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Yeast *cdc35* mutants are defective in adenylate cyclase and are allelic with *cyr1* mutants while *CAS1*, a new gene, is involved in the regulation of adenylate cyclase

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Newly isolated temperature-sensitive *cdc35* mutants of *Saccharomyces cerevisiae* have been characterized. They show the morphology, growth and conjugation characteristics typical of class-A or class-II start mutants. The *cdc35* mutation induces a significant decrease of the intracellular cAMP level and produces a thermolabile adenylate cyclase. By classical genetic criteria the *CDC35* gene is identical with the structural gene of adenylate cyclase, *CYR1*. The results of the mutant selection, the kinetics of macromolecule accumulation and the cell-density change of *cdc35* mutants at the restrictive temperature, indicate that the *CDC35* function may not be cell cycle-specific. A new mutation, *cas1*, was isolated and partially characterized. It mediates the suppression by external cAMP of the unlinked *cdc35* mutation. It causes a slight increase of the intracellular cAMP level and has strong effects on the adenylate cyclase activities, especially on the Mg^{2+}-dependent activity. The data suggest that the *CAS1* protein is a controlling element of adenylate cyclase. The *CAS1* locus is different from the *RAS1* and *RAS2* loci.

Key words: adenylate cyclase/*CAS1*/*CDC35*/*CYR1*/yeast

Introduction

Cell-division-cycle (*cdc*) mutants have provided a major contribution to our knowledge of the process of cellular reproduction in the budding and fission yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, which, in several respects, serve as model systems for investigating the division cycle of the eucaryotic cell (Pringle and Hartwell, 1981; Fantes, 1984). Approximately 60 *S. cerevisiae CDC* genes have been identified (Pringle and Hartwell, 1981; Bedard *et al.*, 1981) on the basis of the behaviour of conditional, mostly temperature-sensitive (*ts*) mutants.

Although study of these mutants has characterized many individual cycle events and allowed us to analyze the controls which regulate their occurrence, only in a few cases has the gene product, altered by a *cdc* mutation, been identified and its physiological and biochemical roles been determined. This was the case for the *CDC8, CDC9, CDC19* (allele of *PYK1*) and *CDC21* (allele of *TMP1*) genes which have been cloned and shown to encode thymidylate kinase (Jong *et al.*, 1984), a DNA ligase (Johnston and Nasmyth, 1978; Barker and Johnston, 1983), pyruvate kinase (Kawasaki, 1979; Burke *et al.*, 1983) and thymidylate synthetase (Game, 1976; Bisson and Thorner, 1977; Taylor *et al.*, 1982), respectively. Four 'start' genes *CDC28, CDC36, CDC37* and *CDC39* have been isolated and their transcriptional products have been characterized (Nasmyth and Reed, 1980; Reed *et al.*, 1982; Breter *et al.*, 1983). The amino acid sequence of the*CDC28* protein, as deduced from the DNA sequence, suggests that it is a protein kinase (Lörincz and Reed, 1984).

Analysis of yeast mutants altered in the production of cyclic AMP (cAMP) and the activity of cAMP-dependent protein kinase, indicates that cAMP-dependent phosphorylation of certain, unidentified, key proteins is required to allow progression of cells through the G1 phase. Cell populations of the cAMP-requiring mutants *cyr1* and *cyr2*, which are altered in adenylate cyclase and in the catalytic subunit of cAMP-dependent protein kinase, respectively, are arrested as unbudded, uninuclear G1 cells, in the absence of external cAMP, at the temperature where the mutations are expressed (Matsumoto *et al.*, 1982a, 1983, 1984; Uno *et al.*, 1983). The same kind of cell cycle arrest, although less conspicuous, is caused by mutations in a third gene, *CYR3*; these mutations also result in a cAMP requirement and they affect the regulatory subunit of protein kinase (Uno *et al.*, 1982). Two other yeast genes, *RAS1* and *RAS2*, which are related to the *ras* genes of mammalian cells, are also involved in cAMP metabolism. Strains in which both *RAS1* and *RAS2* have been disrupted, resemble *cyr1* mutants in several aspects. Membranes from these mutants lack a GTP-dependent activity which would stimulate adenylate cyclase (Toda *et al.*, 1985).

Here we characterize a mutation which is allelic with both *cdc35* and *cyr1*. We also report the isolation and preliminary characterization of mutations which mediate suppression of *cdc35* and *cyr1* by external cAMP and which appear to affect a component of the adenylate cyclase regulatory system.

Results

Isolation and identification of cdc35 mutants

In a search for G1-arrested mutants, we screened a set of 300 newly isolated *ts* mutants by time-lapse photomicroscopy (Hartwell *et al.*, 1970) and by complementation tests with G1-arrested mutants of the classes A (*cdc19, cdc25, cdc33* and *cdc35*) and B (*cdc28, cdc36, cdc37* and *cdc39*) (Pringle and Hartwell, 1981). Our mutants had been isolated by the nystatin-enrichment procedure (Thouvenot and Bourgeois, 1971), after X-ray mutagenesis; the permissive and restrictive temperatures were 26°C and 36°C, respectively.

