entire *cyr1* gene with its open reading frame shown as an open area. The direction of transcription is from left to right. Both pDAC5 and pDAC3 carry defective *cyr1* alleles disrupted by insertion of a *ura4+* cassette. A restriction map of the *Sch. pombe cyr1* locus is shown at the top. B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; N, *Nsi* I; P, *Pvu* II; kb, kilobase.

codon. The two types of disrupted alleles were introduced into diploid strain JY765, and transformants in which one allele of *cyr1* was properly replaced by the disrupted allele were identified. Since the two types of disruption resulted in the same phenotype, we describe below only the analysis of diploid JZ296, which was obtained with pDAC5 and hence has the larger deletion in *cyr1*.

Southern blotting analysis indicated proper integration of the disrupted allele in JZ296 chromosomes (Fig. 2, lane 2). Accuracy of the integration was confirmed by production of a DNA fragment of the expected length in a polymerase chain reaction in which JZ296 DNA was primed with two 20-mer oligodeoxynucleotides: one was complementary to a 5′ upstream region of *cyr1* not included in pDAC5, and the other was complementary to an internal sequence of the *ura4* cassette (data not shown).

Cells with Disrupted *cyr1* Are Viable. Sporulation was induced in JZ296 and progeny spores were dissected. Many

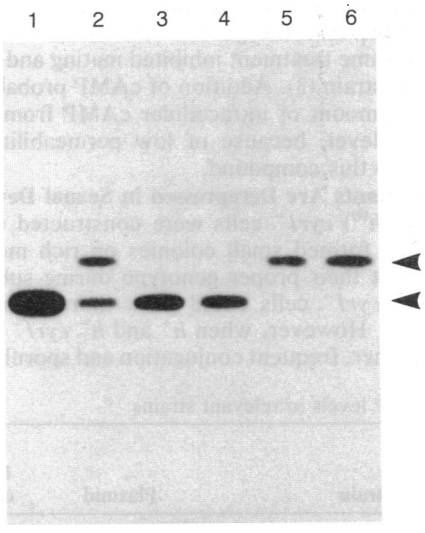

FIG. 2. Southern blotting analysis of *cyr1* disruptants. DNA was extracted from strains indicated below, digested with *Bam*HI and *Bgl* II, electrophoresed in an agarose gel, and probed with a ^{32}P-labeled *Eco*RI–*Pvu* II fragment immediately upstream of the *cyr1* open reading frame (see Fig. 1). Lane 1, L975, a wild-type strain as a control; lane 2, JZ296, a diploid carrying one normal and one disrupted allele of *cyr1*, constructed using pDAC5; lanes 3–6, a complete set of tetrads derived from JZ296. Strains in lanes 3 and 4 were Ura$^-$, whereas those in lanes 5 and 6 were Ura$^+$ and apparently had lost the normal *cyr1* allele. Each lane was loaded with about 0.5 μg of DNA.

Table 2. Adenylyl cyclase activity in *cyr1* disruptants and transformants

Strain	Plasmid	Activity, pmol of cAMP per min per 10^7 cells Exp. 1	Exp. 2
JY333 (*cyr1*$^+$)	None	1.2	1.0
	pDB248′	1.3	1.2
	pEAC1	17.0	18.7
JZ300 (*cyr1*$^-$)	None	<0.1	<0.1
	pDB248′	<0.1	<0.1
	pEAC1	18.6	23.3

tetrads gave four viable spores, two of which were Ura$^+$ and hence possible *cyr1* disruptants. Southern blotting showed that they indeed had the disrupted *cyr1* allele (Fig. 2). To examine whether these cells carried a suppressor mutation that made *cyr1*$^-$ cells viable, they were back-crossed with the wild-type strain. Few inviable segregants appeared in such crosses (data not shown), indicating that *cyr1* disruption does not confer lethality in *Sch. pombe*. This conclusion was further supported by analysis of *cyr1* disruptants constructed independently. The doubling time of the *cyr1*$^-$ haploid strain JZ300 was 178 min at 30°C in YPD medium, whereas that of the wild-type JY333 was 125 min.

Adenylyl Cyclase Activity and cAMP Level in *cyr1*$^-$ Strains. The activity of adenylyl cyclase and the level of intracellular cAMP were measured in haploid *cyr1* disruptants. The adenylyl cyclase activity, maximized by the addition of manganese, was assayed using permeabilized cells. *cyr1*$^-$ cells had no detectable activity of adenylyl cyclase, suggesting that there is no other gene that encodes this enzyme (Table 2). Adenylyl cyclase is therefore dispensable for cell growth in *Sch. pombe*. No cAMP was detected in *cyr1*$^-$ cells (Table 3), indicating that *Sch. pombe* has no bypass reaction for synthesis of cAMP. From these biochemical data we conclude that cAMP is not essential for *Sch. pombe* cell growth, although it is required for maximal growth rate. Addition of 2 mM cAMP and 5 mM caffeine to the medium did not significantly increase the growth rate of *cyr1* disruptants, although the same treatment inhibited mating and meiosis in the wild-type strain (3). Addition of cAMP probably cannot increase the amount of intracellular cAMP from zero to a physiological level, because of low permeability of *Sch. pombe* cells to this compound.

