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Expression in *Escherichia coli* of *BCY1*, the Regulatory Subunit of Cyclic AMP-dependent Protein Kinase from *Saccharomyces cerevisiae*

PURIFICATION AND CHARACTERIZATION*

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The regulatory (R) subunit of cAMP-dependent protein kinase from the yeast *Saccharomyces cerevisiae* was expressed in *Escherichia coli* by engineering the gene for yeast R, *BCY1*, into an *E. coli* expression vector that contained a promoter from phage T7. Oligonucleotide-directed mutagenesis was used to create an *NdeI* restriction site at the natural ATG of the yeast R. This facilitated construction of the T7 expression vector so that the sequence of the protein produced was identical to the natural R subunit. Yeast R was highly expressed in a soluble form. 20 mg of purified yeast R was obtained from 4 liters of *E. coli*. N-terminal amino acid sequencing revealed that the expressed protein began with the natural sequence. 60% of the molecules contained an N-terminal methionine, and 40% initiated with valine, the second amino acid of yeast R.

The protein produced in *E. coli* migrated on a sodium dodecyl sulfate-polyacrylamide gel with an M_r of 52,000. The yeast R bound 2 mol of cAMP/mol of R monomer with a K_d of 76 nM. The protein was treated with urea to remove bound cAMP. Sedimentation values before and after the urea treatment were identical ($s_{20,w}$ = 5.1). Addition of purified R subunit to a preparation of yeast C subunit (*TPK1*) rendered catalytic activity cAMP-dependent with an activity ratio of 4.6. The yeast R was autophosphorylated by yeast C to a level of 0.8 mol of phosphate/mol of R monomer. By these criteria, the R subunit produced in *E. coli* was structurally and functionally identical to the natural yeast R subunit and similar to mammalian type II R subunits.

The regulation of cellular processes by cAMP has been demonstrated in both prokaryotes (1) and eukaryotes (2) and thus represents a fundamental biological control mechanism. In *Escherichia coli*, cAMP participates in gene regulation. The primary cAMP binding protein in *E. coli* is the catabolite activator protein (CAP)[1] (3). In eukaryotes, cAMP serves as a second messenger to control processes such as glycogenolysis, fatty acid synthesis (4), and as recently reported, gene expression (5). The primary intracellular cAMP binding protein in eukaryotes is the regulatory subunit of cAMP-dependent protein kinase. In most eukaryotic systems studied, cAMP-dependent protein kinase is a tetramer comprised of two catalytic (C) subunits and two regulatory (R) subunits (6). An exception is *Dictyostelium discoideum*, in which cAMP-dependent protein kinase consists of a heterodimer of one C and one R subunit (7). Biochemical studies on purified cAMP-dependent protein kinases have demonstrated that, in the absence of cAMP, the R subunit functions to inhibit the activity of the C subunit. Upon addition of cAMP, the R subunits bind cAMP and the holoenzyme dissociates into two (active) catalytic subunits and a dimer of regulatory subunits. Structural studies on the isolated subunits have localized functional domains within each protein (8). The R subunit, for example, has been shown to consist of three clear domains: a dimerization domain in the N-terminal region, a "hinge" region containing a substrate consensus sequence for the C subunit, followed by the cAMP binding domain. The cAMP binding domain has been further characterized by comparison with the structure of the *E. coli* CAP protein (9).

Previously, Hixon and Krebs (10) described the purification and characterization of the R subunit from the yeast *Saccharomyces cerevisiae*. Matsumoto and co-workers (11) isolated mutant yeast strains that required cAMP for growth. Subsequently, suppressors of this mutation were isolated. Of these, the *bcy1* mutation resulted in a reduced level of regulatory subunit (12). Recently, Toda *et al.* (13) and Kunisawa *et al.* (14) cloned the *BCY1* gene. The predicted coding sequence of the *BCY1* gene was homologous to mammalian regulatory subunit and matched N-terminal protein sequence data of yeast R subunit determined by Hixon and Krebs (10). Toda *et al.* (13) further demonstrated that *BCY1* encoded the R subunit using additional protein sequence data from isolated yeast R subunit as well as biochemical analysis of yeast strains lacking the *BCY1* gene. Unlike higher eukaryotes (15, 16), genetic and biochemical evidence suggests that *S. cerevisiae* have only one R subunit gene. In contrast, yeast appear to have three genes, *TPK1-3*, that encode C subunits.[2] The isolation of the genes for both R and C subunits has made it possible to probe structure/function questions of cAMP-dependent protein kinase by molecular biological techniques.

Until recently, characterization of functional domains in cAMP-dependent protein kinase was approached primarily by biochemical means, for example by affinity labeling (17), substrate analogs (18), and limited proteolysis (8). Currently, the comparison of protein structure by computers (19) and the modification of proteins through site-directed mutagene-

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[1] The abbreviations used are: CAP, catabolite activator protein; R subunit, regulatory subunit; C subunit, catalytic subunit; SDS, sodium dodecyl sulfate; IPTG, isopropyl thiogalactoside; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

[2] T. Toda and M. Wigler, unpublished data.

sis (20, 21) have provided new ways to characterize structure/function relationships of proteins. Yeast provides a powerful eukaryotic system to address questions of protein structure and function (22). Its major feature is the ability to study the effect of "altered" proteins in the absence of the wild type by the technique of "gene replacement," by which a wild type gene is replaced in the chromosome with a mutated gene (23).

In this paper, we report the expression of the *BCY1* gene in *E. coli*, purification of the *BCY1* protein (yeast R subunit), and characterization of the isolated protein. Our studies indicate that the protein produced in *E. coli* is structurally and functionally identical to the natural yeast protein. These studies establish the system we will use for *in vitro* analysis of mutations to be constructed subsequently. The *in vivo* effect of these mutated proteins will be studied in parallel by introducing the mutagenized gene into yeast.

EXPERIMENTAL PROCEDURES

Materials—cAMP, riboATP, and histone IIB were purchased from Sigma. [^{3}H]cAMP was obtained from Amersham Corp. cAMP-agarose (type 3), Sephadex G-50, and Sephacryl-200 were purchased from Pharmacia P-L Biochemicals. Kemptide was purchased from Peninsula Laboratories, Inc. Reagents used for cloning were molecular biology grade. Restriction endonucleases and T4 DNA ligase were purchase from New England Biolabs. *E. coli* DNA polymerase (large fragment) was obtained from Bethesda Research Laboratories.