Alleles of each of the four class A mutants were found; among these were eight independently isolated alleles of *cdc35*. No class B mutant allele was detected. Four non-allelic mutants, complementing all of the class A and B mutants, showed first cycle arrest as unbudded cells. One of these is deficient in histidyl-tRNA synthetase; the relevant gene has been cloned and its characterization is in progress (Natsoulis *et al.*, 1984 and in preparation).

Analysis of the meiotic progeny of diploids obtained by crossing three of the *cdc35* mutants isolated (*tsm0007, tsm0185, tsm0447*) with a haploid, wild-type strain showed a typical, Mendelian (2+:2−) segregation for the *ts* phenotype. The allelism of our *cdc35* mutants with the original *cdc35-1* mutant, (Reid, 1979), was confirmed by the fact that the meiotic pro-

ducts (12 complete tetrads analyzed) from the *tsm0185* × *cdc35-1* diploid consisted exclusively of parental ditype tetrads, i.e., 4 *ts versus* 0 wild-type segregants per tetrad.

The mutant isolation procedure we used apparently discriminated between class A and class B mutants and selected for the former. We interpret this as follows. The selection step on minimal medium + $(NH_4)_2SO_4$, at 36°C, is preceded by a nitrogen starvation step on minimal medium without $(NH_4)_2SO_4$, at 26°C. Nystatin is added during the selection step, at the onset of the exponential growth phase. Class A mutant cells do not leave the starvation-induced resting state, after transfer to the selection medium, and thus escape the lethal action of nystatin. In contrast, class B mutant cells are released from the starvation arrest and are engaged in the G1 growth phase preceding the steps mediated by class B genes; in this growing state they would be sensitive to nystatin.

Characterization of the cdc phenotype

Time-lapse photomicroscopy (*op. cit.*) on four *cdc35* alleles showed that cell division was arrested at 36°C after one or two generations and that 80–90% of the cell population accumulated as unbudded, uninuclear cells, with a mean cell volume similar to that of cells grown at 26°C.

To determine the point of arrest in the G1 phase relative to the mating pheromone-sensitive step, reciprocal shift experiments were performed on the *cdc35-10* allele (Hereford and Hartwell, 1974). The mutant cells behaved in these experiments as if the *cdc35* step were interdependent with the mating pheromone-sensitive step.

Conjugation experiments (Reid and Hartwell, 1977) clearly indicated that the stage of arrest of the *cdc35* mutants does not coincide with the stage where conjugation is initiated.

The time course of cellular viability, mass and macromolecule content at the restrictive temperature

Figure 1 shows the time course of total cell mass (as measured by O.D.), of the number of viable cells and of the cellular protein, RNA and DNA contents in a culture of strain *Be333* (α; *cdc35-10*), after a shift from 26°C to 36°C.

About 4 h after the shift to 36°C, total cell mass and the number of viable cells had reached a maximum, ~2.2 times the values at the time of the shift. Total cell mass then slightly decreased while the viable-cell number entered a death phase. Microscopic observation indicated that the death curve was paralleled by the progress of cell lysis, which became apparent 4 h after the shift and affected about half of the cells 3 h later.

Incorporation of radioactive precursors into macromolecules was similar for the three *cdc35* strains studied (*Be333*, *Gx3256* and *Gx3259*). The uncoordinated accumulation of protein, RNA and DNA was characteristic. RNA and DNA contents increased by 45 and 30%, respectively, during the first hour at 36°C; the rates of accumulation then declined and leveled off after 2.5 h when the total increases were 70 and 40%, respectively. By contrast, cellular protein content declined by 10% during the first hour at 36°C and then increased slightly but steadily. The initial decrease seems significant, as it was observed for the three mutant alleles studied, and is probably due to protein degradation.

Density change at the restrictive temperature

Incidently, we had observed a change in density of the cells of a *cdc35-10* mutant after transfer from 26°C to 36°C. To confirm this, we compared a wild-type and three *cdc35* mutant strains (alleles 10, 11 and 12), by measuring the density of cells grown at 26°C and at 36°C. Wild-type and mutant cells, grown exponentially on YEPD at 26°C, formed a single band between

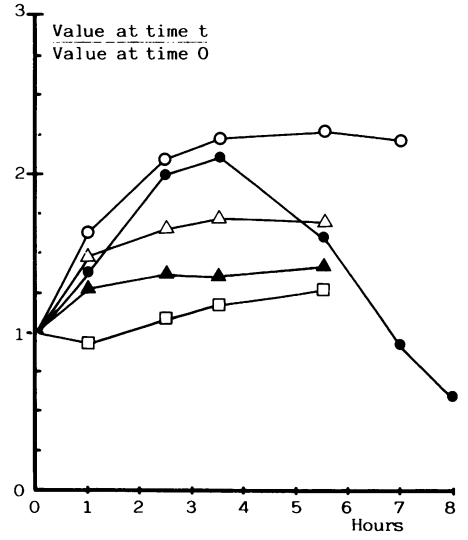

Fig. 1. Time course of optical density (– ○ –), viable cell number (– ● –), protein (– □ –), RNA (– △ –) and DNA (– ▲ –), after a shift from 26 to 36°C, for cultures of the strain *Be333* (α; *cdc35-10*). The different parameters are normalized to a value of 1, at the time of the shift. Cultures on YNB-LL (for protein measurement) and YNB-A (for RNA and DNA measurement) were started at an optical density ~0.002, with cells growing exponentially on YNB, at 26°C. After ~6 generations at 26°C on the labeled media, the cultures were shifted to 36°C. At that time, the following values were recorded: optical density, 0.150; viable-cell number, 1.45 × 10^6/ml; protein, 8250 c.p.m./ml; RNA, 22 500 c.p.m./ml; DNA, 610 c.p.m./ml. These parameters showed the same doubling time (150 min) during the 2 h preceding the shift (data not shown). The changes of optical density and viable cell number were essentially the same on the two labeling media; the data reported here are mean values for the two media. The measurement of the different parameters is described under Materials and methods.

