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Genetics

Isolation of the gene encoding adenylate cyclase in *Saccharomyces cerevisiae*

(*CYR1*/yeast/start/cell cycle)

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ABSTRACT **By complementation of the *cyr1-1* mutation in *Saccharomyces cerevisiae*, we have isolated yeast genomic DNA containing the structural gene that encodes the catalytic unit of adenylate cyclase (EC 4.6.1.1). The isolated DNA restored adenylate cyclase activity to *cyr1-1* mutants and directed integration at the *CYR1* locus. Wild-type strains transformed with *CYR1* DNA on the high copy number vector YEp24 contained 4- to 6-fold more adenylate cyclase activity than strains carrying the plasmid with no insert. This result suggests that expression of the *CYR1* gene product, rather than that of other polypeptide components of the adenylate cyclase system, limits total adenylate cyclase activity in *S. cerevisiae*. *CYR1*-containing plasmids also complemented the temperature-sensitive growth defect of the cell division cycle mutation *cdc35-1*, which confers a phenotype under restrictive conditions similar to that of *cyr1-1* and maps to the same locus. Further, *cdc35-1 cam* mutants, which contain mutations that enable them to take up cAMP from the medium, grew at the restrictive temperature in the presence of exogenous cAMP. These observations support the view that *CDC35* and *CYR1* are allelic and confirm the hypothesis that cAMP synthesis is required for cells to pass through the "start" position of the cell division cycle.**

Adenosine 3',5'-monophosphate (cAMP) is thought to play at least two important regulatory roles in the yeast *Saccharomyces cerevisiae*: As a negative regulator of sporulation in **a**/α diploids (1) and as a positive regulator at the "start" position of the cell division cycle (2). The adenylate cyclase (EC 4.6.1.1) system of *S. cerevisiae* resembles that of mammalian cells. The yeast enzyme synthesizes cAMP from either MgATP or MnATP; GTP and its hydrolysis-resistant analogs guanosine 5'-[β,γ-imido]triphosphate (p[NH]ppG) and guanosine 5'-[γ-thio]triphosphate (GTP[γS]) stimulate cAMP synthesis via a membrane protein or proteins distinct from the catalytic unit of adenylate cyclase (3). The regulatory components of yeast adenylate cyclase probably include either or both of the *RAS1* and *RAS2* products (4), which were isolated by hybridization with a viral *ras* probe (5, 6).

Isolation of yeast strains with mutations in the gene (*CYR1*) encoding the catalytic unit of adenylate cyclase (7, 8) made this protein accessible to study by genetic and molecular biological techniques. Here we report the molecular cloning of genomic DNA containing the *CYR1* gene.

MATERIALS AND METHODS

Strains, Plasmids, and Media. Table 1 shows the yeast strains used. Strain GC26-7B was derived from a cross between HR125-5Da and AM18-5C, followed by two backcrosses of *cyr1-1* segregants to HR125-5Da or 1369. NW23-6D and NW23-9C were similarly obtained from a cross between GC26-7B and 1369 and two additional backcrosses of *cyr1-1* segregants to HR125-5Da or 1369. NW56-8A was obtained as an Ade$^-$ Cyr$^+$ *cam* segregant from a cross between AM3-4B and NW23-9C with three subsequent backcrosses of such segregants to NW23-6D or NW23-9C. NW33-1 was a *ura3-52 cdc35-1* segregant from a cross between BR314-4A and 1369. The *Escherichia coli* strain was HB101.

Plasmids used were YEp24 [pBR322 containing the yeast *URA3* gene and a portion of yeast 2-μm DNA (10)] and YIp5 [pBR322 containing the yeast *URA3* gene (11)].

Rich medium for cultivation of yeast was YEPD (1% yeast extract/2% Bacto-peptone/2% glucose). Defined medium was 6.7 g of yeast nitrogen base without amino acids per liter and 2% glucose, supplemented with appropriate factors to support growth of auxotrophic strains. Solid medium contained 2% Bacto-agar. Media for growth of *cyr1-1* mutants were supplemented with 2 mM cAMP.