Strains—*E. coli* JM101 (Δ*lacpro*, supE, thi1, F′, proAB$^+$, *laci*^{q}, *lacZ* ΔM15, traD36), and MV1193 were used for phagemid propagation and oligonucleotide-directed mutagenesis. These were gifts from J. Messing (Waksman Institute, Piscataway, NJ). *E. coli* MM294 (F$^-$, endA1, hsdR17 (r_k^-, M_k^+) supE44, thi1, λ$^-$) was used for plasmid constructions and isolations. *E. coli* BL21 (DE3) (F$^-$ *hsdS gal*) contained phage DE3 as a lysogen (24). This phage contains the T7 gene 1 (RNA polymerase) under *lac*UV5 control cloned into the int gene. This strain was the gift of W. Studier (Brookhaven National Laboratories, Upton, NY).

Vectors—The parent T7 expression vector, pAR3040, was a gift from W. Studier.[3] pAR3040 was modified to delete the region between the *Sal*I and *Pvu*II sites. The *Sal*I site was destroyed as well. In addition, the *Hin*dIII site was removed by cleavage with *Hin*dIII followed by DNA polymerase fill in and ligation with DNA ligase. A *Hin*dIII linker was then inserted at the *Bam*HI site. pUC118 and pUC119 phagemid vectors were provided by J. Messing (Waksman Institute). For a discussion of phagemids, see Mead *et al.* (25).

Oligonucleotide-directed Mutagenesis—A unique *Nde*I site was engineered into the *BCY1* gene at the initiator methionine. A 3.0-kilobase *Bam*HI to *Eco*RI fragment was inserted into pUC118. Single-stranded DNA was prepared by superinfection with M13KO7 (supplied by J. Messing) and was used as template for oligonucleotide-directed mutagenesis according to Zoller and Smith (26). A 23-mer (5′TAAGAATAAACATATGGTATCTT) was used to construct the *Nde*I site (underlined). Screening for the desired mutation was accomplished by colony hybridization using the mutagenic oligonucleotide as a probe (27). The mutation was verified by DNA sequencing (28).

Construction of pT7/BCY1—As depicted in Fig. 1, a 2.1-kb *Nde*I-*Hin*dIII fragment containing the *BCY1* coding sequence was ligated into *Nde*I and *Hin*dIII-digested pT7 backbone. As described above, the *Nde*I site was engineered into the *BCY1* gene at the natural ATG by changing the sequence ACGATG to CATATG by oligonucleotide-directed mutagenesis. The T7 promoter is 65 nucleotides upstream of the ATG. The ligation mixture was transformed into MM294, and transformed colonies were selected on LB-ampicillin plates (29). Plasmid DNA was prepared from 12 of the resulting colonies and checked for the desired construction by restriction digest mapping and DNA sequencing. Basic cloning procedures were accomplished using procedures described in Maniatis *et al.* (29).

Expression in E. coli—pT7/*BCY1* was transformed into BL21 (DE3) (29). Several transformants were individually grown in LB + ampicillin overnight at 37 °C. 100 μl were added to 5 ml of fresh LB + ampicillin. When the culture reached A_{600} = 0.5, isopropyl thiogalactoside (IPTG) was added to 0.5 mM final concentration, and growth was continued at 37 °C. After 3 h, the cells were harvested by centrifugation, resuspended in 750 μl of SDS sample buffer, and a 20-μl aliquot was electrophoresed on a 12.5% polyacrylamide-SDS slab gel. One of the transformants that exhibited a new protein of the expected size was characterized further. For large scale expression, 2.5 ml of an overnight culture was added to 4 liters of M9 (29) + 100 μg/ml ampicillin. IPTG was added to 0.25 mM when the culture reached A_{600} = 0.5. Growth continued for 3 h and then the cells were harvested.

Purification of BCY1—Purification of yeast R subunit followed a modification of the standard procedure for R subunit (8, 10). Cells from 4 liters (approximately 30 g) were washed with 50 ml of Buffer A (50 mM potassium phosphate, pH 6.5, 100 mM sodium chloride, 1 mM EDTA, 0.1 mM EGTA, 5% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride), harvested by centrifugation at 4 °C, and then resuspended in 10 ml of Buffer A + 1 mg/ml lysozyme. The solution was kept on ice for 30 min, and then the culture was sonicated on ice 5 × 2 min (Branson sonicator, setting 3, microtip). The broken cell extract was transferred to a 15-ml Corex tube and then centrifuged for 15 min at 8000 rpm in an SS-34 rotor at 4 °C. The supernatant was brought to 15% ammonium sulfate, incubated on ice for 60 min, and then centrifuged for 30 min at 8000 rpm in an SS-34 rotor. Following this, the 15% supernatant was diluted to 20 ml with Buffer A, added to 5 ml of cAMP-agarose (Pharmacia P-L Biochemicals type 2), and incubated at 4 °C for 18 h with gentle rotation. The solution was poured into a 2.5-cm wide × 10-cm column to collect the agarose. After collecting the flow-through, the column was washed with 200 ml of Buffer A, 200 ml of Buffer A containing 1 M sodium chloride, and then 50 ml of Buffer A. Yeast R was eluted with 4 × 5 ml of 10 mM cAMP in Buffer A. Bound cAMP was removed by urea treatment according to Builder *et al.* (30). Following this, the protein was concentrated by vacuum dialysis and applied to a 1.5 × 47-cm Sephacryl-200 column equilibrated in Buffer A. 1-ml aliquots were collected and analyzed by absorbance at 280 nm and for [^{3}H]cAMP binding (see below). 10-μl aliquots were analyzed on a 12.5% SDS-polyacrylamide gel. Fractions were pooled from the central part of the protein peak. We have found that the protein can be eluted from the cAMP-agarose column using 10 mM cGMP instead of cAMP. Dialysis of the eluted protein against 4 × 2 liters of Buffer A effectively removes unbound and bound cGMP from the sample.