Table I. Mapping of *CDC35* on chromosome X

Gene pairs	Tetrad types (no.)			Map dist.
	DP	NPD	T	(cM)
cdc35-met3	43	0	1	1.1
cdc35-ilv3	41	0	3	3.4
met3-ilv3	42	0	2	2.3
cdc35-leu2	18	17	8	2.8[a]
met3-leu2	21	14	9	3.8[a]
ilv3-leu2	16	17	11	6.0[a]

The distances were calculated according to Mortimer and Schild (1980, x′ values). PD, NPD, T = parental ditypes, non-parental ditypes and tetratypes, respectively. *leu2* is linked to centromere III.
[a]distances to centromere. cM = centimorgans.

1.108 and 1.114 g/ml. No density change was apparent for wild-type cells harvested 2 h after the shift to 36°C, while, under the same conditions, the mutant cells formed a single, narrow band at 1.145 g/ml. This density change (0.034 g/ml) is considerable as compared with the range of density variation (0.010 g/ml) during the cell cycle for wild-type cells grown in similar conditions (Baldwin and Kubitschek, 1984). The correlation of the density change with the *ts* growth phenotype was ascertained by the analysis of the meiotic progeny of a diploid heterozygous for the *cdc35-10* mutation; the *ts* and density phenotypes co-segregated perfectly in the four tetrads analysed.

Genetic mapping of the CDC35 gene

A *cdc35* strain was crossed with strains carrying centromere markers and segregational analysis was performed. The tetrad

Table II. Adenylate cyclase activities in crude extracts, permeabilized cells and membrane preparations from wild-type, *cdc35*, *cas1* and *cdc35 cas1* mutants

Strain	Genotype	Permeabilized cells			Membrane preparation			Crude extract
		Mn^{2+}	Mg^{2+}	Mg^{2+} + Gpp(NH)p	Mn^{2+}	Mg^{2+}	Mg^{2+} + Gpp(NH)p	Mn^{2+}
Experiment 1								
BeGX2	*CDC35 CAS1*	23.6	5.3	–	41.2	5.6	7.9	6
Be333	*cdc35-10 CAS1*	<0.1	<0.1	–	1.6	<0.1	–	<0.1
Gx3256	*cdc35-11 CAS1*	<0.1	–	–	1.4	–	–	<0.1
Be357	*CDC35 cas1-9*	62.3	19.1	–	22.5	0.3	0.6	11.2
Be333-9	*cdc35-10 cas1-9*	<0.1	<0.1	–	1.1	<0.1	–	<0.1
Experiment 2								
BeGx2	*CDC35 CAS1*	21.2	4.7	5.1	43.3	6.2	7.9	–
Be333	*cdc35-10 CAS1*	<0.1	<0.1	<0.1	1.4	<0.1	<0.1	–
Be362	*CDC35 cas1-16*	43.8	13.9	17.3	18.7	0.2	0.5	–
Be333-16	*cdc35-10 cas1-16*	<0.1	<0.1	<0.1	0.9	<0.1	<0.1	–

Enzyme activities are expressed in units per mg of protein. The assay conditions were as described in Materials and methods. The reaction mixture incubated with the different cellular materials contained 1 mM Mn^{2+} or 3.5 mM Mg^{2+} or 3.5 mM Mg^{2+} and 10 μM Gpp(NH)p. In each of the two experiments and for each strain, the crude extracts, the permeabilized cells and the membranes were prepared from the same culture. The strains are isogenic and their complete genotype is described in Table I. – = not determined.

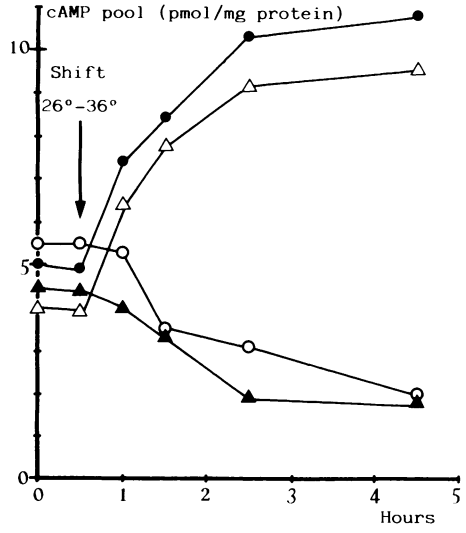

Fig. 2. Time-course of the intracellular concentration of cAMP at 26°C and 36°C of a wild-type strain (*BeGx2*, –△–) and mutant strains carrying *cdc35-10* (*Be333*, –▲–), *cas-16* (*Be362*, –●–) or *cdc35-10 cas1-16* (*Be333-16*, –○–). The strains were grown exponentially on YEPD at 26°C to about one fourth of the log phase; the cultures were then shifted to 36°C (arrow). The cAMP pools were measured on cells harvested at different times before and after the temperature shift.