Preparation of Yeast Particulate Extracts. Strains to be tested for adenylate cyclase activity were grown in defined medium lacking uracil to maintain the *URA3* plasmids. Cells (5×10^8) were harvested at a density of $1–2 \times 10^7$ per ml, washed once with distilled water, once with YMB [100 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.0/0.2 M sorbitol/0.1 mM EDTA] and resuspended in 2 vol of YMB+PI (YMB/1.0 mM phenylmethylsulfonyl fluoride/75 μg of leupeptin per ml) per vol of packed cells. One volume of acid-washed glass beads (B. Braun Melsungen, 0.45–0.50 mm, from Sargent–Welch) was added and the samples were swirled on a Vortex mixer in 1.5-ml centrifuge tubes for 4 min in 30-s cycles. Tubes were kept on ice between cycles. The lysate/glass bead mixture was diluted to 1.5 ml with YMB+PI, the lysate was removed, and the particulate fraction was collected by centrifugation for 15 min in an Eppendorf centrifuge. The pellet was resuspended in 1 ml of YMB+PI and stored at −70°C.

Assays. Adenylate cyclase activity was determined as previously described (3) except that 100 mM Mes served as buffer. Briefly, reaction mixtures consisted of 100 mM Mes at pH 6.0, bovine serum albumin at 0.1 mg/ml, 0.1 mM EGTA, 20 mM creatine phosphate, creatine kinase at 20 units/ml, 2.0 mM 2-mercaptoethanol, 0.4 mM [α-^{32}P]ATP (50–200 cpm/pmol), and 1.0 mM [^{3}H]cAMP (20,000 cpm). The reaction was started by addition of 100 μg of particulate extract and incubated at 30°C. After 30 min the reaction was stopped and [^{32}P]cAMP formed was determined (12).

Protein was determined as described (13).

Enzymes and Reagents. Reagents for adenylate cyclase assays and other chemicals were from Sigma and were of the highest purity available. Media components were from Difco. Restriction endonucleases and T4 DNA ligase were obtained

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Abbreviations: p[NH]ppG, guanosine 5'-[β,γ-imido]triphosphate; Ade$^-$, adenine-dependent; Ura$^+$, uracil-independent; Cyr$^+$, cAMP-independent; kb, kilobases.

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Table 1. Yeast strains

Strain	Source*	Genotype
HR125-5Da	1	**a** *leu2-3 leu2-112 ura3-52 his3-532 his4 trp1-1*
1369	1	α isogenic to HR125-5Da
AM7-11D	2	α *ade6 ade10 amp1 cam1 cam2 cam3*
AM18-5C	3	α *cyr1-1 cam1 cam2 cam3*
BR214-4a	4	**a** *cdc35-1 ade1 his7 ura1 trp1 arg4*
GC26-7B	5	**a** *cyr1-1 cam*† *leu2-3 leu2-112 ura3-52 his3-532 his4*
NW23-6D	5	α *cyr1-1 cam leu2-3 leu2-112 ura3-52 his3-532 his4*
NW23-9C	5	**a** *cyr1-1 cam leu2-3 leu2-112 ura3-52 his3-532 his4 trp1-1*
NW56-8A	5	α *cam ade10 leu2-3 leu2-112 ura3-52 his3-532 his4*
NW33-1	5	α *cdc35-1 ura3-52*
TC20-11-5	5	**a** *cyr1-1*::YIp5:RR1(*URA3*) *ura3-52 leu2-3 leu2-112 his3-532 his4 trp1-1*

*Sources: 1, I. Herskowitz and R. Jensen; 2, K. Matsumoto (9); 3, K. Matsumoto (7); 4, L. Hartwell; 5, this study (see *Materials and Methods*).

†The *cam* mutations allow yeast cells to take up and utilize exogenous cAMP (9). Although all our strains designated as bearing *cam* mutations are capable of taking up exogenous cAMP, we are not certain how many *cam* mutations are required to produce this phenotype. Matsumoto *et al.* (9) reported the presence of three distinct *cam* mutations in AM3-4B, from which these strains were derived. However, in the segregants of a cross between NW56-8A and NW23-6D, many of the adenine-dependent (Ade^-) strains could not utilize cAMP as an adenine source. This was true of both *cyr1-1* and cAMP-independent (Cyr^+) strains (unpublished observation). We thus indicate presence of mutations conferring the ability to take up exogenous cAMP as *cam* without commenting as to their number.

from New England Biolabs or Bethesda Research Laboratories and were used according to suppliers' specifications.