Expression of Yeast C Subunit (TPK1)—Yeast C subunit was expressed in *E. coli* using the T7 system described above.[4] Briefly, the *TPK1* gene was engineered into pT7 and expressed in 200 ml of BL21 (DE3). The cells were resuspended in 1 ml of Buffer A + lysozyme, sonicated as described for yeast R subunit, and then centrifuged at 4 °C for 10 min at 12,000 rpm in an Eppendorf centrifuge. The supernatant fraction was used for catalytic activity. This extract was approximately 10 mg/ml total protein, of which approximately 0.02 mg/ml represented yeast catalytic subunit. Control experiments demonstrated that extracts prepared from cells that did not contain the T7/*TPK1* expression vector contained no detectable phosphotransferase activity when using Kemptide, histone, or yeast R subunit as a substrate.

Sedimentation Coefficient, $s_{20,w}$—Sucrose gradient centrifugation was performed using a 5–20% sucrose gradient in Buffer A in 5-ml polyallomer tubes. Centrifugation was carried out for 40 h at 24,000 rpm at 4 °C in a SW 50 rotor. 0.1 mg of purified R subunit before and after urea treatment and duplicate sets of protein standards (0.1 mg of each protein) were centrifuged. Gradients were fractionated using a Buechler autofractionator. 0.2-ml aliquots were collected, and 10-μl aliquots were analyzed on a 12.5% SDS-polyacrylamide slab gel. Standards were bovine catalase (11.0), yeast alcohol dehydrogenase (7.4), and bovine serum albumin (4.3).

cAMP Binding Constant—cAMP binding to yeast R subunit was measured by the filter binding method of Sugden and Corbin (31). By this method, the stoichiometry of binding was shown to be the same as that determined by equilibrium dialysis. 0.16 μg of protein was incubated with various amounts of [^{3}H]cAMP (specific activity, 6780 cpm/pmol) in 50 mM potassium phosphate (pH 6.8), 1 mM EDTA, 0.5 mg/ml histone, 2 M NaCl in a total volume of 100 μl. The solution was incubated for 60 min at 30 °C and then quantitatively transferred to a vacuum filtration reservoir containing 5 ml of ice-cold wash buffer (10 mM potassium phosphate, 1 mM EDTA, pH 6.8). The protein was bound on nitrocellulose filters (Millipore HAWP) by application of vacuum. The filters were washed with an additional 25 ml of wash buffer, air dried, and then placed in scintillation vials containing 7 ml of Aquasol-2 (Du Pont-New England Nuclear). Filters

[3] W. Studier, submitted to *Gene* (*Amst.*) for publication.

[4] M. Zoller, manuscript in preparation.

were counted using a Beckman scintillation counter. K_d, the apparent binding constant, was calculated from a Scatchard plot (32).

SDS-Polyacrylamide Gel Electrophoresis—SDS slab gels were cast using a Bio-Rad apparatus and solutions according to Laemmli (33). Samples were mixed with an equal volume of 2 × SDS sample buffer (0.1 M Tris-HCl (pH 6.8), 20% glycerol (v/v), 4% SDS (w/v), 5% mercaptoethanol (v/v), and 0.02% bromphenol blue (w/v)), heated at 95 °C for 3 min, applied to gels, and electrophoresed for 3 h at 25 mA. Gels were stained with 0.05% Coomassie Blue in acetic acid/methanol/water (10:10:80) and destained with acetic acid/methanol/water. Molecular weight standards used were bovine serum albumin (66,200), ovalbumin (43,000), and cytochrome *c* (12,300).

Assay for Catalytic Activity—Phosphotransferase activity of yeast C subunit (*TPK1*) was measured exactly as described by Roskoski (34) using Kemptide as a substrate. Extracts containing yeast C subunit were prepared by expression of the *TPK1* gene in *E. coli* using the T7 expression vector as described above. Holoenzyme was formed by the addition of a 10-μl aliquot of C subunit with 0.5 μg of purified R subunit in 50 mM MOPS (pH 6.5), 10 mM $MgCl_2$, and 100 μg/ml bovine serum albumin in a total volume of 50 μl. The mixture was placed at 4 °C for 2 h, and then 10 μl were assayed for phosphotransferase activity at 15 °C in the presence and absence of 10^{-5} M cAMP. (The assay was performed at 15 °C in order to keep the assay in the linear range over this time interval.) At indicated time intervals, aliquots (25 μl) were removed and spotted onto 1 × 2-cm P-81 paper strips (Whatman). The filter papers were washed 3 × 2 min in 250 ml of 75 mM phosphoric acid, air dried, placed in scintillation vials, and then counted in Aquasol-2.

Phosphorylation of R Subunit by TPK1—A reaction mixture was prepared as described above for phosphotransferase assay with R subunit substituting for Kemptide. The specific activity of ATP for these studies was increased to 4000 cpm/pmol. Aliquots (25 μl) were removed at specified time points, spotted onto P-81 paper, and processed as described above. Duplicate aliquots were removed, added to 25 μl of 2 × SDS sample buffer, and electrophoresed on an 12.5% SDS-polyacrylamide gel. The gel was stained, destained, dried, and then placed with XAR-5 film for 18 h at −70 °C with a Du Pont screen.

DNA Sequencing—DNA sequencing was performed according to Sanger *et al.* (28) using phagemid vectors as described in Mead *et al.* (25). Specific oligonucleotide primers were synthesized to sequence the *Nde*I mutation and the construction of the expression vector in the T7 vector. Oligonucleotides were synthesized using an Applied Biosystems 380A DNA synthesizer.

Protein Sequencing—N-terminal sequence analysis was performed on the purified R subunit according to Hunkapillar *et al.* (35) using an Applied Biosystems protein sequenator, 0.2 mg of purified protein was dialyzed *versus* 0.5 M ammonium bicarbonate and then lyophilized to dryness.

Protein Concentration—Protein concentration was determined by the method of Bradford (36) using lysozyme as a standard.