distributions indicated that *cdc35* was tightly linked to the centromere X markers *met3* and *ilv3* (Table I). The analysis of the meiotic progeny of a diploid heterozygous for these two markers and for *cdc35*, indicated the following gene order: centromere X - *cdc35* - *met3* - *ilv3* (Table I). These results were established (summary in Boutelet and Hilger, 1980) when only the genes just mentioned had been mapped in that region. Subsequently, *cyr1* was assigned to the same region (Matsumoto *et al.*, 1982a).

Adenylate cyclase activities and intracellular cAMP pools

The adenylate cyclase-deficient *cyr1* mutants are arrested at the G1 phase of the cell cycle in the absence of external cAMP (Matsumoto *et al.*, 1982a). Since the *cdc35* mutations induced a similar cell cycle arrest and mapped at the same chromosomal region, we measured the intracellular cAMP concentrations and the adenylate cyclase activities in our mutants, in comparison with isogenic wild-type strains.

Figure 2 shows the time course of cAMP pools in wild-type (*CDC35*) and mutant (*cdc35*) cells, grown on YEPD at 26°C and 36°C. In *CDC35* strains, the cAMP concentration increased rapidly during the first 2 h at 36°C and attained a nearly steady level 2 h later, when the total increase was ~100%. The cAMP pools in *cdc35* strains, comparable at 26°C with their wild-type counterparts, rapidly decreased at 36°C, after an initial lag; after 4 h at 36°C they were ~40% of their levels at 26°C.

We next assayed the adenylate cyclase activities in crude extracts, nystatin-permeabilized cells and membrane preparations, from *CDC35* and *cdc35* strains, grown exponentially at 26°C on YEPD. The enzyme was assayed in the presence of either Mn^{2+} or Mg^{2+} or Mg^{2+} + Gpp (NH)p [guanosine-5′(β,γ-imino)triphosphate, a non-hydrolysable analog of GTP]. Table II shows that no activity could be detected in *cdc35* mutants, except in membrane preparations where its level was 20–30 times lower than in *CDC35* membranes. This was the case for the two independently isolated mutants that were tested. Further evidence that the adenylate cyclase deficiency was associated with the thermosensitive growth defect was obtained by analysing three tetrads from a diploid, heterozygous for *cdc35*. In each tetrad two spores were both thermosensitive for growth and deficient in adenylate cyclase, while the other two spores had a wild-type phenotype.

To test whether the adenylate cyclase in mutant cell membranes is distinguishable from that in wild-type membranes by a higher thermolability, we measured the activity in membrane preparations that had been incubated at 45°C for various times. As Figure 3 shows, 99% of the adenylate cyclase activity in mutant membranes was lost after 8 min at 45°C, *versus* 25% in wild-type membranes. That the rapid inactivation of the mutant adenylate cyclase was not caused by the presence of an inhibitor was shown by the fact that a mixture of wild-type and mutant membranes had an inactivation profile intermediate between those of the two types of membranes tested separately.

Recombination and complementation analysis of the cdc35 and cyr1 mutations. Selection and genetic characterization of cas1 mutations

Recombination and complementation tests were performed to establish the allelism, suggested by the previous results, between

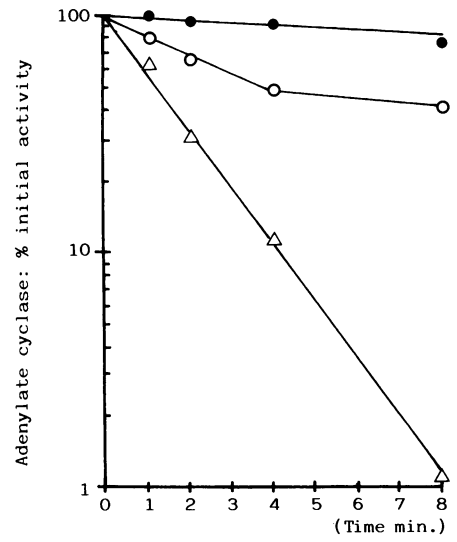

Fig. 3. Thermal inactivation of adenylate cyclase activity in membrane preparations from wild-type and *cdc35* mutant strains. The membranes, suspended in buffer C (Materials and methods: preparation of plasma membranes), were incubated at 45°C for the times indicated on the abscissa. They were then cooled at 0°C and the enzyme was assayed at 28°C in the presence of 1 mM $MnCl_2$. Symbols: ●, membranes from the wild-type strain *BeGx2*; △, membranes from the cdc35-10 mutant strain *Be333*; ○, a mixture of equal amounts (activity units) of membranes from the two mentioned strains.

cyr1 and *cdc35*. These tests were hampered by the following facts: the meiotic progeny from *cyr1* × *cdc35* diploids showed low viability and poor growth and conjugation capacities; the *cdc35* strains were not permeable to external cAMP (see below), contrary to the *cyr1* strains, which carry recessive permeability mutations, designated by 'cam' for the strains made available to us (Matsumoto *et al.*, 1982a, 1982b; Matsumoto, personal communication).

