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Cloning and Characterization of *BCY1*, a Locus Encoding a Regulatory Subunit of the Cyclic AMP-Dependent Protein Kinase in *Saccharomyces cerevisiae*

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We have cloned a gene (*BCY1*) from the yeast *Saccharomyces cerevisiae* that encodes a regulatory subunit of the cyclic AMP-dependent protein kinase. The encoded protein has a structural organization similar to that of the RI and RII regulatory subunits of the mammalian cyclic AMP-dependent protein kinase. Strains of *S. cerevisiae* with disrupted *BCY1* genes do not display a cyclic AMP-dependent protein kinase in vitro, fail to grow on many carbon sources, and are exquisitely sensitive to heat shock and starvation.

In the yeast *Saccharomyces cerevisiae*, *RAS* genes are positive modulators of adenylate cyclase activity (4). An activated form of *RAS2*, *RAS2*^{val19}, causes elevated and improperly regulated adenylate cyclase activity. Strains containing *RAS2*^{val19} display several abnormalities, including aberrations of carbohydrate metabolism, response to nutrient limitation, and cell cycle arrest. These phenotypes are very similar to those associated with the previously described mutation designated *bcy1*. Strains with the *bcy1* mutation fail to make a detectable regulatory subunit of the cyclic AMP (cAMP)-dependent protein kinase (cAPK) (21). Cells containing mutant alleles of *bcy1*, unlike normal yeast cells, do not require functional *RAS* genes (39). To better understand the relationship between *RAS* and the adenylate cyclase pathway in yeast cells, we have begun to identify, clone, and characterize genes involved in the *RAS*-cAMP effector pathway. The possession of the cloned genes facilitated the design of experiments to describe the role of these genes in the regulation of cell growth.

cAMP is known to mediate, in both procaryotes and eucaryotes, a wide variety of cellular responses to external stimuli. In eucaryotes, the effects of cAMP are commonly thought to be due largely, if not entirely, to cAPK. In mammals, these kinases are tetrameric proteins consisting of two regulatory subunits and two catalytic subunits. The regulatory subunits each contain two binding sites for cAMP, which, when occupied, cause the holoenzyme to dissociate two active catalytic subunits, with the regulatory subunits remaining as a dimer (17). At least two regulatory subunits, RI and RII, are known to be present in mammalian cells. The full number of distinct mammalian regulatory and catalytic subunits has not been ascertained, nor has the question of their physiological significance been resolved. In this paper, we describe the cloning of *BCY1*, which was found to encode a regulatory subunit of the cAPK in *S. cerevisiae*, and we present the nucleotide sequence of *BCY1* and explore the consequences of its disruption. Genetic dissection of *BCY1* may allow an analysis of the role of the

cAPK regulatory subunit in mediating the effects of cAMP and *RAS* in *S. cerevisiae*.

MATERIALS AND METHODS

Strains and media. In this study, we used *Escherichia coli* HB101 and *S. cerevisiae* strains (Table 1). The media for yeast cells have been described elsewhere (39).

Genetic techniques and nomenclature. Standard genetic procedures as described by Mortimer and Hawthorn were followed (23). Yeast transformation was done by the method of Ito et al. (13). The YCp50 genomic library was generously provided by M. Rose and G. Fink. The library was a *Sau3A*I partial digest inserted into the unique *Bam*HI site of YCp50. Nomenclature for genotypes and phenotypes follows standard rules. Capital letters designate wild-type alleles or dominant mutant alleles. Lowercase letters designate recessive mutant alleles. *ABC::XYZ* indicates that *XYZ* has been integrated at the *ABC* locus.

DNA. DNA restriction endonucleases, polymerases, and ligases were used under conditions recommended by suppliers (New England BioLabs, Inc., or Bethesda Research Laboratories, Inc.). Nitrocellulose filter blot hybridization was performed as described by Maniatis et al. (18). DNA sequencing was determined by the dideoxy method of Sanger et al. (30) with [α -³⁵S]dATP as a substrate (2).

