Nutritional Control of Nucleocytoplasmic Localization of cAMP-dependent Protein Kinase Catalytic and Regulatory Subunits in *Saccharomyces cerevisiae**

Gerard Griffioen‡, Paola Anghileri‡§, Esther Imre‡, Maurizio D. Baroni§, and Helmut Ruis‡¶

*From the ‡Vienna Biocenter, Institut für Biochemie und Molekulare Zellbiologie der Universität Wien
and Ludwig Boltzmann-Forschungsstelle für Biochemie, Dr. Bohrgasse 9, A-1030 Wien, Austria
and §Sezione di Biochimica Comparata, Dipartimento di Fisiologia e Biochimica Generali,
Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy*

In budding yeast, cAMP-dependent protein kinase (PKA) plays a central role in the nutritional control of metabolism, cell cycle, and transcription. This study shows that both the regulatory subunit Bcy1p and the catalytic subunit Tpk1p associated with it are predominantly localized in the nucleus of rapidly growing cells. Activation of nuclear PKA by cAMP leads to fast entry of a significant part of Tpk1p into the cytoplasm, while the regulatory subunit remains nuclear. In contrast to rapidly proliferating cells, both Bcy1p and Tpk1p are distributed over nucleus and cytoplasm in cells growing on a nonfermentable carbon source or in stationary phase cells. These results demonstrate that at least two different mechanisms determine the subcellular localization of PKA; cAMP controls the localization of Tpk1p, and the carbon source determines that of Bcy1p. The N-terminal domain of Bcy1p serves to target it properly during logarithmic and stationary phase. Studies with Bcy1p mutant versions unable to concentrate in the nucleus revealed that cells producing them are less viable in stationary phase than wild type cells, display delayed reproliferation following transfer to fresh growth medium, and, as diploids, exhibit reduced efficiency of sporulation.

In all classes of living cells, extracellular signals regulate intracellular processes via signal transduction pathways. Numerous such pathways have been identified, and this raises important questions about cross-talk, timing, and specificity of signaling. One level of control to ensure signal specificity is to localize proteins involved in signaling to specific subcellular compartments. Differential compartmentation has been demonstrated for a growing number of transcription factors and protein kinases that translocate from the cytoplasm to the nucleus or *vice versa* in response to extracellular signals (1, 2). Signaling complexes have been identified that are specifically localized by means of scaffold or anchor proteins, respectively. Scaffold proteins bind different components of a signaling pathway, simultaneously ensuring specificity and efficiency of signaling. Ste5p is an example of a scaffolding system in yeast, which coordinates the mitogen-activated protein kinase cascade involved in the mating type response (3). Proteins functioning as anchors may target signaling molecules to specific subcellular sites. In mammalian cells, A-kinase anchor proteins (AKAPs)[1] function as adaptor proteins targeting RII type cAMP-dependent protein kinase (PKA) to various specific intracellular structures (4, 5). Detailed analysis revealed that these proteins are in fact multivalent platforms for association not only of PKA but also of other signaling molecules (4–6).

This study deals with the intracellular localization of PKA in the yeast *Saccharomyces cerevisiae*. PKA holoenzyme is a tetramer consisting of a homodimer of two regulatory subunits (encoded by the gene *BCY1* (4–8)) and two catalytic subunits (encoded by the genes *TPK1*, *TPK2*, and *TPK3* (9)). Activation of PKA takes place by binding of two cAMP molecules to each regulatory subunit, resulting in dissociation and thereby activation of the catalytic subunits (8, 10, 11). The only well established extracellular trigger of the Ras/adenylate cyclase pathway, the signaling cascade responsible for production of the second messenger cAMP (12), identified so far is glucose. The addition of this sugar to derepressed cells leads to a transient increase in cAMP levels (13) and consequently to activation of PKA. This transient activation is supposed to facilitate the transition from respiratory to fermentative growth (14, 15). Studies with mutants with high or low PKA activity, respectively, confirm the role of this protein kinase in nutritional signaling. For instance, *bcy1Δ* mutants, which display a constitutive high activity of PKA, do not accumulate reserve carbohydrates, cannot grow on carbon sources other than glucose, are stress-sensitive, do not sporulate, and have very low viability in stationary phase. Most of these phenotypes are reversed to the opposite extreme in mutants with constitutive low PKA activity (16). A subset of these phenotypes is explained, at least partially, by defective regulation of gene transcription in the respective PKA mutants. Most notably, transcription of stress-induced genes bearing stress response elements in their promoter region is repressed by PKA activity (17, 18). These *cis*-acting elements are occupied only under stress conditions by at least two functionally redundant transcription factors, Msn2p and Msn4p, which mediate stress-induced transcription (19). Recently, regulation of the localization of these factors by PKA was described. Low PKA activity allows entry of Msn2p and Msn4p into the nucleus, while high PKA activity stimulates export and inhibits import (20).