Among the viable meiotic products (35%) from *cyr1* × *cdc35* diploids, 45% showed poor growth in any condition; their phenotype was unidentifiable. Among the remaining 64 tractable segregants, none showed the recombinant wild phenotype (i.e., growth at 36°C on YEPD), which is evidence for tight linkage between *cdc35* and *cyr1*. About 40% of these segregants were more or less able to grow at 36°C on YEPD + cAMP. An unambiguous identification of the *cyr1* and *cdc35* phenotypes was impossible for most of these segregants, because of their poor conjugation capacity, of the unavailability of cAMP-permeable *cdc35* strains for complementation tests and, probably also, because of the reassortment of the 'cam' mutations with respect to *cdc35* and *cyr1*.

To obtain a valid complementation test, we attempted to isolate cAMP permeability mutations in our *cdc35* strains, by selecting for clones able to grow at 36°C, in the presence of cAMP. From a population of $\sim 10^{10}$ cells of strain *Be333* (*α cdc35-10*), spread on YEPD + 5 mM cAMP, 23 colonies emerged after 4 days at 36°C. Among these, 10 showed the desired phenotype, i.e., no growth at 36°C on YEPD; growth at 36°C on YEPD + cAMP. The selected mutations had deleterious effects, since the generation time at 26°C on YEPD was 2–3 times higher and the viability of cells from stationary cultures at 26°C on YEPD was 2–4 times lower for the selected mutants than for the *cdc35* mutant, from which they were derived. Segregational analysis showed that the selected mutations were monogenic (2+:2− segregation). The phenotype of the diploids indicated that the mutations were recessive. Complementation tests revealed that the 10 mutations selected were allelic and the gene they define was designated *CAS1*. The *cas1* mutations showed similar cAMP-mediated suppressor effects on the three different *cdc35* alleles that were tested. *cdc35* and *cas1* are not linked (12 PD:14 NPD:42 T; for symbols, see Table I).

The diploids, resulting from crosses between *cyr1* '*cam*' strains and the selected *cdc35 cas1* strains, were able to grow on YEPD + cAMP at 36°C; they did not grow on YEPD at 36°C. This is evidence that *cyr1* and *cdc35* are allelic and that the *cas1* mutation is allelic with one of the '*cam*' mutations, carried by the *cyr1* '*cam*' strains. These diploids showed growth and viability characteristics similar to those described above for the *cas1* strains.

An attempt to characterize the cas1 mutations biochemically

To understand the mechanism by which *cas1* mediates the suppression of the adenylate cyclase deficiency in *cdc35* strains by exogenous cAMP, we first tested whether *cas1* allowed the uptake of cAMP from the medium, as is the case for the *cyr1* strains we used. Two kinds of observations indicated that *cas1* does not induce permeability to cAMP. First, external cAMP (5 mM) did not compensate for an adenine requirement in *cas1 ade6* strains. Second, experiments attempting to detect the uptake of external [^{3}H]cAMP, at 26°C and 36°C on YEPD, gave similar results for mutant (*cas1*) and wild-type (*CAS1*) strains, i.e., no significant penetration of external cAMP into the cells could be detected.

We next tested if *cas1* had an effect on the intracellular cAMP pool, by comparing isogenic wild-type and mutant strains. As Figure 2 shows, *cas1*-16 induced an increase of 15–25% in the cAMP levels at 26°C and 36°C, whether *cdc35* was present or not. This increase seems significant as it was observed repeatedly for two other *cas1* alleles.

As the cellular cAMP pool depends on the activities of cAMP-specific phosphodiesterases and of adenylate cyclase, we assayed these enzymes in crude extracts of *cas1* and *CAS1* strains; no significant difference in phosphodiesterase activities was observed. We assayed adenylate cyclase in crude extracts, permeabilized cells and membrane preparations, in the presence of Mn^{2+} or Mg^{2+} or Mg^{2+} + Gpp(NH)p, following Casperson *et al.* (1983), who showed that guanine nucleotide regulation of adenylate cyclase can be conveniently assayed in yeast membranes by measuring its activity with its physiological substrate MgATP, in the presence or absence of GTP or its analog Gpp(NH)p. Under these assay conditions, we observed striking effects of the *cas1* mutation (Table II). Crude extracts from a *cas1* mutant showed a Mn^{2+}-dependent activity which was nearly twice that measured in wild-type extracts. This difference was even higher (2- to 3-fold), when the activities were assayed in permeabilized cells. As expected, in the presence of Mg^{2+}, the adenylate cyclase activities were lower than in the presence of Mn^{2+}; again, these activities were much higher (3- to 4-fold) in *cas1* than in wild-type permeabilized cells. No less striking, although in the opposite direction, were the effects of *cas1* on the adenylate cyclase activities displayed by membrane preparations. *cas1* induced the loss of ~50% of the Mn^{2+}-dependent activity and at least 95% of the Mg^{2+}-dependent activity. Surprisingly, we did not observe the spectacular stimulatory effects of Gpp(NH)p that have been reported for yeast strains from other stocks (Casperson *et al.*, 1983; Matsumoto *et al.*, 1984; Toda *et al.*, 1985); this might be related to strain-specific traits. However, the stimulatory effect of Gpp(NH)p was significantly higher for strains carrying *cas1* than for the wild-type strain.