***cyr1* Disruptants Are Derepressed in Sexual Development.** Homothallic (h^{90}) *cyr1*$^-$ cells were constructed by genetic crosses. They formed small colonies on rich medium and apparently lost their proper genotype during subculturing. Heterothallic *cyr1*$^-$ cells could grow more stably, as described above. However, when h^+ and h^- *cyr1*$^-$ cells were cultured together, frequent conjugation and sporulation were

Table 3. cAMP levels in relevant strains

Strain	Plasmid	cAMP, pmol/mg of protein
JZ300 (h^- *cyr1*$^-$)		<0.1
JY450 (h^{90} *cyr1*$^+$)	pDB248′	2.2
	pEAC1	2.1
JY362 (h^+/h^- *cyr1*$^+$/*cyr1*$^+$)	pDB248′	2.4
	pEAC1	2.6
JZ222 (h^{90} *ScCYR1* integrant)	None	13.0
JY919* (h^+/h^- *cyr1*$^+$/*cyr1*$^+$)	None	2.5
JY919, nitrogen starved*	None	1.3

*Cells were grown to 5 × 10^6 per ml in PM medium. Half of the culture was harvested immediately and the remainder was starved for nitrogen for 2 hr before harvest.

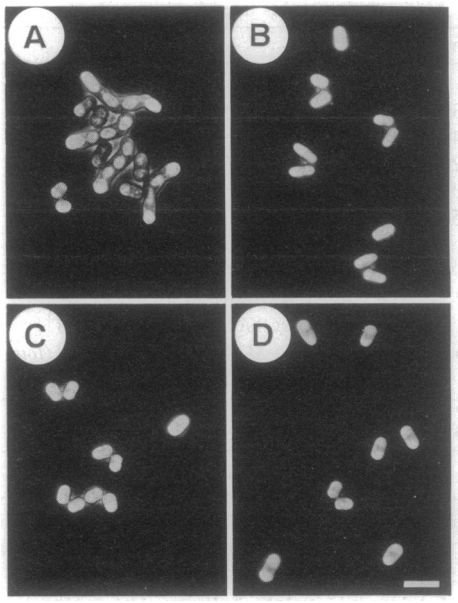

FIG. 3. Mating and sporulation shown by *cyr1*$^-$ strains in the presence of nitrogen. (*A*) JZ298 (h^+ *cyr1*$^-$) × JZ300 (h^- *cyr1*$^-$). (*B*) JZ298 (h^+ *cyr1*$^-$) × JY333 (h^- *cyr1*$^+$). (*C*) JY334 (h^+ *cyr1*$^+$) × JZ300 (h^- *cyr1*$^-$). (*D*) JY334 (h^+ *cyr1*$^+$) × JY333 (h^- *cyr1*$^+$). Each strain was cultured in PM medium to a concentration of 10^7 cells per ml. Aliquots (0.5 ml) of each were mixed pairwise as indicated above and incubated at 28°C for 24 hr with shaking. Mating and sporulation took place under these conditions only when both strains were *cyr1*$^-$ (*A*). (Phase-contrast micrographs; bar = 10 μm.)

observed even in nitrogen-rich medium (Fig. 3). Conjugation did not take place if either of the partners was *cyr1*$^+$ (Fig. 3). Thus, *Sch. pombe* cells apparently become derepressed in sexual development when intracellular cAMP is depleted.

Effects of *cyr1* Gene Dosage and Nitrogen Starvation on the Level of Intracellular cAMP. The effect of an increase in gene dosage of *cyr1*$^+$ was examined. The amount of *cyr1* mRNA increased >10-fold in transformants carrying pEAC1, in both haploid and diploid cells (Fig. 4). An ≈15-fold increase in the adenylyl cyclase activity was observed in a *cyr1*$^+$ strain carrying pEAC1 (Table 2). The adenylyl cyclase activity was similarly high in a *cyr1* disruptant carrying the plasmid (Table 2). Similar gene-dosage effects have been reported for *S. cerevisiae* (29).