RESULTS

Isolation of Genomic DNA Fragments that Complement the *cyr1-1* Mutation. *cyr1-1* mutants require cAMP for growth (9). Complementation of this defect allowed us to clone the *CYR1* gene. Strain GC26-7B (*cyr1-1 ura3-52*), which required both uracil and cAMP for growth, was transformed (14) with a clone bank of yeast genomic DNA fragments inserted into the *URA3* vector YEp24 (15). Uracil-independent (Ura^+), Cyr^+ transformants were selected in a single step by plating on medium lacking both uracil and cAMP. Of approximately 15,000 Ura^+ transformants (determined by titering on plates lacking uracil but containing cAMP), six grew on plates lacking cAMP.

For each of the six Cyr^+ transformants (designated TC1 through TC6), complementation of the *cyr1-1* mutation required the presence of the *URA3* plasmid. Loss of the plasmid by mitotic segregation (detected by a return to uracil auxotrophy) invariably resulted in a return to the Cyr^- phenotype. DNA isolated from transformants TC1 through TC6 transformed *E. coli* to ampicillin resistance. The recovered plasmids (denoted YEp24:CYR1-1 through -6, derived from TC1 through -6, respectively) transformed GC26-7B to Ura^+ and, in each case, to Cyr^+.

Restriction endonuclease mapping of the recombinant plasmids (Fig. 1) revealed five independent, overlapping genomic inserts; YEp24:CYR1-2 and YEp24:CYR1-4 appeared identical. Inserts ranged in size from 8.5 to 10 kb and shared a common region of 8 kb. This indicates that the inserts are derived from the same chromosomal locus and that the complementing activity is probably encoded by a gene within the common region.

In addition to requiring cAMP for growth, *cyr1-1* mutants lack detectable adenylate cyclase activity (3, 7). If the recombinant plasmids contain the *CYR1* gene, they should also restore adenylate cyclase activity to *cyr1-1* mutants. Indeed, *cyr1-1* mutants harboring each of the six recombinant plasmids also contained detectable adenylate cyclase activity, whereas mutants carrying only the vector YEp24 did not (Table 2). In addition, Cyr^+ strains carrying the recombinant plasmids contained 4- to 6-fold more adenylate cyclase activity than did strains containing YEp24 (Table 2).

Cloned DNA Directs Integration at the *CYR1* Locus. Complementation of the Cyr^- phenotype, restoration of adenylate cyclase activity in *cyr1-1* mutants, and increased levels of adenylate cyclase activity in Cyr^+ strains harboring the recombinant plasmids strongly suggested that the plasmids contain *CYR1* DNA. To test this hypothesis, we determined the site of chromosomal integration directed by a fragment of the isolated DNA.

We subcloned an *Eco*RI fragment from the common region of the genomic insert of YEp24:CYR1-2 in the integrating *URA3* vector YIp5. This plasmid was designated YIp5:RR1 (see Fig. 1). YIp5:RR1 transformed NW23-9C (**a** *cyr1-1 ura3-52*) to Ura^+ and the transformants retained the Cyr^- phenotype. The transformants were mated with NW56-8A (α Cyr^+ *cam*); the resulting diploids were induced to sporulate and the asci were dissected. Of nine complete tetrads analyzed all were parental ditypes (2 Ura^+ Cyr^-: 2 Ura^- Cyr^+). In 64 additional tetrads with less than four viable segregants the Ura^+ and Cyr^- phenotypes cosegregated, as did the Ura^- and Cyr^+ phenotypes, in 126 of 127 segregants (Table 3). For 42 of the incomplete tetrads, genotypes could be assigned to the inviable spores on the basis of the genotypes of viable segregants from the same ascus and assuming Mendelian segregation of *CYR1* and *URA3*. Of

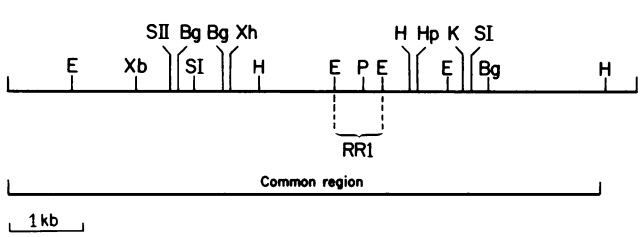

FIG. 1. Restriction endonuclease cleavage sites in the genomic DNA insert of YEp24:CYR1-2. Abbreviations are: E, *Eco*RI; H, *Hin*dIII; Bg, *Bgl* II; SI, *Sst* I; SII, *Sst* II; Xb, *Xba* I; P, *Pvu* I; Xh, *Xho* I; Hp, *Hpa* I; K, *Kpn* I. RR1 designates the *Eco*RI fragment subcloned in YIp5 to produce the plasmid YIp5:RR1. Common region indicates the area shared by all five recombinant plasmids isolated. Enzymes with no cleavage sites in this insert include *Sal* I and *Pvu* II. kb, kilobases.