Here we describe the localization of both regulatory and

¶ To whom correspondence should be addressed. Tel.: 43-1-427752815; Fax: 43-1-42779528; E-mail: HR@abc.univie.ac.at.

[1] The abbreviations used are: AKAP, A-kinase anchor protein; PKA, protein kinase A; PCR, polymerase chain reaction; NES, nuclear export signal; GFP, green fluorescent protein; DAPI, 4,6-diamidino-2-phenylindol; HA, hemagglutinin; FACS, fluorescence-activated cell sorting; WT, wild type.

TABLE I
Plasmids used

Plasmid	Description
33pAGT	*GFP-TPK1* fusion under control of a truncated *ADH1* promoter in *CEN,ARS,URA3* vector YCplac33
33pAT	*TPK1* under control of a truncated *ADH1* promoter in *CEN, ARS, URA3* vector YCplac33
33pAGHBwt	*GFP-HA-BCY1* fusion under control of a truncated *ADH1* promoter in *CEN,ARS,URA3* vector YCplac33
33pAGHBΔN1	Plasmid 33pAGHBwt with Bcy1p deleted for amino acids 1–48
33pAGHBΔN2	Plasmid 33pAGHBwt with Bcy1p deleted for amino acids 1–124
33pAGHBΔN3	Plasmid 33pAGHBwt with Bcy1p deleted for amino acids 1–179
33pAGHBΔC3	Plasmid 33pAGHBwt with Bcy1p deleted for amino acids 49–416
33pAGHBΔC1	Plasmid 33pAGHBwt with Bcy1p deleted for amino acids 125–416
33pAGHBΔC2	Plasmid 33pAGHBwt with Bcy1p deleted for amino acids 180–416
313GHBwt	*GFP-HA-BCY1* fusion under control of the *BCY1* promoter in *CEN,ARS,HIS3* vector pRS313
313HBwt	*HA-BCY1* fusion under control of the *BCY1* promoter in *CEN,ARS,HIS3* vector pRS313
313HBΔN1	Plasmid 313HBwt with Bcy1p deleted for amino acids 1–48
313HBΔN2	Plasmid 313HBwt with Bcy1p deleted for amino acids 1–124
313NES-HBwt	Plasmid 313HBwt with the PKI NES fused to *HA-BCY1*

catalytic subunits of PKA in budding yeast. We found that the localization of Bcy1p is regulated according to the nutritional status of yeast cells and have identified the N-terminal domain of this protein as sufficient for proper targeting. Mutants with constitutively cytoplasmic PKA are less viable in stationary phase than wild type cells and are delayed in the reentry into the cell cycle upon resuspension in fresh growth medium.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Growth Conditions—Yeast strains used are as follows: W303-1A (*MATa ade2 can1 his3 leu2 trp1 ura3*), MR1 (*W303-1A bcy1::LEU2*), JW1 (*W303-1A bcy1::URA3*), EI1 (*W303-1A/ W303-1A bcy1::URA3 bcy1::LEU2*), PA2F2 (*W303-1A bcy1::LEU2 pde2::TRP1*), PA3F3 (*W303-1A bcy1::LEU2 pde2::TRP1-* [313HBΔN2]), and TH5 (*MATa leu2–3 leu2–112 ura3–52 trp1–1 his3– 532 his4 cyr1::LEU2 CAM*) (21). EI1 was generated by crossing MR1 and JW1. TH5 was a generous gift from W. Heideman. Yeast media were prepared as described (22).

To obtain cells in stationary phase, cultures were grown in YPD (supplemented with 60 mg/liter adenine sulfate) or SC medium (at least) 5 days at 30 °C. For reproliferation experiments, cells were resuspended in fresh growth medium at A_{600} of 0.25. To achieve sporulation, diploid cells were grown in YPD to A_{600} of 1.0; subsequently, glucose was added to a final concentration of 10% (w/v), and cells were grown for an additional 6 h. They were then inoculated in YP-acetate at A_{600} of 0.1, grown overnight, washed, and resuspended in sporulation medium. Sporulation efficiency was determined after 48 h of incubation.