RESULTS

Expression of BCY1 in E. coli—The coding region of *BCY1* was cloned into a novel expression vector that uses the promoter from phage T7 (24). The location of the initiator methionine codon in the *BCY1* gene was known from protein sequence of purified R subunit from *S. cerevisiae* (10, 13). Fig. 1 shows the scheme by which the expression vector was constructed. In order to produce the natural protein, oligonucleotide-directed mutagenesis was performed to place an *Nde*I restriction endonuclease site at the start codon. The correct construction was verified by DNA sequencing. An *Eco*RI site at the 3′ end of the gene was converted to a *Hin*dIII site by insertion of a linker. Thus, the *BCY1* coding sequence could be inserted into the T7 expression vector to produce the natural protein. This plasmid was introduced into *E. coli* BL21 (DE3) which harbors the gene for T7 RNA polymerase in the *E. coli* chromosome. As depicted in Fig. 2, IPTG stimulates the production of the T7 RNA polymerase, which in turn transcribes the *BCY1* gene. Fig. 3 compares total cellular extracts from control cells without pT7/*BCY1* and cells containing pT7/*BCY1*. The cells were induced with IPTG and then lysed under nondenaturing conditions, and

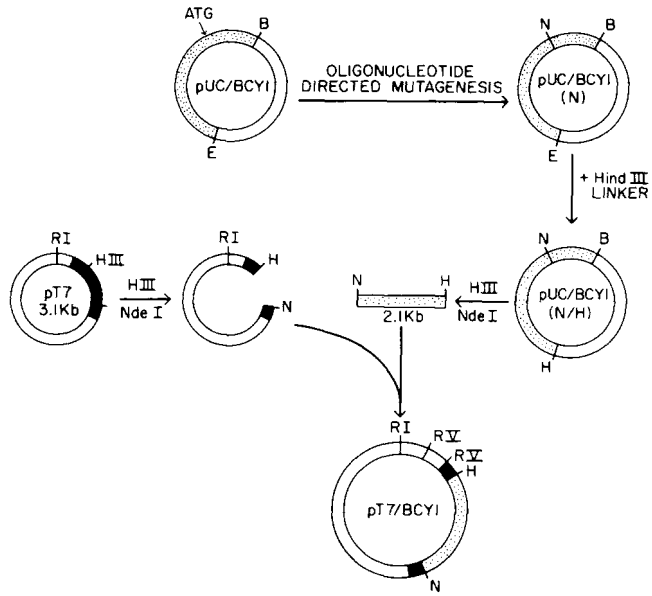
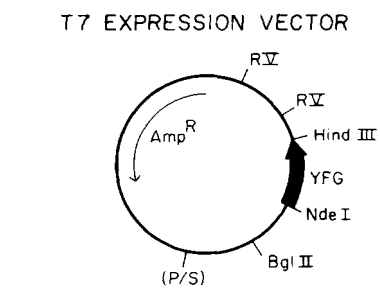


FIG. 1. **Construction of pT7/*BCY1* expression vector.** Oligonucleotide-directed mutagenesis was used to construct an *Nde*I site at the natural ATG of the yeast R subunit. *Hin*dIII site was placed at the 3′ end of the *BCY1* gene by conversion of an *Eco*RI site. The 2.1-kilobase *Nde*I-*Hin*dIII fragment containing the *BCY1* coding sequence was inserted into pT7. This construction produces the natural R subunit protein. *N*, *E*, and *H* represent sites for *Nde*I, *Eco*RI, and *Hin*dIII restriction enzymes, respectively.



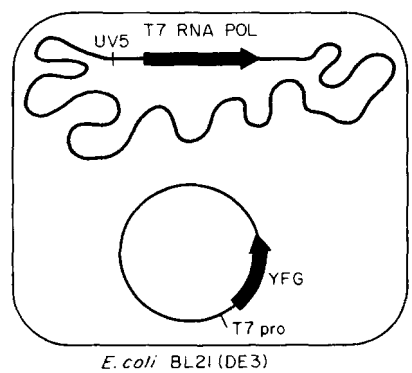


FIG. 2. **Expression of proteins using the pT7 system (24).** The expression vector pT7/*BCY1* was inserted into *E. coli* BL21 (DE3), which contains the gene for T7 RNA polymerase under the control of lac UV5 promoter. Only T7 RNA polymerase will recognize the T7 promoter. In the absence of IPTG, T7 RNA polymerase is not produced. Upon addition of IPTG to the media, induction of the T7 RNA polymerase is induced, which results in transcription of the *BCY1* gene. *YFG* is your favorite gene, *T7 pro* is T7 promoter sequence, and *T7 RNA POL* is the RNA polymerase from T7.

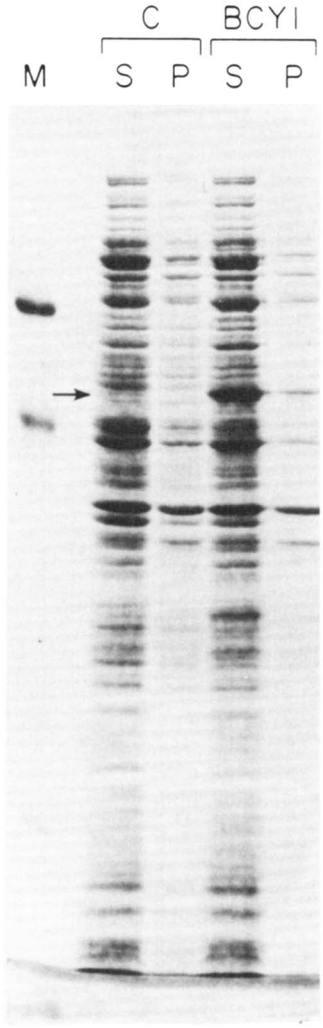


FIG. 3. **Expression of yeast R subunit in *E. coli*.** pT7 without the *BCY1* gene and pT7/*BCY1* expression plasmid were each transformed into BL21 (DE3) and expressed as described under "Materials and Methods." Following induction with IPTG, the cells were harvested, resuspended in 750 μl of lysis buffer, and lysed by sonication. The cell extracts were centrifuged for 5 min at 10,000 × *g* at 4 °C. The supernatants were separated from the pellets, and the pellets were resuspended in 750 μl of buffer. 10-μl aliquots were analyzed by SDS-polyacrylamide gel electrophoresis. *C*, control cells containing pT7 (no insert) supernatant (*S*) and pellet (*P*), respectively. *BCY1*, cells containing pT7/*BCY1* supernatant (*S*) and pellet (*P*). *M* represents markers (bovine serum albumin, ovalbumin, and cytochrome *c*). The *arrow* shows the position of yeast R subunit.

the extracts were separated into supernatant and pelleted fractions. A new protein of approximately 52,000 daltons was observed only in the cells containing the *BCY1* expression plasmid and only in the presence of IPTG (data not shown). The expressed protein was primarily in the soluble fraction and represented approximately 5–10% of soluble protein based on Coomassie staining. In addition, we observed that expression was approximately 2-fold better in minimal M9 media compared to rich LB broth (data not shown).