Preparation of peptide fragments of the cAPK regulatory subunit. The cAPK regulatory subunit was prepared from *S. cerevisiae* as previously described (31). Limited proteolysis of the cAPK regulatory subunit (20 nmol) was performed with chymotrypsin for 2 h at 30°C at an enzyme-to-substrate ratio of 1:1,000 in a solution of 1% (wt/vol) NH_4HCO_3 (pH 7.8). After digestion, the sample was lyophilized, and the peptides were fractionated on a Synchropak R-PP C18 reverse-phase column equilibrated in 1% (wt/vol) trifluoroacetic acid. Elution was achieved by increasing acetonitrile concentration. Citraconylation of the cAPK regulatory subunit (150 nmol) was performed as described by Titani et al. (38). The modified protein was digested for 1 h at 37°C with trypsin (enzyme-to-substrate ratio, 1:100). After diges-

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TABLE 1. *S. cerevisiae* strain descriptions

Strain	Genotype	Source
AM203-1B	<i>MATα his7 bcy1-1</i>	K. Matsumoto
M76-3C	<i>MATα leu2 his3 cyr1-1</i>	J. Szostak
T58-B	<i>MATα leu2 his3 bcy1-1</i>	Segregant from AM203-1B/M76-3C
T16-11A	<i>MATα his3 leu2 ura3 trp1 bcy1-1</i>	Segregant from T58-B/KPPK-1D
TTS121	<i>MATα his3 leu2 ura3 trp1 ade8 can1 bcy1::URA3</i>	Transformant of SP1 with <i>Bam</i> HI fragment of <i>pbcy1::URA3</i>
TTS122	<i>MATα his3 leu2 ura3 trp1 ade8 can1 bcy1::URA3</i>	Transformant of SP1 with <i>Bam</i> HI fragment of <i>pbcy1::URA3</i>
SP1	<i>MATα his3 leu2 ura3 trp1 ade8 can1</i>	39
DC124	<i>MATα his4 leu2 ura3 trp1 ade8</i>	Cold Spring Harbor Laboratory
KPPK-1D	<i>MATα his3 leu2 ura3 trp1 ras1::HIS3</i>	39
S7-5D	<i>MATα his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1^a</i>	
S17-5	<i>MATα his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1 bcy1::LEU2^a</i>	
TTS5501	<i>MATα/MATα his3/+ his4/+ leu2/leu2 ura3/ura3 trp1/trp1 ade8/ade8 can1/+ bcy1::URA3/+</i>	Transformant of SP1/DC124 with <i>Bam</i> HI fragment of <i>pbcy1::URA3</i>
TTS1501	<i>MATα his3 leu2 ura3 trp1 ade8 can1 BCY1::pbcy1::URA3</i>	Transformant of SP1 with <i>pbcy1::URA3</i> integrated into the <i>BCY1</i> locus (see Materials and Methods)

^a *TPK1*, *TPK2*, and *TPK3* each encode catalytic subunits of the cAPK system in *S. cerevisiae* (Toda et al., unpublished results). The *TPK* genes had been disrupted by the indicated markers.

tion, the sample was lyophilized and suspended in 500 μ l of 6 M guanidine hydrochloride before fractionation on two TSK SW 3000 columns (21 by 600 mm) coupled in tandem at a flow rate of 1 ml/min. Each peak was pooled, and individual peptides were purified to homogeneity by reverse-phase high-pressure liquid chromatography. Early-eluting peaks from fractionation on the TSK SW 3000 columns contained larger peptides and were further purified on an Altex RPSC C3 column, while later-eluting fractions were separated with a Synchropak R-PP C18 column. In both cases, the buffer was 1% (wt/vol) trifluoroacetic acid, and elution was achieved by increasing acetonitrile concentration.