Table 2. Adenylate cyclase activity in particulate extracts of strains carrying recombinant plasmids

Strain	Plasmid	cAMP formed, pmol/30 min per mg of protein*		
		Mg^{2+}	Mg^{2+} p[NH]ppG	Mn^{2+}
HR125-5Da (Cyr^+)	YEp24:CYR1-1	204	702	471
	YEp24:CYR1-2	252	843	525
	YEp24:CYR1-3	102	342	600
	YEp24:CYR1-5	180	690	510
	YEp24:CYR1-6	240	819	600
	YEp24	45	186	102
NW23-9C (*cyr1-1*)	YEp24:CYR1-1	174	543	423
	YEp24:CYR1-2	189	645	336
	YEp24:CYR1-3	84	297	318
	YEp24:CYR1-5	132	483	324
	YEp24:CYR1-6	42	198	51
	YEp24	<1.5	<1.5	<1.5

*Assays were performed as described in *Materials and Methods* with additions as shown above: Mg^{2+}, 10 mM $MgCl_2$; p[NH]ppG, 100 μM; Mn^{2+}, 5 mM $MnCl_2$. Values are means of three or four determinations. Standard errors were less than 10% of the mean in each case.

these asci, 41 were parental ditypes and one was a tetratype. These results indicated that the genomic DNA fragment in YIp5:RR1 directed integration at the *CYR1* locus.

Rescue of a *cdc35* Mutant by cAMP and *CYR1* DNA. Mutation at *CDC35* results in a phenotype similar to that of *cyr1-1*: cells with mutations in either gene become arrested in G_1 under restrictive conditions (2, 7, 16). **a**/α diploids homozygous for mutations in either *CDC35* or *CYR1* are capable of sporulating in either rich medium or sporulation medium under restrictive conditions (1, 17). Further, mutations in *CDC35* and *CYR1* map near the same chromosomal locus (7, †). We therefore asked whether *cdc35-1* temperature-sensitive mutants could be rescued by exogenous cAMP or *CYR1*-containing plasmids.

NW33-1 (α *cdc35-1*) was mated to GC26-7B (**a** *cam*). The resulting diploid (NW40) was induced to sporulate and the asci were dissected. One of three temperature-sensitive segregants was capable of growth at the restrictive temperature (35°C) in the presence of cAMP. Poor viability of segregants from this cross precluded a more complete analysis.

To test for complementation of the temperature-sensitive growth defect of *cdc35-1* by *CYR1* DNA, NW33-1 (*cdc35-1 ura3-52*) was transformed with YEp24:CYR1-2. All Ura^+ transformants were capable of growth at both 25°C and 35°C. The ability of the transformants to grow at 35°C required the presence of the plasmid: of 40 clones that lost the plasmid by mitotic segregation (i.e., that became Ura^-), all were temperature sensitive.

DISCUSSION

We report here molecular cloning of genomic DNA containing the *CYR1* gene of *Saccharomyces cerevisiae*. The isolated DNA complemented the cAMP requirement of *cyr1-1* mutants, restored their adenylate cyclase activity, and directed integration at the *CYR1* locus.

Matsumoto and co-workers (7, 8) concluded that *CYR1* encodes the catalytic unit of adenylate cyclase, on the basis of two observations: (*i*) In gene dosage experiments, adenylate cyclase activity in particulate extracts increased with the number of wild-type *CYR1* alleles in tetraploid strains; (*ii*) *cyr1-2* temperature-sensitive mutants contained thermolabile adenylate cyclase activity (8). In agreement with this conclusion, particulate extracts of haploid Cyr^+ strains transformed with the isolated DNA on a high copy number plasmid contained 4- to 6-fold more adenylate cyclase activity than those carrying only the YEp24 vector (Table 2).