Interestingly, no increase in the level of intracellular cAMP was observed in any of the *Sch. pombe* strains transformed with pEAC1 (Table 3). However, the cAMP level was elevated when the catalytic domain of adenylyl cyclase of *S. cerevisiae* was expressed in *Sch. pombe* (Table 3), suggesting that the cAMP level is not strongly regulated by degradation. Thus, a strict feedback mechanism at the enzyme level is likely to regulate the activity of adenylyl cyclase, which can overcome even a 15-fold increase in the amount of enzyme.

Intracellular cAMP in *Sch. pombe* decreased by ≈50% under nitrogen starvation (Table 3). Repeated analysis showed that nitrogen starvation always results in such a decrease, the detailed kinetics of which will be published elsewhere (N.M. and M.Y., unpublished data). Preliminary results indicate that the amount of *cyr1* mRNA is not affected by nitrogen starvation (data not shown), suggesting that the adenylyl cyclase activity is also controlled at the enzyme level in this case, although a small reduction in the amount of *cyr1* mRNA cannot be ruled out.

DISCUSSION

This work has demonstrated that adenylyl cyclase as well as its product, cAMP, are dispensable for cell growth in *Sch.*

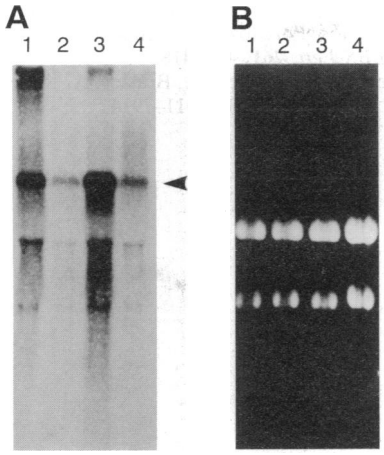

FIG. 4. RNA blotting analysis of the *cyr1* transcripts in transformants carrying multiple copies of *cyr1*. A diploid strain, JY362, and a homothallic haploid strain, JY450, were transformed with either pEAC1 or vector pDB248′. JY362 transformants were grown to 5×10^6 cells per ml in PM medium and harvested, whereas JY450 transformants were grown to 10^7 cells per ml and harvested. Total RNA was extracted from them and was subjected to a standard RNA blotting analysis. Each lane was loaded with 10 μg of RNA. After blotting, the *cyr1* transcripts were probed with the *Bgl* II fragment within the *cyr1* open reading frame (Fig. 1). (*A*) Autoradiograph showing the hybridization patterns. The major transcript of *cyr1* was detected as a band of 5.8 kilobases. (*B*) RNA patterns before blotting, stained with ethidium bromide. Two rRNA bands are seen. Lane 4 may be slightly overloaded, although our staining procedure is not precisely quantitative. Lane 1, JY362 carrying pEAC1; lane 2, JY362 carrying pDB248′; lane 3, JY450 carrying pEAC1; lane 4, JY450 carrying pDB248′.

pombe. This contrasts with the important role of cAMP in cell cycle control in *S. cerevisiae*. These two yeasts species are only distantly related, and the physiological roles of cAMP may have diverged accordingly. From this and previous studies (1–4), we propose that cAMP mainly regulates sexual development in *Sch. pombe*. It has been shown that nitrogen starvation induces a set of genes required for sexual development in *Sch. pombe* and that addition of cAMP represses the induction of these genes (3). Thus, a simple interpretation is that nitrogen starvation results in a reduction in cAMP, which then serves as a signal for induction of such genes.

This work further suggests that *Sch. pombe* controls the cAMP level by modifying the enzymatic activity of adenylyl cyclase. As described in *Results*, it appears unlikely that the level of intracellular cAMP is mainly regulated by the activity of phosphodiesterase, a cAMP-degrading enzyme. We thus assume that *Sch. pombe* has a molecular mechanism that detects nutritional conditions and regulates adenylyl cyclase as an effector. In *S. cerevisiae*, adenylyl cyclase is coupled to Ras proteins and is under their control (13). However, such coupling is unlikely to exist in *Sch. pombe* (4). In mammalian cells, adenylyl cyclase is regulated by heterotrimeric guanine nucleotide-binding proteins G_s and G_i; (reviewed in ref. 30). It is possible that *Sch. pombe* adenylyl cyclase is likewise regulated by G protein(s). However, the structure of *Sch. pombe* adenylyl cyclase is more similar to the *S. cerevisiae* enzyme than to the mammalian enzyme (17–20). This leaves open the question which protein regulates adenylyl cyclase in *S. pombe*.