Sequence determination for peptides. Amino acid sequence determination for peptides was carried out as previously described by Scott et al. (31) with a gas-phase sequenator (AB50; Applied Biosystems) or a liquid-phase sequenator (890 C; Beckman Instruments, Inc.). Identification of phenylthiohydantoin amino acids was done on complementary high-pressure liquid chromatography systems as described by Brigden et al. (4) and Ericsson et al. (10).

Linkage test. Wild-type strain SP1 was transformed by *Bgl*II-digested DNA from *pbcy1::URA3*. This integration duplicated the cloned locus and marked it with *URA3*. One resulting haploid (TTS1501) was then crossed to T16-11A, a strain carrying the *bcy1-1* allele, and tetrad analysis was performed. The *bcy1-1* allele caused only mildly defective germination, which enabled tetrad analysis to be carried out (see Results). Uracil prototrophy and a heat-shock-resistant phenotype cosegregated in 11 complete tetrads. No Ura⁺ heat-shock-sensitive haploids were obtained, thus showing very tight linkage (<4 centimorgan) between the cloned sequences and the *BCY1* locus.

Complementation test. A haploid (TTS121) disrupted at *BCY1* was crossed to wild-type strain DC124. The diploid thus formed was sporulation competent and resistant to heat shock, indicating that phenotypes observed in disrupted haploids are recessive. In contrast, crossing TTS121 to T16-11A produced a sporulation-deficient, heat-shock-sensitive diploid. These results indicate that the disrupted locus in TTS121 and the *bcy1-1* gene of T16-11A constitute a single complementation group.

Preparation of extracts for assays of cAPK activity. One liter of yeast cells was grown to approximately 10^7 cells per ml in minimal medium supplemented with required amino acids. Cells were washed once with buffer A (50 mM Tris [pH 7.4], 2 mM EDTA, 1 mM β -mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride) and then lysed in 10 ml of buffer A containing 1 μ g of soybean trypsin inhibitor per ml by passage through a French press at 12,000 lb/in². Lysates were spun at 20,000 $\times g$ for 1 h. Supernatants were loaded onto columns of DEAE-Sephacel (2 by 6 cm) equilibrated with buffer A. The columns were eluted with buffer A in a series containing NaCl at concentrations from 50 to 300 mM in 50 mM increments. Two 2-ml fractions were collected at each step. All procedures were performed at 4°C. Protein concentrations were determined by the method of Bradford (3) by using a protein mix as the standard.

Protein kinase assay. The standard reaction mixture for assays of protein kinase activity contained, in a total volume of 50 μ l, 50 mM MOPS (3-[*N*-morpholino]propanesulfonic acid; pH 7.0), 10 mM MgCl₂, 250 μ g of bovine serum albumin per ml, 100 μ M [γ -³²P]ATP at 200 cpm/pmol, 150 μ M Kemptide, 5 μ l of extract, and, where indicated, 10 μ M cAMP. Kemptide is a synthetic phosphate acceptor peptide with the sequence Leu-Arg-Arg-Ala-Ser-Leu-Gly-NH₂. Reactions were initiated by adding 15 μ l of ATP-Kemptide-cAMP to 35 μ l of enzyme-buffer solution. Reactions were terminated after 8 min at 30°C by spotting 5 μ l of reaction mixture onto phosphocellulose paper (1 by 2 cm; P-81; Whatman Inc.) and immersing the paper in 75 mM phosphoric acid. Filters were washed five times for 2 min with phosphoric acid, rinsed with acetone, dried in air, and counted. Where indicated, 100 ng of *BCY1* protein purified from *E. coli* as described below was included in the buffer-enzyme mix.