Purification of Yeast R Subunit—Purification of yeast R from *E. coli* consisted of a modified procedure used for isolation of mammalian R subunit (8, 10). The steps were 1) total cell lysis, 2) 15% ammonium sulfate, 3) cAMP-agarose affinity chromatography, 4) Sephadex G-50 chromatography with urea treatment to remove bound cAMP, 5) Sephadex G-50 chromatography to refold protein and remove urea, and 6) Sephacryl S-200 chromatography to remove small impurities and breakdown products of yeast R. Fig. 4 shows an SDS-polyacrylamide gel of aliquots from each purification step. It should be noted that the urea step was done to yield a cAMP-free preparation of R subunit and that this step can be omitted. Both the urea-treated and untreated R subunit migrated with sedimentation coefficients of 5.1 S (see Fig. 5). This indicated that the protein produced in *E. coli* is a dimer. Approximately 20 mg of purified protein was obtained from 4 liters of cells grown in minimal media. This represents ap-

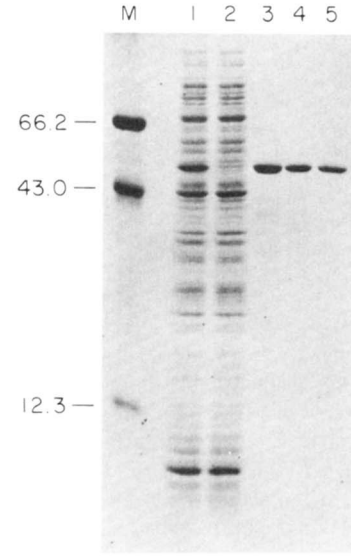


FIG. 4. **Purification of yeast R expressed in *E. coli*.** Yeast R subunit was expressed in 4 liters of M9 and purified as described under "Materials and Methods." Aliquots of each purification step were electrophoresed on SDS-polyacrylamide gel electrophoresis. *Lane 1*, total soluble extract; *lane 2*, non-bind fraction from cAMP-agarose column; *lane 3*, eluted protein from cAMP-agarose column; *lane 4*, urea-treated R subunit following renaturation by Sephadex G-50 chromatography; *lane 5*, yeast R subunit following Sephacryl-200 chromatography.

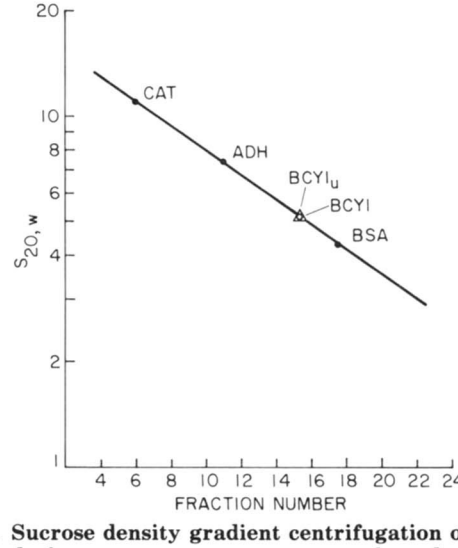


FIG. 5. **Sucrose density gradient centrifugation of yeast R before and after urea treatment to remove bound cAMP.** 100 μl of yeast R (approximately 100 μg) before and after urea treatment were applied to a 5–20% sucrose gradient in 5 ml of buffer A. Centrifugation proceeded for 40 h at 24,000 rpm at 4 °C. Standards were run in duplicate tubes. Following centrifugation, tubes were fractionated in 0.2-ml aliquots, and 10 μl from each fraction were analyzed on a SDS-polyacrylamide gel to locate peak tubes. Standards were bovine catalase (*CAT*), yeast alcohol dehydrogenase (*ADH*), and bovine serum albumin (*BSA*). *BCY1* and $BCY1_u$ are untreated R subunit and urea-treated R subunit, respectively.

TABLE I

Physical characteristics and functional properties of various R subunits

	Skeletal muscle RI[a]	Cardiac muscle RII[b]	*D. discoideum* R[c]	Yeast R[d]	*E. coli*-produced yeast R[e]
Total residues	379	400	327	416	416
Subunit molecular weight (SDS-PAGE)	48,000	55,000	39,000	50,000	52,000
Calculated molecular weight (sequence)	42,804	45,084	36,794	47,226	47,226
Native aggregative state	Dimer	Dimer	Monomer	Dimer	Dimer
Sedimentation coefficient ($s_{20,w}$)	4.7	4.6	3.5	5.0	5.1
mol cAMP bound/mol monomer	2	2	1	1	2
K_d (nM)	0.65	2	4	60–150	76
Inhibits catalytic subunit	Yes	Yes	Yes	Yes	Yes
Phosphorylated by C subunit	No	Yes	No	Yes	Yes

[a] Data obtained from Refs. 30, 39, and 40.
[b] Data obtained from Refs. 30, 41, and 42.
[c] Data obtained from Refs. 7 and 43.
[d] Data obtained from Refs. 10, 13, and 44.
[e] Data obtained from this work.

proximately 70% yield from crude extract. An additional 4 mg were released from the cAMP-agarose column by addition of a solution of 8 M urea.