Construction of Plasmids—All plasmids used in this study are shown in Table I. Plasmid 33pAGT was generated as follows. A PCR fragment of *TPK1* was generated using oligonucleotides 5′-CTT CAG GAT CCG TCG ACT GGC GGC CGC ACT GAA GAA CAA AAT GGA G-3′ and 5′-CAA GAG GAA TTC TTA CCC CCA CAT TCT AAT AAT AA-3′ for a PCR with yeast genomic DNA as template and was cloned in *Bam*HI– *Eco*RI in YCplac33 (23). This introduced a *Not*I site directly after the start of the *TPK1* coding region. Then a *GFP Not*I cassette (20) was introduced in the *Not*I site, and subsequently a *Spe*I–*Hin*dIII fragment from plasmid Ycp50-TPK1 (a generous gift from H. de Winde) corresponding to the downstream part of *TPK1* was inserted. Finally, the *ADH1* promoter was amplified from yeast genomic DNA as template with primers 5′-CTA AAC CGC GGA ATA TTT CGG GAT ATC-3′ and 5′-ACC ATG GTA TAT GAG ATA GTT GAT TGT ATG C-3′. This PCR fragment was cut with *Nco*I–*Hin*dIII and placed upstream of *GFP-TPK1*, bringing the fusion gene under control of the *ADH1* promoter. Plasmid 33pAGHT was generated by introducing a triple copy of the HA epitope (24) into the *Not*I site of 33pAGT directly downstream of GFP. *BCY1* was amplified by PCR using a plasmid-borne copy as template with primers 5′-GAC TGG ATC CAT GGT ATC TTC TTT GCC C-3′ and 5′-GAT GGA ATT CAT CGA TCT GTG TGG ATA GGGG-3′, cut with *Bam*HI–*Eco*RI, and subcloned correspondingly in plasmid 33pAGHT using a unique *Bam*HI site directly after the second HA epitope and thereby replacing *TPK1* by *BCY1*. This resulted in plasmid 33pAGHB. Deletions of *BCY1* were made as follows. PCR fragments were generated using the forward primers ΔN1 (5′-CGC GGA TCC AAG GCC AGG GAG CCT G-3′), ΔN2 (5′-CGC GGA TCC ACT AGA GAA AAG ACA TCA AC-3′), and ΔN3 (5′-CGC GGA TCC CGT AAT AAC TT CTG TTC AA-3′) and reverse primer 5′-GAT GGA ATT CAT CGA TCT GTG TGG ATA GGG G-3′, resulting in DNA fragments corresponding to deletions

ΔN1, ΔN2, and ΔN3 (see Fig. 3*A*). These were cut with *Eco*RI and *Bam*HI and cloned in plasmid 33pAGHB. Similarly, PCR fragments were made using reverse primers ΔC1 (5′-TTT CTG CAG TTA ATG CTG TTG TTC TTC CTG-3′), ΔC2 (5′-TTT CTG CAG TTA GAT CGA TTT TTC CAG TCT TTG-3′), and ΔC3 (5′-TTT CTG CAG TTA CTT GAG GAA CGC TCT CTG-3′) with forward primer 5′-GAC TGG ATC CAT GGT ATC TTC TT GCC C-3′, resulting in DNA fragments corresponding to ΔC1, ΔC2, and ΔC3 (see Fig. 3*A*). These were cut with *Pst*I and *Bam*HI and cloned in plasmid 33pAGHBwt. To bring the *GFP-HA2-BCY1* fusion under control of the authentic *BCY1* promoter, this region was amplified from yeast genomic DNA as template using oligonucleotide primers 5′-AGA TGA TCG ATG CAT GCC ACC ACG AC-5′ and 5′-CCG GAA TTC CAT GGA TCC CGT TTA TTC TTA CTG TTG TC-3′, cut with *Eco*RI and *Cla*I, and cloned in pRS313 (25). Then a double-stranded linker oligonucleotide 5′-CATG GGC GGC CGC GAA TTC GAG CT-3′ and 5′-CCG CCG GCG CTT AAG C-3′ was inserted (*Nco*I–*Sac*I) directly downstream of the *BCY1* promoter region, resulting in plasmid 313pBL. Subsequently, the *Nco*I–*Eco*RI DNA fragment containing *GFP-HA-BCY1* from plasmid 33pAGHBwt was subcloned in 313pBL, resulting in plasmid 313GHBwt. Plasmids 313HBwt, 313HBΔN1, and 313HBΔN2 were generated by subcloning the *Not*I– *Eco*RI fragments from the corresponding 33pAGHB deletion series in 313pBL. In 313GHBwt, a double-stranded oligonucleotide obtained by annealing oligonucleotides 5′-ATG AAT GAA TTA GCC TTG AAA TTA GCA GGT CTT GAT ATC AAC AAG ATG CAT GC-3′ and 5′-CAT GGC ATG CAT CTT GTT GAT ATC AAG ACC TGC TAA TTT CAA GGC TAA TTC ATT-3′, which encode the cAMP-dependent protein kinase inhibitor nuclear export signal (NES) flanked by sequences required for cloning and determination of orientation, was inserted in the *Nco*I site. This resulted in plasmid 313NES-HBwt, in which the NES is placed at the N terminus of the HA tag.

GFP Fluorescence Microscopy—Cells were used for fluorescence microscopy directly without fixation. Nuclei were stained by the addition of 5 μg/ml 4,6-diamidino-2-phenylindol (DAPI) to the cell suspension. All cells were viewed using a Zeiss Axioplan 2 fluorescence microscope. Images were taken with a Quantix CCD camera using IP LAB software, processed in Adobe Photoshop 4.0, and printed on an Epson Stylus Photo printer.