Expression and purification of *BCY1* protein. *BCY1* protein was expressed in *E. coli* by using a modified T7 expression vector (35) and purified by using cAMP-agarose affinity chromatography (K. Johnson, S. Cameron, M. Wigler, and M. Zoller, manuscript in preparation). Bound cAMP was removed from the purified protein by the procedure of Builder et al. (5). After these steps, the protein was esti-

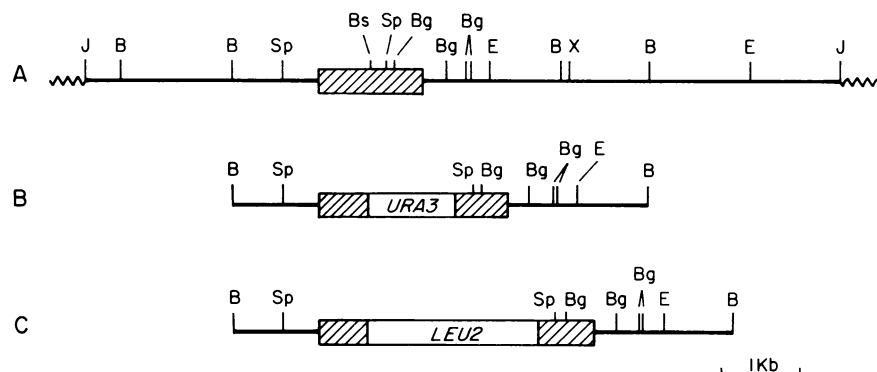


FIG. 1. Structure and disruption of the *BCY1* gene. (A) Restriction map of the original *BCY1* clone (YCpBCY1). J, Junction between an insert DNA and a *Bam*HI site of the vector YCp50 DNA. The 2.6-kilobase (kb) *Sph*I-*Eco*RI fragment was sequenced by the M13 dideoxy method (30). Both strands of the single long open reading frame were completely sequenced, and the coding sequences for the *BCY1* gene are indicated (▨). (B) Structure of *pbcyl::URA3* disruption plasmid. The 4.2-kb *Bam*HI fragment of *BCY1* was inserted into the *Bam*HI site of pUC8 (41), thereby creating *pBCY1*. The plasmid was linearized at a unique *Bst*EII site, which was filled in with Klenow fragment and deoxynucleoside triphosphates. The 1.1-kb *Hind*III-Klenow-treated *URA3* fragment (▨) was inserted into the *Bst*EII site. (C) Structure of the *pbcyl::LEU2* disruption plasmid. The Klenow-treated 2.2-kb *Sall*-*Xho*I fragment of *LEU2* was inserted into the *Bst*EII site of *BCY1* as described above. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; Sa, *Sall*; Sp, *Sph*I; and X, *Xho*I. Only the 4.2-kb *Bam*HI fragment was mapped for all of these sites.

mated to be more than 95% pure as judged by a Coomassie blue-stained sodium dodecylsulfate-polyacrylamide gel.

RESULTS

Cloning the *BCY1* locus. The *bcy1* mutations were originally isolated by Matsumoto and co-workers (21). Biochemically, cells with *bcy1* mutations do not appear to synthesize a functional regulatory subunit of the cAPK (21). It therefore seemed plausible that *BCY1* encoded a cAPK regulatory subunit (see Discussion). We set about to clone *BCY1* by complementation screening. The *bcy1* strain AM203-1B, obtained from K. Matsumoto, was repeatedly backcrossed into our strain background to create strain T16-11A, which contained the additional genetic markers *his3 leu2 ura3 trp1* (strain descriptions are in Table 1). For clarity, we refer to the allele of *bcy1* in AM203-1B as *bcy1-1*. These strains of *S. cerevisiae* have numerous phenotypic defects, including sensitivity to starvation (19) and heat shock, which are consequences of the *bcy1-1* mutation. Cells from strain T16-11A were transformed with a library of yeast genomic DNA carried on the shuttle vector YCp50 (see Materials and Methods). *Ura*⁺ transformants were picked and screened by replica plating for nitrogen starvation resistance. Nitrogen-starvation-resistant transformants were isolated and tested for vector dependence. Several strains which were nitrogen starvation resistant in a vector-dependent manner were thus identified, and their vector plasmids were isolated by transforming *E. coli*. Analysis of the resulting plasmids indicated that all contained one insert from the locus shown in Fig. 1. Genetic experiments, described in Materials and Methods, indicated that the locus we cloned was tightly linked to the *bcy1-1* mutation, and disruptions of this locus fell into the same complementation class as *bcy1-1*. We have, therefore, cloned the *BCY1* locus.

