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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, RAS2val19, causes elevated and improperly regulated adenylate cyclase activity. Strains containing RAS2val19 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 Sau3AI partial digest inserted into the unique BamHI 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-32P]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) NH4HCO3 (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-
TABLE 1. S. cerevisiae strain descriptions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM203-1B</td>
<td>MATα his7 bcy1-1</td>
<td>K. Matsumoto</td>
</tr>
<tr>
<td>M76-3C</td>
<td>MATα leu2 his5 cyr1-1</td>
<td>J. Szostak</td>
</tr>
<tr>
<td>T58-B</td>
<td>MATα leu2 his3 bcy1-1</td>
<td>Segregant from AM203-1B/M76-3C</td>
</tr>
<tr>
<td>T16-11A</td>
<td>MATα his1 leu2 ural3 trpl bcy1-1</td>
<td>Segregant from T58-B/KPPK-1D</td>
</tr>
<tr>
<td>TTS121</td>
<td>MATα his1 leu2 ural3 trpl ade8 can1 bcy1::URA3</td>
<td>Transformant of SP1 with BamHI fragment of pbcyl::URA3</td>
</tr>
<tr>
<td>TTS122</td>
<td>MATα his1 leu2 ural3 trpl ade8 can1 bcy1::URA3</td>
<td>Transformant of SP1 with BamHI fragment of pbcyl::URA3</td>
</tr>
<tr>
<td>SP1</td>
<td>MATα his1 leu2 ural3 trpl ade8 can1</td>
<td>39 Cold Spring Harbor Laboratory</td>
</tr>
<tr>
<td>DC124</td>
<td>MATα his4 leu2 ural3 trpl ade8</td>
<td>39</td>
</tr>
<tr>
<td>KPPK-1D</td>
<td>MATα his3 leu2 ural3 trpl ras1::HIS3</td>
<td></td>
</tr>
<tr>
<td>S7-5D</td>
<td>MATα his3 leu2 ural3 trpl ade8 tpk2::HIS3 tpk3::TRP1m</td>
<td></td>
</tr>
<tr>
<td>SI7-5</td>
<td>MATα his3 leu2 ural3 trpl ade8 tpk2::HIS3 tpk3::TRP1b bcy1::LEU2m</td>
<td></td>
</tr>
<tr>
<td>TTS5501</td>
<td>MATα/MATα his3/* his4/* leu2/leu2 ural3/ural3 trpl/trpl ade8/adde8 can1/* bcy1::URA3/*</td>
<td></td>
</tr>
<tr>
<td>TTS5501</td>
<td>MATα his3 leu2 ural3 trpl ade8 can1</td>
<td></td>
</tr>
</tbody>
</table>

* 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.

Preparation of extracts for assays of cAPK activity. One liter of yeast cells was grown to approximately 10⁸ 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 μg of soybean trypsin inhibitor per ml by passage through a French press at 12,000 lb/ft². Lysates were spun at 20,000 × g for 1 h. Supernatants were loaded onto columns of DEAE-Sepharose (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 μl, 50 mM MOPS (3-[N-morpholino]propanesulfonic acid; pH 7.0), 10 mM MgCl₂, 250 μg of bovine serum albumin per ml, 100 μM [γ-³²P]ATP at 200 cpm/pmol, 150 μM Kemptide, 5 μl of extract, and, where indicated, 10 μM 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 μl of ATP-Kemptide-cAMP to 35 μl of enzyme-buffer solution. Reactions were terminated after 8 min at 30°C by spotting 5 μl 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-
RESULTS

Cloning the BCYI locus. The bcyl mutations were originally isolated by Matsumoto and co-workers (21). Biochemically, cells with bcyl mutations do not appear to synthesize a functional regulatory subunit of the cAPK (21). It therefore seemed plausible that BCYI encoded a cAPK regulatory subunit (see Discussion). We set about to clone BCYI by complementation screening. The bcyl 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 bcyl in AM203-1B as bcyl-I. These strains of S. cerevisiae have numerous phenotypic defects, including sensitivity to starvation (19) and heat shock, which are consequences of the bcyl-I 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 bcyl-I mutation, and disruptions of this locus fell into the same complementation class as bcyl-I. We have, therefore, cloned the BCYI locus.

BCYI encodes cAPK regulatory subunit. Subcloning experiments indicated which region of the BCYI 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 BCYI 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 BCYI 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 BCYI 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 BCYI encodes a regulatory subunit of the cAPK in S. cerevisiae. To determine the effect of BCYI disruption on cAPK activity, extracts were prepared from cells containing a disrupted bcyl 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 bcyl disruption and
three functional catalytic subunits. Strains with a disrupted bcyl locus were constructed as described below. The extracts were fractionated on a DEAE-Sephacl column, and fractions were assayed for cAP kinase activity by using the phosphatase acceptor peptide Kemptide as substrate. The results for strains containing a functional TPK1 gene but lacking TPK2 and TPK3 are shown in Fig. 3. Cells containing a wild-type TTP1 gene had a wild-type pattern on acetate, but that pattern was dependent on TTP1 expression in each strain. The phenotype of the TTP1 gene was determined by a cAMP-dependent cAPK activity that is regulated by the cAMP-dependent protein kinase A (cAPKA).

**Phenotypes of TTP1 gene disruptions.** Strains carrying the bcyl-1 mutation isolated by Matsumoto and co-workers (19) have a number of distinguishing phenotypic features, including no G1 (first gap phase) arrest during starvation, sensitivity to starvation, and sensitivity to heat shock. Since the exact molecular basis for this behavior is unknown, we decided to examine the function of TTP1 by studying the phenotypes of cells carrying a bcyl-1 gene with a known disruption. For this purpose, we used the plasmid pbcyl:URA3, which contains the URA3 gene within the coding domain of the TTP1 gene (Fig. 1). The BamHI DNA fragment was used to transform diploid ura3::ura3 strains from Ura" prototrophy. Southern hybridization confirmed in each case that integration of the fragment occurred within one copy of the BCYJ gene, thus presumably completely disrupting the function of that gene. Diploid strains carrying a single disrupted locus were then sporulated, dissected, and germinated on rich medium. From seven tetrad, 14 Ura" and therefore bcyl-disrupted, spores were expected, but none were obtained. Each tetrad produced only two viable Ura" spores. The observation that haploid strains carrying the bcyl-1 allele are viable (21), led us to attempt the direct transformation of a haploid strain carrying a bcyl disrupted locus. Normal frequencies of Ura" transformants were obtained. Transformants were isolated, and disruption of the bcyl-1 locus was confirmed by Southern hybridization (data not shown). This finding suggests that BCYJ is not an essential gene product, but that spores which lack it germinate poorly or not at all. This conclusion is supported by work with strains containing mutant TPK alleles (S. Cameron, T. Toda, and M. Wigler, unpublished data). In this strain background, spores with disrupted bcyl genes germinated as efficiently as wild-type spores.

Wild-type strains with disrupted bcyl 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 bcyl strains were sensitive to heat shock and nitrogen starvation and were unable to grow on acetate. Additional experiments indicated that these bcyl 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 bcyl 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-Sepharose 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) bcyl 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 bcyl-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 subunit. Moreover, addition of the purified BCY1 product made in E. coli to fractions from bcyl 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 bcyl 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 bcyl-1 CYR3 double mutant, as in bcyl-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
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, 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), b-tubulin (25), actin (11, 26), and the cyt ochromes (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 catalobolite 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 G1-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 allele 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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LITERATURE CITED