Table 3. Segregation of *cyr1* and *ura3*

Ascus type	No. of asci				
	Total	No. of viable spores in ascus			
		4	3	2	1
Parental ditype	50	9	14	27	0
Tetratype	1	0	1	0	0
Nonparental ditype	0	0	0	0	0
Unassigned	22	0	0	6	16

NW59 [**a**/α *cyr1-1*::YIp5:RR1(*URA3*)/+ *ura3/ura3 cam/cam*] was induced to sporulate and the asci were dissected. Genotypes of nonviable spores were assigned on the basis of the genotypes of the viable segregants from the same ascus, assuming Mendelian segregation of *cyr1* and *ura3*. Those asci for which genotypes could not be unambiguously assigned to the nonviable spores are listed as unassigned. All viable segregants from the unassigned tetrads were *cyr1* Ura^+ or Cyr^+ *ura3*.

The latter result suggests an additional conclusion with respect to the stoichiometry of polypeptide components of the adenylate cyclase system in normal yeast cells: the regulatory components of the adenylate cyclase system are present in excess relative to the *CYR1* gene product. Particulate extracts of strains carrying the *CYR1* gene in high copy number [presumably 30–50 copies per cell (18)] showed increased adenylate cyclase activity with MgATP or MnATP as substrate. The amount of this increase was approximately the same with either substrate, and guanine nucleotide stimulation (measured in the presence of Mg^{2+}) also increased proportionately (Table 2). The catalytic unit of adenylate cyclase probably utilizes MnATP as substrate independent of the guanine nucleotide-binding regulatory protein (G), whereas utilization of MgATP and guanine nucleotide regulation depend upon interaction of the catalytic unit with G (3, 4). In this context, the adenylate cyclase measurements (Table 2) suggest that the guanine nucleotide-binding regulatory components of yeast adenylate cyclase are present in excess relative to the polypeptide encoded by *CYR1*.

The cell cycle mutation *cdc35-1* causes phenotypes resembling those of *cyr1-1* in the following respects (1, 2, 16, 17): Haploids become arrested as unbudded, nongrowing cells in G_1, and homozygous diploids sporulate in rich medium containing nutrients that prevent sporulation of wild-type cells. Apparent complementation of the *cdc35-1* mutation by *CYR1* DNA and rescue of *cdc35-1* mutant cells by exogenous cAMP strongly suggest that *CDC35* and *CYR1* are allelic. If *CDC35* and *CYR1* are alleles, *cdc35-1* mutants might be expected to show diminished or temperature-sensitive adenylate cyclase activity *in vitro*. This is the case for temperature-sensitive *cyr1-2* mutants, which exhibited no adenylate cyclase activity when grown at 35°C and only 19% of wild-type activity when grown at 25°C (8). Extracts of *cdc35-1* mutants, however, showed approximately wild-type amounts of adenylate cyclase activity, whether grown at 25°C or arrested by incubation at 35°C (unpublished observation).

Despite this result, *CDC35* will probably prove to be allelic to *CYR1* or to encode a component of the adenylate cyclase system of yeast. Because of instability of the *cdc35-1* allele in

†Boutelet, F. & Hilger, F., Tenth International Conference on Yeast Genetics and Molecular Biology, Sept. 8–12, 1980, Louvain-la-Neuve, Belgium, p. 177 (abstr.).

diploids, we have been unable to perform complementation tests.

Two additional genes may encode components of the adenylate cyclase system of yeast: *TSM0185*, recently isolated by Masson *et al.* (19), and *CDC25*. Like *CDC35*, mutations in *TSM0185* map to the *CYR1* locus.† *CDC25* mutants share the phenotypes of G_1 arrest and hypersporulation (16, 17) with *CDC35* and *CYR1* mutants. Identification of the biochemical lesions in such mutants will advance our understanding of the regulation of both the cell cycle and sporulation.

In addition to aiding in elucidation of important yeast cell functions, isolation of the yeast adenylate cyclase gene may open avenues to more precise understanding of the cognate gene product in mammalian cells. Mammalian adenylate cyclase, a pivotal enzyme in neural and endocrine regulation, has so far resisted biochemical purification (20). Despite elegant biochemical characterization of the purified stimulatory and inhibitory GTP-binding components of the mammalian enzyme, G_s and G_i (21–23), little is known of the molecular mechanisms by which these regulatory proteins control activity of the catalytic unit. *CYR1* DNA may prove useful as a probe for isolating cognate genes from other organisms. Furthermore, the nucleotide sequence of the *CYR1* coding region (and the deduced amino acid sequence of the encoded protein) will provide structural information about the catalytic protein not readily obtainable by other means. This structural information will be of particular interest to investigators of mammalian adenylate cyclase.

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