Characterization of the Purified Protein—Table I lists the properties of the purified yeast R expressed in *E. coli* compared to the properties of mammalian RI and RII, *D. discoideum* R subunit, and natural yeast R subunit. The yeast R protein produced in *E. coli* migrated on an SDS-polyacrylamide gel with a molecular weight of M_r = 52,000. This is somewhat anomalous compared to the calculated molecular weight from the translation of the *BCY1* gene, 47,226 (13), but has been observed for a number of mammalian R subunits (see Table I). A Scatchard plot (Fig. 6) of cAMP binding reveals that the protein produced in *E. coli* binds 2 mol of cAMP/mol of R subunit with a K_d of 76 nM. This plot indicates that the two sites bind cAMP with approximately the same affinity.

Inhibition of Yeast C Subunit by R Subunit—The ability of R subunit expressed in *E. coli* to complex and inhibit yeast C subunit is shown in Fig. 7. Holoenzyme was formed by mixing R and C together, and then phosphotransferase activity was measured in the presence and absence of cAMP. Addition of R subunit to yeast C subunit inhibited catalytic activity. In the presence of cAMP, full catalytic activity was observed. A similar result was observed upon addition of *E. coli*-produced R subunit to purified bovine C subunit (data not shown). In a recent report, Toda *et al.* (13) added *E. coli*-produced R subunit to yeast extracts prepared from strains of *S. cerevisiae* that completely lacked R subunit. In the absence of exogenous

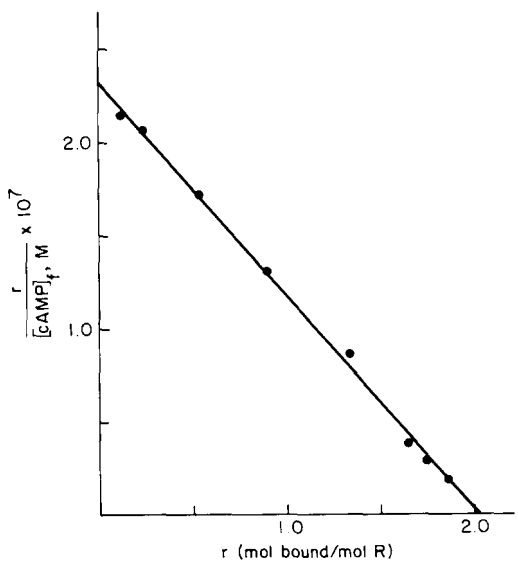


FIG. 6. **Stoichiometry and K_d of cAMP binding.** 0.16 μg of purified R subunit was incubated with various concentrations of [^{3}H] cAMP in 50 mM potassium phosphate (pH 6.8), 1 mM EDTA, 0.5 mg/ml histone, 2 M sodium chloride in a total volume of 100 μl as described under "Materials and Methods." After 1 h at 30 °C, the samples were quantitatively transferred to a vacuum filtration device and bound on Millipore filters as described by Sugden and Corbin (31). The data are plotted as a Scatchard plot according to Fersht (32). Each point represents the average of duplicate assays. r is mol of cAMP bound per mol of R subunit. $[cAMP]_f$ is the concentration of free cAMP. The slope of the line is $-1/K_d$, where K_d is the apparent dissociation constant for cAMP. The maximum stoichiometry of cAMP binding is determined from the intercept with the abscissa.

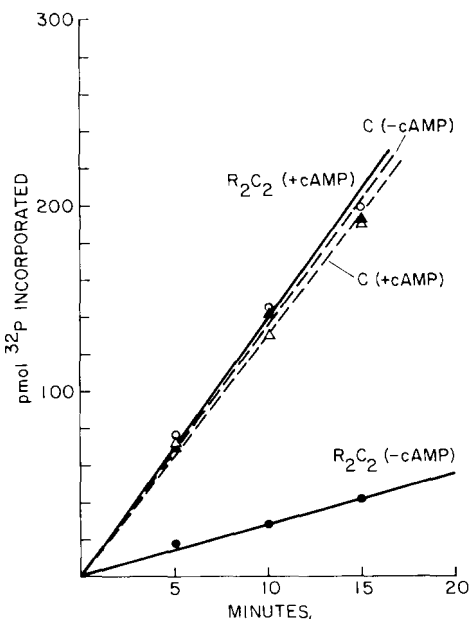


FIG. 7. **Inhibition of yeast C subunit (*TPK1*) by *E. coli*-produced yeast R subunit.** 10 μl of *TPK1* extract (approximately 0.2 μg of yeast C) were incubated alone or with 0.5 μg of yeast R in a total volume of 50 μl containing 50 mM MOPS (pH 6.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μg/ml bovine serum albumin. After 2 h at 4 °C, 10-μl aliquots were removed and assayed for phosphotransferase activity. The C subunit was incubated with the addition of R and then assayed in the presence (○) and absence (●) of cAMP. The C subunit incubated without R was then assayed in the presence (△) and absence (▲) of cAMP. Phosphotransferase activity was measured using Kemptide as described under "Materials and Methods." R_2C_2 is holoenzyme.

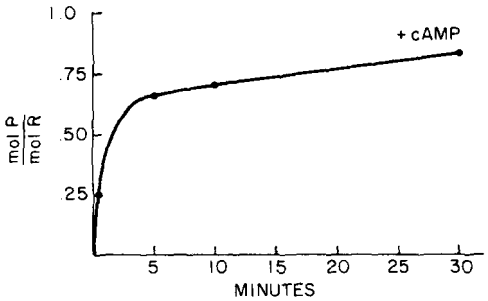


FIG. 8. **Phosphorylation of yeast R by yeast C subunit (*TPK1*).** 0.5 μg of yeast R was incubated with 1 μl of yeast C subunit (approximately 0.02 μg) in a total volume of 100 μl containing 50 mM MOPS, 10 mM $MgCl_2$, 100 μg of bovine serum albumin, 0.1 mM [^{32}P] rATP (4000 cpm/pmol), and 10^{-5} M cAMP. The reaction was initiated by addition of ATP. 10-μl aliquots were withdrawn at the indicated time points, spotted onto P-81 paper, and processed as described for the phosphotransferase assay.

R subunit, protein kinase activity was cAMP-independent. Upon addition of exogenous R subunit, kinase activity was fully cAMP-dependent.