***BCY1* encodes cAPK regulatory subunit.** Subcloning experiments indicated which region of the *BCY1* locus was essential for complementing activity, and this region was then sequenced by the dideoxynucleotide method. One open reading frame of 416 codons, initiated by ATG, was found. An in-frame stop codon was found 6 nucleotides upstream

from this ATG. The nucleotide sequence and the predicted amino acid sequence of the open reading frame are indicated in Fig. 2. The N-terminal sequence of the encoded protein was identical to the previously reported N-terminal sequence of the yeast cAPK regulatory subunit at 19 of 20 positions (12). This result strongly suggests that *BCY1* encoded the cAPK regulatory subunit. The experiments described below prove this.

The purification of the regulatory subunit of the cAPK from *S. cerevisiae* has been previously described (12). The partial amino acid sequence of this protein was established by following the procedures described in Materials and Methods. Various chymotrypsin and trypsin proteolytic fragments were purified by high-pressure liquid chromatography fractionation and were sequenced. Thirteen fragments were aligned with the predicted amino acid sequence of the *BCY1* gene product, covering 77% of residues (Fig. 2). There was excellent agreement between the predicted and derived amino acid sequences, with discrepancies at only three positions. Two cysteinyl residues were identified at positions 199 and 267 during protein sequencing; the *BCY1* nucleotide sequencing, however, predicts aspartyl groups at these positions. This difference is readily explained, as phenylthiohydantoin cysteine and phenylthiohydantoin aspartic acid elute at nearly identical times from the high-pressure liquid chromatography systems used for their identification (10). The third discrepancy was at position 293, where nucleotide sequencing predicts lysine and protein sequencing yielded isoleucine. This discrepancy may have resulted from differences in the yeast strains used for gene cloning and protein purification.

These results confirm that *BCY1* encodes a regulatory subunit of the cAPK of *S. cerevisiae*. To determine the effect of *BCY1* disruption on cAPK activity, extracts were prepared from cells containing a disrupted *bcy1* gene and were compared with corresponding extracts from parental strains. For this comparison, we used *S. cerevisiae* strains which lacked two of the three genes (*TPK1*, *TPK2*, and *TPK3*) which encode the catalytic subunits of the cAPK. These strains were used because of their improved growth and viability relative a strain that contains a *bcy1* disruption and

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Wild-type strains with disrupted *bcy1* alleles were examined for several phenotypes, including the ability to survive heat shock or nitrogen starvation and the ability to utilize carbon sources other than glucose. These phenotypes were assayed for by a replica plating method (Fig. 4). The results (Fig. 4) clearly indicate that the Ura⁺ (and therefore *bcy1*) strains were sensitive to heat shock and nitrogen starvation and were unable to grow on acetate. Additional experiments indicated that these *bcy1* strains could not grow on the carbon sources (other than glucose) which we tested, including raffinose, galactose, glycerol, pyruvate, and acetate. Diploid strains homozygous for *bcy1* disruption were also