All these observations are a strong indication that *cas1* affects a component of the adenylate cyclase regulatory system. Casperson *et al.* (1983) report that the factor that confers guanine nucleotide regulation and the ability to utilize MgATP on yeast adenylate cyclase is highly thermolabile. We, therefore, tested whether the activity, observed in *cas1* permeabilized cells, was lost more rapidly by a heat treatment than the activity in wild-type permeabilized cells. This was the case in a striking way. The experimental conditions were the same as for the thermodenaturation experiment, described above, on membranes of a *cdc35* and a wild-type strain. The adenylate cyclase in *cas1* cells was inactivated according to a biphasic pattern: an exponential decrease (similar to that shown in Figure 3 for *cdc35-10*), during the first 4 min of incubation at 45°C, to a level of ~20% of the initial activity; this residual activity then decayed slowly, in a way comparable with that observed for wild-type cells, the activity of which declined uniformly during the heat treatment by 10% in 10 min (details to be published). The properties of the *cas1* mutants are under continued study.

Toda *et al.* (1985) reported recently that mutations in the yeast *RAS1* and *RAS2* genes caused modifications in the intracellular cAMP levels and in the GTP-regulated, Mg^{2+}-dependent adenylate cyclase activities of membrane preparations. The study strongly suggested that the *RAS* proteins are involved in the regulation of adenylate cyclase. To test whether *cas1* was allelic with *ras1* or *ras2*, we performed linkage tests between *cas1* and chromosome markers (*tsm8740* on chromosome XV and *met4* on chromosome XIV) tightly linked to the *ras* loci (Tatchell *et al.*, 1984; Kataoka *et al.*, 1984). This analysis clearly indicated that *cas1* is not linked to one of the *ras* loci.

Discussion

According to the current functional sequence map of the yeast cell cycle (Pringle and Hartwell, 1981), the cycle begins with steps, denoted A and B, which are operationally defined by the points of arrest of nutrient-deprived cells, of mating pheromone-treated cells and of certain *cdc* mutants, the so-called start mutants. Step-A start mutants arrest as unbudded, uninucleate, non-growing cells whose spindle pole bodies do not carry satellites. Step-B start mutants arrest as unbudded, uninucleate cells which continue growth and whose spindle pole bodies carry satellites. Reed (1980) defines two classes of start mutants, denoted I and II, by the same criteria and adds the conjugation-competence criterium. Class I mutants have the properties mentioned above for class B mutants and are able to conjugate in the arrested state; class II mutants are similar to class A mutants and are unable to conjugate in the arrested state. *cdc35* mutants had been ranged in class A (Pringle and Hartwell, 1981). The new *cdc35* alleles studied here show the morphology and growth characteristics of class A mutants.

Class A mutant cells show an arrest phenotype similar to that of wild-type cells arrested by nutrient deprivation (Pringle and Hartwell, 1981). The results of our mutant selection are indicative of a coincidence of the step(s) affected by class A mutations and the nutrient deprivation-sensitive step(s). Our selection procedure, in principle, selected for mutant clones unable to resume growth on complete medium at 36°C, after an arrest induced by nitrogen starvation at 26°C. The procedure, in fact, led to the isolation of alleles of the four genes recognized as controlling class A events (Pringle and Hartwell, 1981); it did not produce class B mutations.

The cell density increase and the unbalanced and poor synthesis of macromolecules by *cdc35* mutants, after the shift to the restrictive condition, indicate that *cdc35*-arrested cells are in a physiological state different from that of normal G1 cells or, in other words, that *cdc35* imposes a block which is not cell cycle specific. It is striking that the cell number increases nearly 2-fold during the 2.5 h following the temperature shift, while protein accumulation is arrested. A situation similar to this has been observed by Johnston *et al.* (1977) for nitrogen-starved cultures of wild-type strains. These authors showed that, in fact, the overall protein synthesis is not prevented by nitrogen starvation but is counterbalanced by extensive degradation. This seems to occur also to some extent in the *cdc35* mutant cells, as indicated by the slight decrease of the protein content during the first hour at 36°C. Johnston *et al.* (1977b) also showed that RNA undergoes the same process as protein, in nitrogen-starved cells. This is not apparent in our experiments; RNA increases by 50% during the first 2.5 h at 36°C. The density increase occurring in the *cdc35* mutant cells within 2 h after the temperature shift is 3.4 times the maximum density difference during the cycle at normal conditions (Baldwin and Kubitschek, 1984). The density effect of *cdc35* is all the more striking as, at the same time, no protein and relatively little RNA and DNA are accumulated; there is no apparent reduction in cell volume as the number of viable cells almost doubles. The elucidation of the mechanisms of the density effects of *cdc35* needs further analysis. A similar effect has been observed for inositol-requiring yeast mutants upon starvation for inositol (Henry *et al.*, 1977). On the basis of the reciprocal-shift experiment, designed to determine the relative order of cycle-specific events, the formal conclusion was drawn that the *cdc35* step was interdependent with the mating pheromone-sensitive step (class B step). As the *cdc35* block does not seem cycle-specific, the result obtained might not be significant.