Indirect Immunofluorescence—Fixation and staining was carried out as described by Nasmyth *et al*. (26). HA-tagged Bcy1p was detected by anti-HA antibodies (12CA5 mouse IgG), and indirect immunofluorescence was achieved by using goat anti-mouse IgG CYC3-conjugated secondary antibodies (Sigma).

Flow Cytometric DNA Analysis (FACS)—Samples were drawn containing about 10^7 cells and fixed immediately in 70% ethanol. Cells were washed and resuspended in 50 mM Tris-HCl, pH 7.8, and treated with RNase (0.4 mg/ml) for 4 h at 37 °C. Then cells were resuspended in 0.5 ml of 5 mg/ml pepsin dissolved in 55 mM HCl, and incubated for 30 min at 37 °C with shaking. DNA was stained by resuspending the cells in a buffer containing 180 mM Tris-HCl, pH 7.5, 190 mM NaCl, 70 mM MgCl$_2$, and 50 μg/ml propidium iodide and sonicated briefly. For each time point taken, 10,000 cells were measured for DNA content with a Becton Dickinson FACScan using LysisII software.

RESULTS

In Rapidly Growing Cells the Regulatory Subunit Bcy1p Is Nuclear, while the Localization of the Catalytic Subunit Tpk1p Is Regulated by cAMP—To study the localization of PKA in
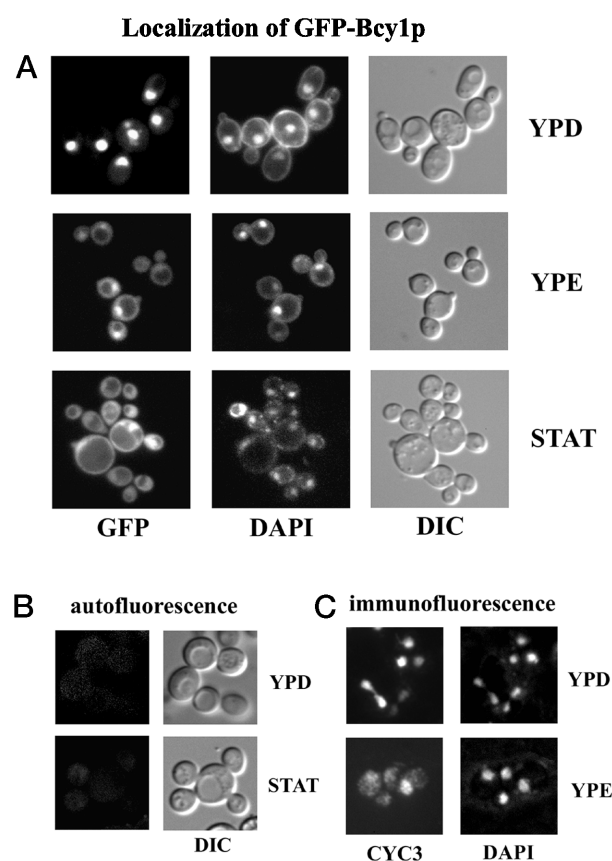
GFP-Bcy1p in strain TH5 (*CAMcyr1*)
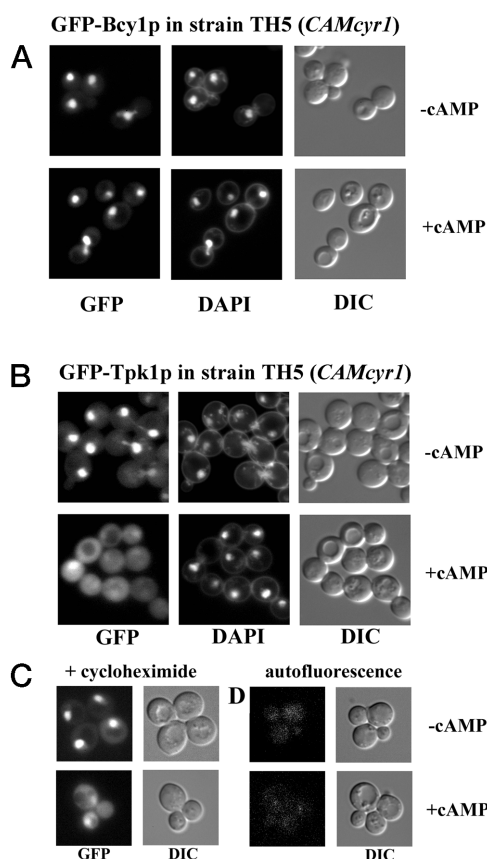
FIG. 1. **Subcellular localization of GFP-Bcy1p and GFP-Tpk1p in the absence and presence of cAMP.** Strain TH5 (*cyr1 CAM*) cells transformed with the GFP-Bcy1p-encoding plasmid 33pAGHBwt (*A*), with the GFP-Tpk1p-encoding plasmid 33pAGT (*B* and *C*) or with a control plasmid 33pAT (*D*) were pregrown to logarithmic phase on glucose-based synthetic medium supplemented with 3 mM cAMP. Cells were washed with medium without cAMP, and pictures were taken after 10 min (−*cAMP*). Subsequently, cAMP was added to a final concentration of 3 mM, and pictures were taken after 10 min (+*cAMP*). The experiment shown in *C* was carried out in the presence of 100 μg/ml cycloheximide added 40 min before the withdrawal of cAMP.