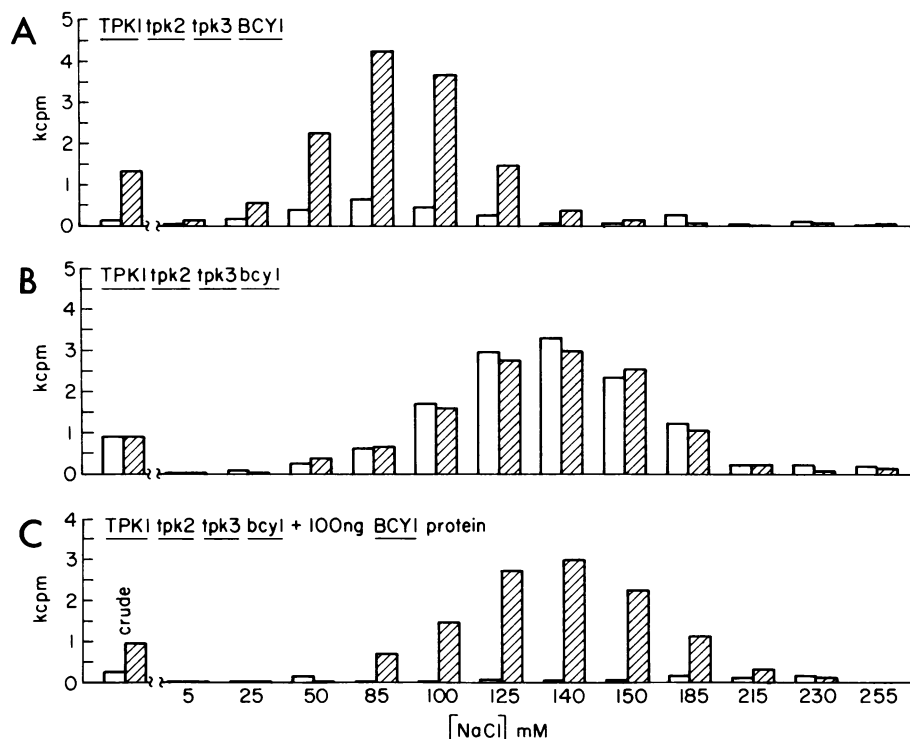


FIG. 3. cAPK activity in fractionated yeast extracts. Protein (9 μg) from the indicated yeast cell lysates was fractionated on a DEAE-Sephacel column eluting with a NaCl step gradient and was assayed for cAPK activity as described in Material and Methods. Fractions were assayed for cAPK activity in the absence (□) or presence (▨) of 10 μM cAMP by using the synthetic peptide Kemptide as substrate. (A) Strain S7-5D, with a wild-type *BCY1* gene. (B) *bcy1* disruptant S17-5. (C) Data were obtained by adding 100 ng of *BCY1* protein to the fractions from S17-5 and by assaying as described in the text. *BCY1* protein was purified from an *E. coli* expression system as described in Materials and Methods. NaCl concentrations were determined from fraction conductivities. kcpm, Kilocounts per minute.

unable to sporulate, a phenotype previously seen with *bcy1-1* homozygous diploids (20).

DISCUSSION

We have cloned and sequenced the *BCY1* gene of the yeast *S. cerevisiae*. The predicted amino acid sequence of the encoded protein is in excellent agreement with the amino acid sequence determined from the purified cAPK regulatory

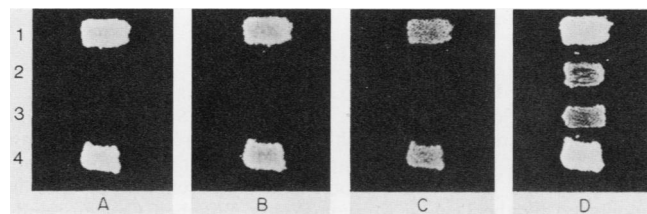


FIG. 4. Phenotypes of *bcy1* disruptant strains. Two-day-old patches of strains on a YPD (39) plate were replica plated to the following media. (A) A YPD plate, which was immediately incubated at 55°C for 30 min and then cultured at 30°C overnight (test for heat shock sensitivity). (B) YNB-N (39), a minimal medium with all sources of nitrogen omitted. After 7 days at 30°C, this plate was replica plated to YPD and incubated at 30°C for 24 h (test for nitrogen starvation sensitivity). (C) YPA (39), a rich medium with 2% potassium acetate as a carbon source (test for carbon sources utilization). (D) YPD, which was incubated at 30°C (control). Strains shown are (row 1) SP1, a wild-type strain; (rows 2 and 3) TTS121 and TTS122, respectively, which are haploid disruptants of *BCY1*; and (row 4) TTS5501, a diploid heterozygous for disruption of *BCY1*.