The cAMP-requiring *cyr1* mutants carry lesions in the structural gene for adenylate cyclase (Matsumoto *et al.*, 1982a and 1984). These mutants also have a *cdc* phenotype typical of class A mutants (Matsumoto *et al.*, 1983). The phenotypic and biochemical properties shown by our *cdc35* mutants are similar to those of *cyr1* mutants, namely class A *cdc* phenotypes, lowered intracellular cAMP concentration at the restrictive temperature, undetectable or low adenylate cyclase activities, high thermolability of the mutant enzyme activity. Our genetic study shows that the *cdc35* maps close to or at the same locus as *cyr1*, that the two mutations do not complement each other and that no wild-type recombinants are observed among the meiotic progeny from diploids heterozygous for the two mutations. We conclude that *cdc35* and *cyr1* are mutations in the same gene.

The *cas1* mutation we isolated mediates the suppression of the phenotypic effects of *cdc35* when external cAMP is provided. Our analysis of the *cas1*-carrying mutants suggests that they have a biochemical defect in the cAMP effector pathway. The Mn^{2+}-dependent adenylate cyclase activities in crude extracts and permeabilized cells were significantly higher for *cas1* than for the wild-type strains; this increase seemed amplified when Mg^{2+} replaced Mn^{2+}. The enzyme activity in permeabilized cells, which was in excess in *cas1* strains with respect to wild-type strains, was highly thermolabile. When the enzyme activities were measured on membrane preparations, the effects of *cas1* were a significant decrease of the Mn^{2+}-dependent activity and an almost complete loss of the Mg^{2+}-dependent activity. A simple and plausible interpretation is that the *CAS1* gene encodes a component of the regulatory system involving GTP and Mg^{2+} (Casperson *et al.*, 1983; Gilman, 1984). The alteration due to *cas1* would have two effects: (i) a permanent stimulation of the

adenylate cyclase in the state in which it exists in crude extracts and permeabilized cells; (ii) a modification of the interactions of the *CAS1* protein with membrane components such that the treatment used to prepare membranes causes the loss of the altered *cas1* protein or destroys its interaction with adenylate cyclase.

In contrast to the striking effects mentioned above, the *cas1* mutation has little effect on the intracellular cAMP levels. *cas1* by itself does not raise the *cAMP* level sufficiently to compensate for the defective adenylate cyclase activity in *cdc35* mutants at 36°C. In fact, *cas1* suppresses the *cdc35* phenotype only when cAMP is present in the culture medium. A simple explanation of the role of external cAMP would be that it is taken up in amounts too low to be detected by our procedures, but sufficient, together with the slight surplus due to *cas1*, to overcome the deficiency caused by *cdc35*. An alternate explanation would be to assume that the adenylate cyclase activity is sensitive to external cAMP and that the *CAS1* protein is part of the regulatory system mentioned above, with a role similar to that of the G proteins in vertebrate cell membranes (Gilman, 1984) in the transmission of external signals to adenylate cyclase. The effects of the *cas1* mutation would be to amplify the signalling of external cAMP and to raise cAMP production. Taken together the observed effects of the *cas1* mutation are consistent with the hypothesis that the *CAS1* protein is a controlling element of adenylate cyclase, a role also assigned to the *RAS1* and *RAS2* proteins (Toda *et al.*, 1985). However the *CAS1* gene does not correspond to one of the *RAS* loci, as shown by our linkage tests. In addition the *cas1* mutations are phenotypically distinct from the *ras* mutations, especially the RAS^{val19} allele.

Materials and methods

Yeast strains and genetic procedures

The wild-type strains used in this study were *1278b* and *3962c*, of the mating-types α and a, respectively (Hilger, 1973). the *cdc35* isolates were derived from *1278b*; the four alleles investigated here are *tsm0185*, *tsm0007*, *tsm0302* and *tsm0447*, to which we assigned the allele number *10, 11, 12* and *13*, respectively. The other strains, carrying one of the *cdc35* alleles (*Be333* = α *cdc35-10*; *Be334* = *a cdc35-10*; *Gx3068* = *a cdc35-10 arg1*; *Gx3256* = *a cdc35-11 arg1*; *Gx3259* = α *cdc35-12 arg1*), are segregants from crosses between the original *cdc35* isolates and the wild-type strains mentioned above or isogenic strains carrying *arg1* (*MG685* = α *arg1*; *BeGx2* = *a arg1*) (Hilger and Mortimer, 1980). The original *cas1* mutants (*Be333-9* = α *cdc35-10 cas1-9*; *BE333-16* = α *cdc35-10 cas1-16*) were selected from *Be333* (= α *cdc35-10*). *Be357* (= α *cas1-9*) and *Be362* (= α *cas1-16*) are segregants from the crosses (*BeGx2* × *Be333-9*) and (*BeGx2* × *Be333-16*), respectively. The markers for the mapping of *cdc35* were provided by strains from the 'Yeast Genetic Stock Center' (Berkeley). The *cdc* mutants used for the complementation study were obtained from the same source and from Dr. L. H. Hartwell. The *cyr1-1* strains (*AM116-1B* and *AM116-2A*), which carried *ade6* or *ade10* and had the Cam^+ phenotype (i.e., cAMP can be utilized as adenine source), were provided by Dr. Matsumoto. General genetic manipulations were carried out according to Mortimer and Hawthorne (1969).