Localization of GFP-Bcy1p

FIG. 2. **Subcellular localization of GFP-Bcy1p (*A*) or HA-Bcy1p (*C*) in cells grown on glucose or ethanol and in stationary phase cells.** Fluorescence microscopy was carried out with strain MR1 transformed with GFP-Bcy1p-encoding plasmid 313GHBwt (*A*) or with control plasmid 313HBwt (*B*) grown to logarithmic phase on YPD or YPE and to stationary phase on YPD. *C*, indirect immunofluorescence of MR1 cells transformed with 313HBwt, using anti-HA antibody.

living cells, we fused GFP to the N termini of the regulatory subunit Bcy1p and one of the catalytic subunit isoforms, Tpk1p. These fusion proteins were found to be functional, since GFP-Bcy1p produced in a *bcy1Δ* strain restored the ability of this mutant to grow on carbon sources other than glucose. GFP-TPK1 expressed in a *tpk1 tpk2 tpk3* triple deletion strain complemented the otherwise lethal phenotype of such a strain (data not shown).

Since cAMP is known to cause dissociation of Tpk1p and Bcy1p, we determined whether intracellular cAMP levels regulate the localization of these fusion proteins. The strain TH5 used for this experiment allows manipulation of intracellular cAMP levels due to a deletion of the adenylate cyclase gene (*CYR1*). It bears an additional mutation (*CAM*), allowing uptake of cAMP (21). GFP-Bcy1p was found nuclear both in the presence and absence of cAMP (Fig. 1*A*). Although GFP-Bcy1p production in this experiment was driven by a truncated *ADH1* promoter (up to position −410 relative to the transcription start, lacking its main upstream activating sequence (27)), a similar localization of this fusion protein was detected when it was produced from its own promoter in cells grown on glucose (Fig. 2*A*).

In the case of GFP-Tpk1p, expression from the authentic *TPK1* promoter was too weak for us to observe fluorescence (data not shown). To increase expression, the GFP-TPK1 fusion

was expressed under control of a similar truncated *ADH1* promoter as for GFP-Bcy1p, resulting in a moderate overexpression. When cells expressing this construct were briefly starved for cAMP, GFP-Tpk1p was found predominantly localized in the nucleus, but subsequent addition of cAMP led to redistribution of Tpk1p throughout the cell within about 10 min (Fig. 1*B*). This effect is independent of *de novo* protein sythesis, since it occurs also in the presence of cycloheximide (Fig. 1*C*). Fig. 1*D* shows that autofluorescence of cells studied is very low, indicating that the observed fluorescence is derived specifically from the GFP derivatives.

The results summarized in Fig. 1 show that, under the experimental conditions used here, catalytically inactive PKA holoenzyme resides in the nucleus. On the other hand, when the catalytic Tpk1p subunit is released from the regulatory subunit upon activation by high intracellular cAMP, it does partly enter the cytoplasm.