Phosphorylation of Yeast R Subunit by Yeast C Subunit—As demonstrated by Hixon and Krebs (10), yeast R subunit can be phosphorylated by bovine C subunit. Fig. 8 shows a time course of phosphorylation of yeast R subunit by yeast C subunit (*TPK1*). In the presence of cAMP, approximately 0.8 mol of phosphate/mol of R subunit was incorporated. A similar result was obtained using purified bovine heart C subunit (data not shown). Unlike mammalian RII, the migration of yeast R on an SDS gel did not change upon phosphorylation (data not shown).

Protein Sequence Analysis of the E. coli-expressed Protein—N-terminal sequence analysis of the purified protein revealed that 60% of the molecules initiated with methionine and 40% initiated with valine. No other amino acid residue was observed in a significant amount, indicating that the protein without the methionine was resistant to further N-terminal degradation. Each of the following steps showed 2 residues and yielded the same sequence offset by one position. The sequences exactly followed the protein sequence of the yeast regulatory subunit and the translation of the *BCY1* gene as reported by Hixon and Krebs (10) and by Toda *et al.* (13): (Met)-Val-Ser-Ser-Leu-Pro-Lys-Glu-Ser-Gln-Ala-Glu-Leu-Gln-Leu.

DISCUSSION

This paper reports the expression in *E. coli*, purification, and characterization of the *BCY1* protein, the regulatory subunit of cAMP-dependent protein kinase from the yeast *S. cerevisiae*. We used oligonucleotide-directed mutagenesis to engineer the *BCY1* gene into the T7 vector such that translation would start at the natural N terminus. Expression using the T7 vector yielded a high level of yeast R subunit. Approximately 20 mg of purified protein could be isolated from 4 liters of *E. coli*, about 30 g of cells. Hixon and Krebs (10) reported a yield of 10–15 mg of R subunit isolated from 6.8 kg of pressed bakers' yeast. Thus, the *E. coli* expression of yeast R subunit is much more efficient than expression in yeast.

The functional properties of the yeast R subunit expressed in *E. coli* were indistinguishable from the characteristics of the protein isolated from yeast (10). The isolated protein inhibited both the yeast and the bovine catalytic subunits in the absence of cAMP. The protein isolated from *E. coli* was an unphosphorylated form and could be phosphorylated *in vitro*. We did find a discrepancy regarding the stoichiometry of phosphorylation of yeast R subunit by catalytic subunit and the stoichiometry of binding cAMP. Hixon and Krebs (10) reported the incorporation of 2 mol/mol R subunit using bovine C subunit. In contrast, phosphorylation of R subunit by yeast C subunit (*TPK1*) expressed in *E. coli* resulted in the incorporation of approximately 1 mol of phosphate/mol of R subunit. It is possible that bovine C subunit phosphorylates yeast R subunit at two positions, though we feel this is unlikely upon inspection of the amino acid sequence. Recently, the major phosphorylation site of R subunit isolated from yeast and phosphorylated *in vitro* was sequenced.[5] Correlation with the complete protein sequence determined by Toda *et al.* (13) positioned the phosphorylated residue as Ser-145. This is homologous to Ser-95 of bovine RII (41). Site-directed mutagenesis of Ser-145 confirmed that this is the major site of autophosphorylation by yeast C subunit.[4] Further studies are underway to determine the role of this specific phosphorylation. Unlike bovine RII, phosphorylation of the *E. coli*-produced yeast R subunit did not change its electrophoretic mobility on an SDS-polyacrylamide gel.

Hixon and Krebs (10) reported a stoichiometry of binding cAMP of 1 mol/mol monomer. In this case, cAMP binding to yeast R subunit was conducted using the Millipore filtration assay under conditions of low sodium chloride concentration. These conditions have been shown to result in a stoichiometry of binding of only 1 mol of cAMP/mol of R subunit for the mammalian R subunits. The present report used conditions developed by Sugden and Corbin (31) that correctly assays cAMP binding when using the Millipore filtration method. This is the most likely explanation for the discrepancy. It is clear from inspection of the sequence of yeast R subunit that it contains tandemly repeated cAMP binding domains similar to the mammalian proteins.

This report is the second example of expression of R subunit in *E. coli* and the first example of the expression of a "type II-like" R subunit, which can be autophosphorylated. Saraswat *et al.* (37) reported recently the expression of bovine type I R subunit in *E. coli* using an expression vector derived from the plasmid pUC7 (38) that uses the lac promoter. In this case, the RI cDNA was inserted into the polylinker region of the expression vector and resulted in the production of an RI protein with 10 additional amino acids N-terminal of the natural N terminus. While the functional and apparent physical characteristics of the *E. coli*-produced protein were similar to the properties of the natural protein, it is possible that the additional amino acids altered the structure of the protein yet were not detected. In addition, the fusion protein and the natural protein may crystallize in different ways. To circumvent such problems, we used oligonucleotide-directed mutagenesis to construct an expression vector that produces a protein initiating with the natural ATG. Protein sequence analysis confirmed that the expressed protein initiated correctly. 60% of the R subunit still contained the initiator methionine. The remainder of the purified protein contained an N-terminal valine, the natural N terminus of the protein isolated from yeast. We are investigating whether this ratio varies from one preparation to another.

A comparison between the primary sequences of the yeast R subunit and the bovine type I and II R subunits was made previously in Toda *et al.* (13). The yeast and mammalian proteins show approximately 40% sequence identity. Upon inspection of the aligned sequences, the yeast protein can be broken down into the same three domains that comprise the mammalian proteins. 1) The yeast N-terminal region, shown

[5] J. Scott and E. G. Krebs, personal communication.

to function in the mammalian proteins as a dimerization domain, exhibits no homology with its mammalian counterparts. In addition, when aligned with the mammalian proteins, the N terminus of the yeast protein is 40–50 amino acids longer than the N termini of the mammalian R subunits. 2) The "hinge" region of the yeast R subunit is related to the type II R subunits, characterized by the substrate consensus sequence Arg-Arg-*X*-Ser. 3) The C-terminal region consists of tandemly duplicated cAMP binding domains, homologous to the mammalian R subunits as well as the cAMP binding domain of the *E. coli* CAP protein. It is significant to note that, once the yeast and mammalian primary sequences have been aligned for maximal sequence identity, the yeast protein terminates 7 amino acids before the type I protein and 18 amino acids prior to the type II R subunit. This is a significant difference because a major contact for cAMP binding in mammalian R subunit has been proposed to reside within the region missing from the yeast sequence (9). Affinity labeling studies, site-directed mutagenesis, and crystallographic studies are in progress to understand the importance of this region for cAMP binding. In addition, future studies will be aimed at an understanding of the role of phosphorylation of the R subunit both *in vivo* and *in vitro*.