Media

YEPD medium consisted of 1% yeast extract, 2% Bacto-peptone and 2% glucose. YNB medium contained 0.67% yeast nitrogen base without amino acids (DIFCO), 2% glucose, 10.5% citric acid, 0.4% KOH, the pH being adjusted to 6.0 with NaOH. YNB-LL = YNB medium plus 0.3 mM L-leucine and L-lysine. YNB-A = YNB + 0.15 mM adenine. Solid media contained 2% agar.

Cellular parameters

The number of viable cells was determined by plating appropriate dilutions on solid YEPD and by counting the colonies after 4 days of incubation at 26°C. Total cell mass was estimated by measuring the optical density at 560 nm with a Beckmann, Model 25, spectrophotometer. For the analysis of cell volumes, measurements of ~200 cells were made on microphotographs at a magnification of 1000. Cell buoyant density was measured by isopycnic banding of cells in spontaneously generated 'Percoll' gradients (Pharmacia Fine Chemicals). 10^7 cells from cultures on YEPD were collected in 0.4 ml of 0.25 M sucrose and 10 mM NaN_3, and suspended in 12 ml of a mixture containing 9 volumes of 'Percoll' (1.131 g/ml) and 1 volume of 2.5 M sucrose and 100 mM NaN_3. The suspension was centrifuged at 35 000 *g* for 40 min at 4°C. The 'Pharmacia' density markers were used for the calibration of the density gradients. The number of nuclei per cell was determined by the Giemsa staining according to Sherman *et al.* (1983).

Preparation of mating pheromone (α factor)

α factor was partially purified by following the methods described by Bücking-Throm (1973) and Duntze *et al.* (1973). The preparation was used at a concentration that allowed a growth arrest of *a* cells during 8 h.

Incorporation of labelled precursors into protein, RNA and DNA

The procedures used were essentially as described by Johnston *et al.* (1977). Samples were withdrawn from cultures on YNB-LL medium, containing 0.2 μCi/ml L-[4,5-^{3}H]leucine (50.5 Ci/mmol) and 0.2 μCi/ml L-[4,5-^{3}H]lysine (40 Ci/mmol) for the determination of protein content, and from cultures on YNB-A medium, containing 0.2 μCi/ml [8-^{14}C]adenine (53 mCi/mmol) for the determination of RNA and DNA contents. When these procedures were applied to a wild-type strain, growing exponentially for at least five generations on the labelled media, the time course of labelling was essentially the same for protein, RNA and DNA; it was also parallel to the time course of optical density and cell number (data not shown).

Preparation of cell-free extracts

All operations were carried out at 0 – 4°C. Cells grown exponentially to 0.8 – 1.2 × 10^7 cells/ml on YEPD were harvested by centrifugation and washed with 25 mM Pipes-HCl, pH 6,2, 0.1 mM EDTA, and suspended in the same buffer + 1 mM mercaptoethanol and 0.5 mM phenylmethylsulfonyl fluoride. The cell suspension was homogenized in a 'FRENCH' press (Aminco 4-3398A) at 5.500 p.s.i. Unbroken cells and large debris were removed by centrifugation at 1000 *g* for 10 min.

Preparation of permeabilized cells and plasma membranes

Cells were permeabilized by the nystatin procedure (Hilger *et al.*, 1973). Free permeation of the substrates and products of the reactions catalyzed by ornithine transcarbamylase (*op. cit.*) and adenylate cyclase was demonstrated by the fact that, at saturating concentrations of the substrates, the amounts of products formed were proportional, within reasonable limits, to the quantities of permeabilized cells incubated and to the incubation time. The specific enzyme activities of permeabilized cells were estimated by considering that 1 unit optical density at 560 nm contains 0.2 mg of protein. Preparation of plasma membranes was performed according to Liao and Thorner (1980).

Adenylate cyclase and phosphodiesterase assays

Adenylate cyclase was assayed as described by Uno *et al.* (1981), except for the following: the concentrations of ATP and $MnCl_2$ were 5 and 1 mM, respectively; the pH of the buffer was 6.2; the reaction temperature was 28°C. cAMP was measured with Amersham's assay kit. The assays in the presence of $MgCl_2$ and Gpp(NH)p were performed with appropriate controls to estimate the interference of these molecules with cAMP binding. Phosphodiesterase was assayed as described by Uno *et al.* (1983). One unit of activity of these enzymes was defined as the amount of enzyme that produced 1 pmol cAMP or 5'-AMP, respectively, in 1 min at 28°C.

Cellular cAMP pools

The cellular cAMP pools were measured by the procedure of Uno *et al.* (1981). cAMP was measured on acetylated samples by the New England Nuclear cAMP[^{125}I]RIA assay kit.

Protein estimation

Protein was measured by the method of Lowry *et al.* (1951); bovine serum albumin was the standard.

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