The Localization of Bcy1p Is Determined by the Carbon Source Used—We followed the subcellular localization of a GFP-Bcy1p fusion, making use of the native *BCY1* promoter, under different physiological conditions. Similar to the situation shown in Fig. 1, GFP-Bcy1p was found predominantly nuclear in wild type cells (W303-1A) grown on glucose (Fig. 2*A*). However, in cells grown on ethanol (or on various other nonfermentable carbon sources) a significant portion of the fusion protein was found extranuclear, an effect that became even more pronounced in stationary phase cells (Fig. 2*A*; data obtained for the other carbon sources not shown). The fluorescence observed is derived specifically from GFP fusion protein,
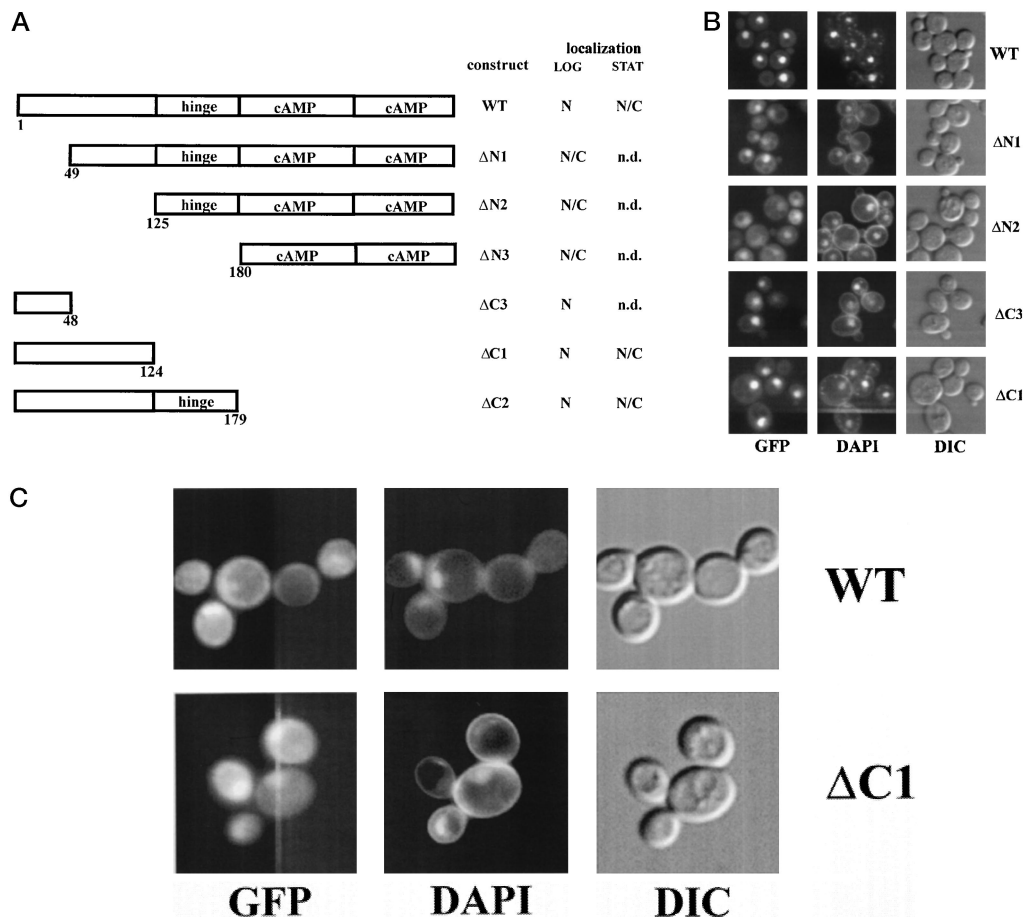
FIG. 3. **Identification of the nuclear localization domain of Bcy1p.** *A*, schematic representation of Bcy1p and its deletion derivatives. The localization in logarithmic (*LOG*) or stationary phase (*STAT*) cells determined by fluorescence microscopy of the different deletions fused at their N terminus with GFP is indicated. *N*, predominant nuclear fluorescence; *N/C*, nucleocytoplasmic distribution of fluorescence with no or weak nuclear accumulation; *n.d.*, not determined. *B*, fluorescence microscopy of W303-1A cells transformed with 33pAGHBwt (wild type GFP-Bcy1p), 33pAGHBΔN1 (ΔN1*)*, 33pAGHBΔN2 (ΔN2), 33pAGHBΔC3 (ΔC3), or 33pAGHBΔC1 (ΔC1). Cells examined were grown to logarithmic phase on glucose-based synthetic medium. *C*, fluorescence microscopy of stationary phase W303-1A cells transformed with 33pAGHBwt (WT) and 33pAGHBΔC1 (ΔC1).

since the levels of autofluorescence were found to be very low in logarithmic and stationary phase cells (Fig. 2*B*). The observations made with GFP-Bcy1p were confirmed by indirect immunofluorescence of fixed cells using a HA-tagged Bcy1p (Fig. 2*C*). Also with this method, we found that in glucose-grown cells HA-Bcy1p is predominantly nuclear and distributed over nucleus and cytoplasm in cells grown on ethanol (this method did not allow us to study stationary phase cells, since such cells could not be spheroplasted). The cytoplasmic localization of Bcy1p observed seemed to be a specific growth phase and/or carbon source effect, since treatment of glucose-grown cells with stress agents (salt or ethanol), starvation for nitrogen overnight, or mating factor arrest did not have any influence on nuclear localization (data not shown). These observations rule out the possibility that cytoplasmic localization of Bcy1p is caused by a decrease of general metabolic activity or by a cell cycle arrest *per se*. In summary, the results obtained demonstrate that Bcy1p is predominantly nuclear in rapidly growing cells but is distributed over nucleus and cytoplasm in cells grown on ethanol and in stationary phase cells.