subunit. Moreover, addition of the purified *BCY1* product made in *E. coli* to fractions from *bcy1* cells restores a cAPK activity. Several lines of evidence suggest that *BCY1* encodes the only regulatory subunit in *S. cerevisiae*. Biochemical evidence described previously (21) and in this paper indicates that kinase activity in extracts from cells lacking a functional *BCY1* gene is wholly unresponsive to cAMP. In the many genetic screens performed in this laboratory, no mutant with the characteristics expected of a second regulatory subunit has appeared. The cAMP affinity column used to purify *BCY1* protein from yeast cell extracts yielded only *BCY1* protein. Finally, drastic overproduction of cAMP in yeast cells produces a phenotype virtually indistinguishable from that of *bcy1* disruptants (15, 16; J. Nikawa, et al., unpublished data). Nevertheless, we cannot completely exclude the existence of a second gene which may encode a minor regulatory element.

Mutations in *BCY1* were originally isolated as bypass mutations of cAMP-requiring, or *cyr*, yeast strains (21). A partially dominant mutation, *CYR3*, was isolated by Uno and co-workers (40). Cells with the *CYR3* mutation do not synthesize a wild-type cAPK regulatory subunit, but synthesize instead a regulatory subunit with a lower affinity for cAMP and an altered mobility in two-dimensional gels. In the *bcy1-1* *CYR3* double mutant, as in *bcy1-1* cells, no binding of the cAMP photoactivatable analog 8-azido cAMP was observed. Because of this finding, Uno and co-workers suggested that *BCY1* was required for the production of the regulatory subunit, which they postulated was encoded by the *CYR3* gene (40). Since we have shown that it is *BCY1* which encodes the structural gene for the cAPK regulatory

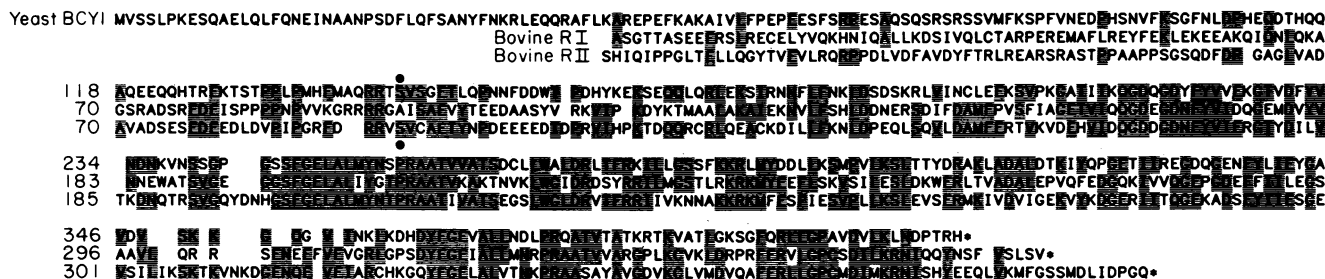


FIG. 5. Sequence comparison of the yeast *BCY1* protein and bovine RI and RII regulatory subunits. Amino acid identities are shown (■). The asterisk at serine 145 of the yeast *BCY1* protein indicates the site of phosphorylation by the catalytic subunit of the kinase. The sequences of RI and RII are from Titani et al. (38) and Takio et al. (36). Sequences were compared by the method of Dayhoff et al. (9).

subunit, their suggestion cannot be correct. It is possible, however, that *CYR3* encodes a product which covalently modifies the regulatory subunit.