Adenylyl cyclase from *Sch. pombe* is not activated by the addition of GTP *in vitro*, either in a membrane fraction (19, 31) or in permeabilized cells (31), whereas this enzyme from *S. cerevisiae* can be activated (32). Engelberg *et al.* (31) interpret this observation to suggest that *Sch. pombe* adenylyl cyclase is not regulated by G proteins. We have also failed to activate the enzyme with GTP. However, we interpret these results as an indication that the assay system for *Sch. pombe* adenylyl cyclase *in vitro* does not reflect the precise *in vivo* situation. It appears unlikely that manganese ions, which are currently the only effective activator of *Sch. pombe* adenylyl cyclase *in vitro*, play a key role in regulation of the enzyme activity in response to physiological conditions. Identification and characterization of *Sch. pombe* G proteins should answer this question.

Although *Sch. pombe* cells can survive without any adenylyl cyclase activity, their growth rate is considerably reduced under such conditions. This may mean that adenylyl cyclase is involved in cell cycle control as an auxiliary element. It remains an interesting question whether *Sch. pombe cyr1* disruptants stay longer in a specific phase of the cell cycle.

We thank Dr. Tohru Kozasa for instruction on the adenylyl cyclase assay, Dr. Yoshito Kaziro for helpful discussion, and Dr. David A. Hughes for critical reading of the manuscript. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

1. Calleja, G. B., Johnson, B. F. & Yoo, B. Y. (1980) *Plant Cell Physiol.* **21,** 613–624.
2. Beach, D., Rodgers, L. & Gould, J. (1985) *Curr. Genet.* **10,** 297–311.
3. Watanabe, Y., Iino, Y., Furuhata, K., Shimoda, C. & Yamamoto, M. (1988) *EMBO J.* **7,** 761–767.
4. Fukui, Y., Kozasa, T., Kaziro, Y., Takeda, T. & Yamamoto, M. (1986) *Cell* **44,** 329–336.
5. Egel, R. & Egel-Mitani, M. (1974) *Exp. Cell Res.* **88,** 127–134.
6. Brickman, E., Soll, L. & Beckwith, J. (1973) *J. Bacteriol.* **116,** 582–587.
7. Rickenberg, H. V. (1974) *Annu. Rev. Microbiol.* **28,** 353–369.
8. Matsumoto, K., Uno, I., Oshima, Y. & Ishikawa, T. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2355–2359.
9. Toda, T., Cameron, S., Sass, P. & Wigler, M. (1988) *Genes Dev.* **2,** 517–527.
10. Pastan, I., Johnson, G. S. & Anderson, W. B. (1975) *Annu. Rev. Biochem.* **44,** 491–522.
11. Dumont, J. E., Jauniaux, J.-C. & Roger, P. P. (1989) *Trends Biochem. Sci.* **14,** 67–71.
12. Nurse, P. (1990) *Nature (London)* **344,** 503–508.
13. Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K. & Wigler, M. (1985) *Cell* **40,** 27–36.
14. Beckner, S. K., Hattori, S. & Shih, T. (1985) *Nature (London)* **317,** 71–72.
15. Birchmeier, C., Broek, D. & Wigler, M. (1985) *Cell* **43,** 615–621.
16. Levitzki, A., Rudick, J., Pastan, I., Vass, W. C. & Lowy, D. W. (1986) *FEBS Lett.* **197,** 134–138.
17. Kataoka, T., Broek, D. & Wigler, M. (1985) *Cell* **43,** 493–505.
18. Yamawaki-Kataoka, Y., Tamaoki, T., Choe, H.-R., Tanaka, H. & Kataoka, T. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 5693–5697.
19. Young, D., Riggs, M., Field, J., Vojtek, A., Broek, D. & Wigler, M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 7989–7993.
20. 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.
21. Sherman, F., Fink, G. R. & Hicks, J. B. (1986) *Methods in Yeast Genetics: Laboratory Course Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
22. Gutz, H., Heslot, H., Leupold, U. & Loprieno, N. (1974) in *Handbook of Genetics*, ed. King, R. D. (Plenum, New York), Vol. 1, pp. 395–446.
23. Beach, D., Piper, M. & Nurse, P. (1982) *Mol. Gen. Genet.* **187,** 326–329.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
25. Becker, J. M., Enari, E. & Levitzki, A. (1988) *Biochim. Biophys. Acta* **968,** 408–417.
26. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.

27. Thomas, P. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5201–5205.
28. Grimm, C., Kohli, J., Murray, J. & Maundrell, K. (1988) *Mol. Gen. Genet.* **215,** 81–86.
29. Casperson, G. F., Walker, N. & Bourne, H. R. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 5060–5063.
30. Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56,** 615–649.
31. Engelberg, D., Poradosu, E., Simchen, G. & Levitzki, A. (1990) *FEBS Lett.* **261,** 413–418.
32. Casperson, G. F., Walker, N., Brasier, A. R. & Bourne, H. R. (1983) *J. Biol. Chem.* **258,** 7911–7914.