The N Terminus of Bcy1p Is Necessary and Sufficient for Localization—As a first step toward identification of the molecular mechanism of control of Bcy1p localization, we decided to identify the protein domain responsible for subcellular targeting. It has been reported previously (8) that Bcy1p exhibits high sequence conservation to the C-terminal cAMP binding pockets and to the catalytic subunit association domain (or hinge region) of its mammalian counterparts (Fig. 3*A*). The yeast N terminus, however, does not exhibit any homology and is longer than that of mammalian RII regulatory subunits (8). Moreover, the extreme N terminus of mammalian RII interacts with AKAPs that localize this protein to various subcellular locations (28). We therefore carried out a deletion analysis of the Bcy1p part of a GFP-Bcy1p fusion. Removal of the N-terminal extension (ΔN1) led to a significant decrease of nuclear accumulation of Bcy1p, but deletion of the whole nonconserved N terminus (ΔN2) resulted in an almost even nucleocytoplasmic distribution of fluorescence in logarithmic cells (Fig. 3*B*). An even more extended deletion, including the hinge region (ΔN3), gave results similar to those obtained with the ΔN2 deletion (data not shown). We then tried to address whether the N-terminal domain is sufficient for proper targeting by determining the subcellular location of C-terminal deletions of Bcy1p (ΔC1–C3). Fusion of the first 48 N-terminal amino acids to GFP (ΔC3) was sufficient for nuclear accumulation although somewhat less efficient than in the case of full-length Bcy1p. When the whole N-terminal region was fused to GFP (ΔC1) or when the hinge region was also present (ΔC2), efficient nuclear concentration was observed (Fig. 3*B* and data not shown). Moreover, GFP fusions containing only the Bcy1p N-terminal domain are also distributed over nucleus and cytoplasm upon transition into stationary phase (Fig. 3*C*). Based on these results, we conclude that the first 124 N-terminal amino acids of Bcy1p are necessary and sufficient for
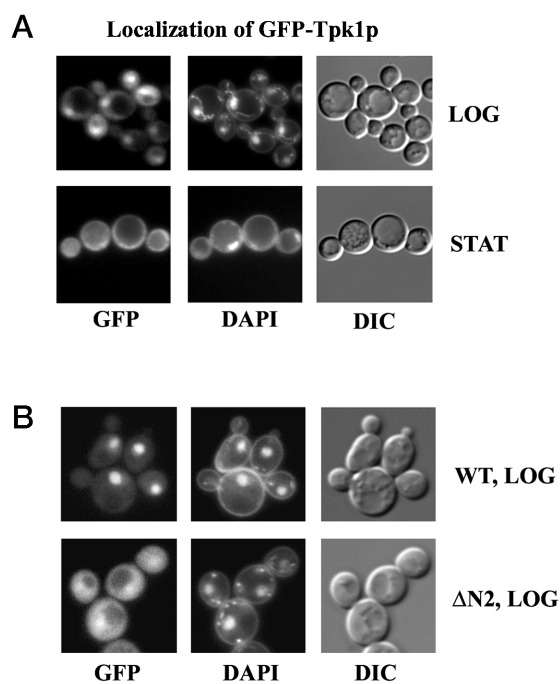
A   Localization of GFP-Tpk1p

GFP   DAPI   DIC

B

GFP   DAPI   DIC

FIG. 4. **Subcellular localization of GFP-Tpk1p in cells bearing wild type or an inappropriately localized Bcy1p.** *A*, fluorescence microscopy of W303-1A cells transformed with 33pAGT grown logarithmically on glucose or to stationary phase. *B*, fluorescence microscopy of strain PA2F2 (*bcy1Δ pde2Δ* [313HBwt]) (WT) and PA3F3 (*bcy1Δ pde2Δ* [313HBΔN2]) (ΔN2) cells transformed with 33pAGT. Both strains were grown to logarithmic phase on glucose ($A_{600} = 1$).

proper nutrient-regulated subcellular targeting.

Bcy1p Directs Nuclear Localization of Tpk1p—In wild type cells growing on glucose, GFP-Tpk1p was found to be concentrated in the nucleus, with a significant portion being present in the cytoplasm (Fig. 4*A*). The fact that GFP-Tpk1p is concentrated in the nucleus (although cAMP levels are supposed to be relatively high under these conditions) probably indicates that a subpopulation of GFP-Tpk1p is still bound to Bcy1p, since the latter protein is exclusively nuclear under these conditions (Figs. 1 and 2). Importantly, in stationary phase, when Bcy1p is evenly distributed through the nucleus and cytoplasm, this nuclear concentration of GFP-Tpk1p is not observed (Fig. 4*A*). To study whether the nuclear accumulation of GFP-Tpk1p is indeed dependent on nuclear Bcy1p, we created two strains, PA2F2 and PA3F3, which are deleted for chromosomally encoded *BCY1* but express a plasmid-borne copy of full-length *BCY1* (strain PA2F2) and a mutant allele of *BCY1* expressing an N-terminal truncated version of Bcy1p (ΔN2; strain PA3F3), respectively. Both strains were transformed with a plasmid encoding GFP-Tpk1p. As shown in Fig. 4*B*, strain PA2F2 (with wild type Bcy1p) displayed nuclear accumulation of GFP-Tpk1p. In contrast, in strain PA3F3 (Bcy1pΔN2), GFP-Tpk1p has lost most of its nuclear accumulation. These results demonstrate that nuclear concentration of GFP-Tpk1p in rapidly growing cells is dependent on correspondingly localized Bcy1p. If nuclear concentration of GFP-Tpk1p is dependent on association with nuclear Bcy1p, then this would be abolished by high intracellular cAMP levels comparable with what is shown in Fig. 1*A*, where nuclear accumulation of GFP-Tpk1p is lost after the addition of cAMP. Strain PA2F2 (with full-length Bcy1p) used in the experiment shown in Fig. 4*B* bears a deletion of *PDE2*. This makes it responsive to extracellular cAMP. The addition of 3 mM cAMP to this strain abolished nuclear accumulation of GFP-Tpk1p, resulting in a nucleocytoplasmic distribution of GFP-Tpk1p (results not shown), similar to the