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REFERENCES

1. Zubay, G., Schwartz, D., and Beckwith, J. (1970) *Proc. Natl. Acad. Sci. U. S. A.* **66,** 104–110
2. Steinberg, R. A. (1983) in *Biochemical Actions of Hormones* (Litwak, G., ed) Vol. 11, pp. 25–65, Academic Press, Orlando, FL
3. Ullman, A., and Danchin, A. (1983) *Adv. Cyclic Nucleotide Res.* **15,** 1–53
4. Krebs, E. G., and Beavo, J. A. (1979) *Annu. Rev. Biochem.* **48,** 923–960
5. Waterman, M., Murdoch, G. H., Evans, R. M., and Rosenfeld, M. G. (1985) *Science* **229,** 267–269
6. Walsh, D. A., Perkins, J. P., and Krebs, E. G. (1968) *J. Biol. Chem.* **243,** 3763–3765
7. de Gunzburg, J., and Veron, M. (1982) *Embo J.* **1,** 1063–1068
8. Potter, R. L., and Taylor, S. S. (1979) *J. Biol. Chem.* **254,** 2413–2418
9. Weber, I. T., Steitz, T. A., Bubis, J., and Taylor, S. S. (1987) *Biochemistry* **26,** 343–351
10. Hixson, C. S., and Krebs, E. G. (1980) *J. Biol. Chem.* **255,** 2137–2145
11. Matsumoto, K., Uno, I., Oshima, Y., and Ishikawa, T. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 2355–2359
12. Uno, I., Matsumoto, K., and Ishikawa, T. (1982) *J. Biol. Chem.* **257,** 14110–14115
13. Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J. D., McMullen, B., Hurwitz, J., Krebs, E. G., and Wigler, M. (1987) *Mol. Cell. Biol.* **7,** 1371–1377
14. Kunisawa, R., Davis, T. N., Urdea, M. S., and Thorner, J. (1987) *Nucleic Acids Res.* **15,** 368–369
15. Jahnsen, T., Hedin, L., Lohmann, S. M., Walter, U., and Richards, J. S. (1986) *J. Biol. Chem.* **261,** 6637–6639
16. Scott, J. D., Zoller, M. J., Glaccum, M. B., Helfman, D., and Krebs, E. G. (1986) *Fed. Proc.* **45,** 1799
17. Kerlavage, A. R., and Taylor, S. S. (1980) *J. Biol. Chem.* **255,** 8483–8488
18. Corbin, J. D., Sugden, P. H., West, L., Flockhart, D. A., Lincoln, T. M., and McCarthy, D. (1978) *J. Biol. Chem.* **253,** 3997–4003
19. Barker, W. C., and Dayhof, M. O. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 2836–2839
20. Leatherbarrow, R. J., and Fersht, A. R. (1986) *Protein Engineering* **1,** 7–16
21. Zoller, M. J. (1987) in *Methods of Protein Sequence Analysis* (Walsh, K., ed) Humana Press, NY, in press
22. Struhl, K. (1983) *Nature* **305,** 391–397
23. Winston, F., Chumley, F., and Fink, G. R. (1983) *Methods Enzymol.* **101,** 211–227
24. Studier, F. W., and Moffatt, B. A. (1986) *J. Mol. Chem.* **189,** 113–130
25. Mead, D. A., Szczesna-Skorupa, E., and Kemper, B. (1986) *Protein Engineering* **1,** 67–74
26. Zoller, M. J., and Smith, M. (1984) *DNA* **3,** 479–488
27. Zoller, M. J. (1987) *Methods Enzymol.* **154,** in press
28. Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H., and Roe, B. A. (1980) *J. Mol. Biol.* **143,** 161–178
29. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
30. Builder, S. E., Beavo, J. A., and Krebs, E. G. (1980) *J. Biol. Chem.* **255,** 3514–3519
31. Sugden, P. H., and Corbin, J. D. (1976) *Biochem. J.* **159,** 423–437
32. Fersht, A. (1985) in *Enzyme Structure and Mechanism,* p. 191, Freeman Publications, San Francisco
33. Laemmli, U. K. (1970) *Nature* **227,** 680–685
34. Roskoski, R. (1983) *Methods Enzymol.* **99,** 3–6
35. Hunkapillar, M. W., and Hood, L. E. (1983) *Methods Enzymol.* **91,** 486–493
36. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
37. Saraswat, L. D., Filutowicz, M., and Taylor, S. S. (1986) *J. Biol. Chem.* **261,** 11091–11096
38. Viera, J., and Messing, J. (1982) *Gene (Amst.)* **19,** 259–268
39. Takio, K., Sasagawa, T., Ericsson, L. H., Kumar, S., Smith, S. B., Krebs, E. G., and Walsh, K. A. (1984) *Biochemistry* **23,** 4193–4199
40. Zoller, M. J., Kerlavage, A. R., and Taylor, S. S. (1979) *J. Biol. Chem.* **254,** 2408–2412
41. Takio, T., Smith, S. B., Krebs, E. G., Walsh, K. A., and Titani, K. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 2544–2548
42. Erlichman, J., Rubin, C. S., and Rosen, O. M. (1973) *J. Biol. Chem.* **248,** 7607–7609
43. Mutzel, R., Lacome, M.-L., Simon, M.-N., de Gunzburg, J., and Veron, M. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 6–10
44. Uno, I., Matsumoto, K., and Ishikawa, T. (1982) *J. Biol. Chem.* **257,** 14110–14115