We have compared the amino acid sequence of the yeast cAPK regulatory subunit (*BCY1*) with those of the bovine regulatory subunits RI and RII (Fig. 5). Although the yeast and bovine regulatory subunits were very similar overall (40% cumulative identity), there was great structural divergence at the N terminus. The *BCY1* protein contained 47 more amino acids at the N terminus than did the bovine proteins. Peptide sequencing of the *BCY1* protein purified from yeast extracts included this additional segment, indicating that it was present in the functional regulatory subunit. As is the case for RI and RII (36, 40), the yeast cell regulatory subunit can be considered to comprise three domains, including an amino-terminal 160-amino-acid domain associated with dimerization and phosphorylation and two regions having the characteristics of internal gene duplication. An amino-terminal domain of *BCY1* is homologous to the N-terminal domains of both RI and RII and includes a site of phosphorylation by the catalytic subunit at serine 145. This serine residue is phosphorylated by the cAPK holoenzyme (J. D. Scott, et al., unpublished data). Residues surrounding this site are likely to be essential for interaction with the catalytic subunit, since homologous structures in RI, RII, and PKI, the heat stable inhibitor of the mammalian cAPK, are important for modulation of the catalytic subunit of the kinase (27, 32, 37; J. D. Scott, M. B. Glaccum, E. H. Fischer, and E. G. Krebs, Proc. Natl. Acad. Sci. USA, in press). Residues 194 to 295 and 302 to 412 of the *BCY1* product are 35% identical. In the mammalian regulatory subunits, these domains have been shown to bind one molecule of cAMP each (28, 29, 45), and it is reasonable to assume the same is true in yeasts. Thus, the yeast cell cAPK regulatory subunit appears to have the same overall primary structure as its mammalian counterparts. This similarity is perhaps not surprising, since the yeast cell regulatory subunit is capable of interacting with and regulating the bovine catalytic subunit (12). However, the constraints on the evolution of the cAPK regulatory subunit have not been as strict as those on some proteins, such as histones (6, 34, 42), β -tubulin (25), actin (11, 26), and the cytochromes (25, 33).

Recent reports have suggested functions for the regulatory subunit independent of its regulation of kinase activity. The RII regulatory subunit has been reported to have topoisomerase activity (7) and to inhibit Mg(II)-ATP-dependent phosphoprotein phosphatase (14). There is considerable homology between the cAMP-binding domains of the regulatory subunits and the *E. coli* cAMP-binding catabolite activator protein (1, 8, 44). This activator protein

directly regulates gene expression by binding to specific sites in DNA, and some workers have suggested an analogous function for the regulatory subunits in gene expression (24, 43). Thus, there is uncertainty as to whether all the effects of cAMP are mediated through the interaction of the regulatory and catalytic subunits of the cAPK. The genetic analysis of the yeast genes that encode the regulatory and catalytic subunits of the cAPK may help to resolve some of these questions.

We have confirmed the *in vivo* role of the *BCY1* gene product in cellular regulatory processes. Cells lacking a functional *BCY1* gene do not survive heat shock or nitrogen starvation. We attribute these defects to an inability of *bcy1* cells to properly attain a G_1 -phase growth arrest state. These phenotypes are similar to those previously described by Matsumoto and co-workers for their *bcy1-1* allele (20). However, strains containing a disrupted *bcy1* are much more sensitive to restricted growth conditions than those containing the *bcy1-1* allele. Our strains with complete disruptions of *BCY1* could not grow on any carbon source tested other than glucose and displayed severe defects in germination. Based on the data presented, we cannot decide whether all the phenotypic defects of *bcy1* strains were due entirely to the unbridled action of the cAPK catalytic subunit or whether some of these effects were a direct consequence of the loss of the *BCY1* gene product itself. Results from work in progress with the cloned genes encoding the catalytic subunits strongly suggest that the phenotypes resulting from disruption of the regulatory subunit are due to the resulting activation of the catalytic subunits.

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