TABLE II
Characterization of strains with mislocalized Bcy1p

Relevant genotype[a]	μ		Viability in stationary phase[b]	Sporulation efficiency[c]
	Glucose	Ethanol		
	h^{-1}		%	%
bcy1Δ[313HBwt]	0.22	0.07	36 ± 4.5	33 ± 5.4
bcy1Δ[313HBΔN1]	0.22	0.07	ND[c]	ND
bcy1Δ[313HBΔN2]	0.21	0.06	13 ± 1.5	6.1 ± 3.2
bcy1Δ[313NES-HBwt]	0.21	0.07	9.3 ± 3.8	10 ± 3.9

[a] MR1 (*bcy1*) strain transformed with indicated plasmids. Cells were grown on YPD ("Glucose" column) or on YPE ("Ethanol" column), respectively. Values below represent the growth rate.
[b] Values are the mean percentage of viable cells ± S.D. after 8 days incubation.
[c] Values are the mean percentage of tetrads ± S.D. obtained by sporulation of diploid strain EI1 (*bcy1/bcy1*) transformed with the plasmids indicated for MR1.
[c] ND, not determined.

results shown in Fig. 1*B*. These findings illustrate that Tpk1p does not possess an intrinsic capacity to concentrate in the nucleus but that its accumulation in the nucleus is dependent on interaction with Bcy1p localized correspondingly.

Regulated Localization of Bcy1p Is Required for Viability, Efficient Sporulation, and Fast Reproliferation from Stationary Phase—Strain MR1 (*bcy1*) transformants bearing a plasmid-borne wild type *BCY1* allele or *BCY1* alleles deleted for the N-terminal nuclear targeting region (ΔN1 and ΔN2) display equal growth rates on both glucose and ethanol (Table II), demonstrating that mislocalization of PKA does not have a significant impact on culture growth under these conditions. Transcription of a number of PKA-regulated genes was studied in such cells by Northern analysis. The increase of mRNA levels of several genes controlled via stress response elements or similar elements (*HXK1*, *HSP104*, *DDR2*, *SSA3*) (29, 30) was not affected in mutant cells grown into stationary phase. The level of ribosomal protein *L25* gene transcript, which is positively controlled by PKA (31, 32), was also completely unaffected in Bcy1p mislocalization mutants (data not shown). Consistent with the data on stress response element-controlled genes, we could not observe any difference in the subcellular localization of an Msn2p-GFP fusion (20) under a variety of conditions known to affect this subcellular localization (data not shown). Since Bcy1p is evenly distributed between nucleus and cytoplasm in stationary phase cells but is predominantly nuclear in rapidly growing cells, we studied the effects of mislocalized Bcy1p on culture growth under changing nutritional conditions. To this end, we determined the kinetics of reproliferation of stationary phase cells upon transfer to fresh growth medium. Compared with wild type cells, cells with an N-terminally truncated Bcy1p reached stationary phase with similar kinetics (data not shown) but displayed a profound delay in increase of cell number, as determined by measuring A_{600} when transferred to fresh medium (Fig. 5*A*). This delay appears specific for stationary phase, since when cells that were growing logarithmically were inoculated to fresh growth medium, they did not display a significant delay in growth (Fig. 5*B*). Also, the increase in growth rate upon the addition of glucose to a culture growing on ethanol was not affected (data not shown). In principle, this delay in regrowth could be explained by mutant cells in stationary phase being delayed in leaving stationary phase and/or in entering the mitotic cell cycle. Alternatively, such cells may exhibit reduced viability. The viability of stationary phase cells was therefore determined using dyes that allow staining of living and dead cells. This showed that viability of mutant stationary phase cells is indeed decreased 3–4-fold (Table II). FACS analysis of cells reproliferating from stationary phase, however, showed a delayed increase of mu-
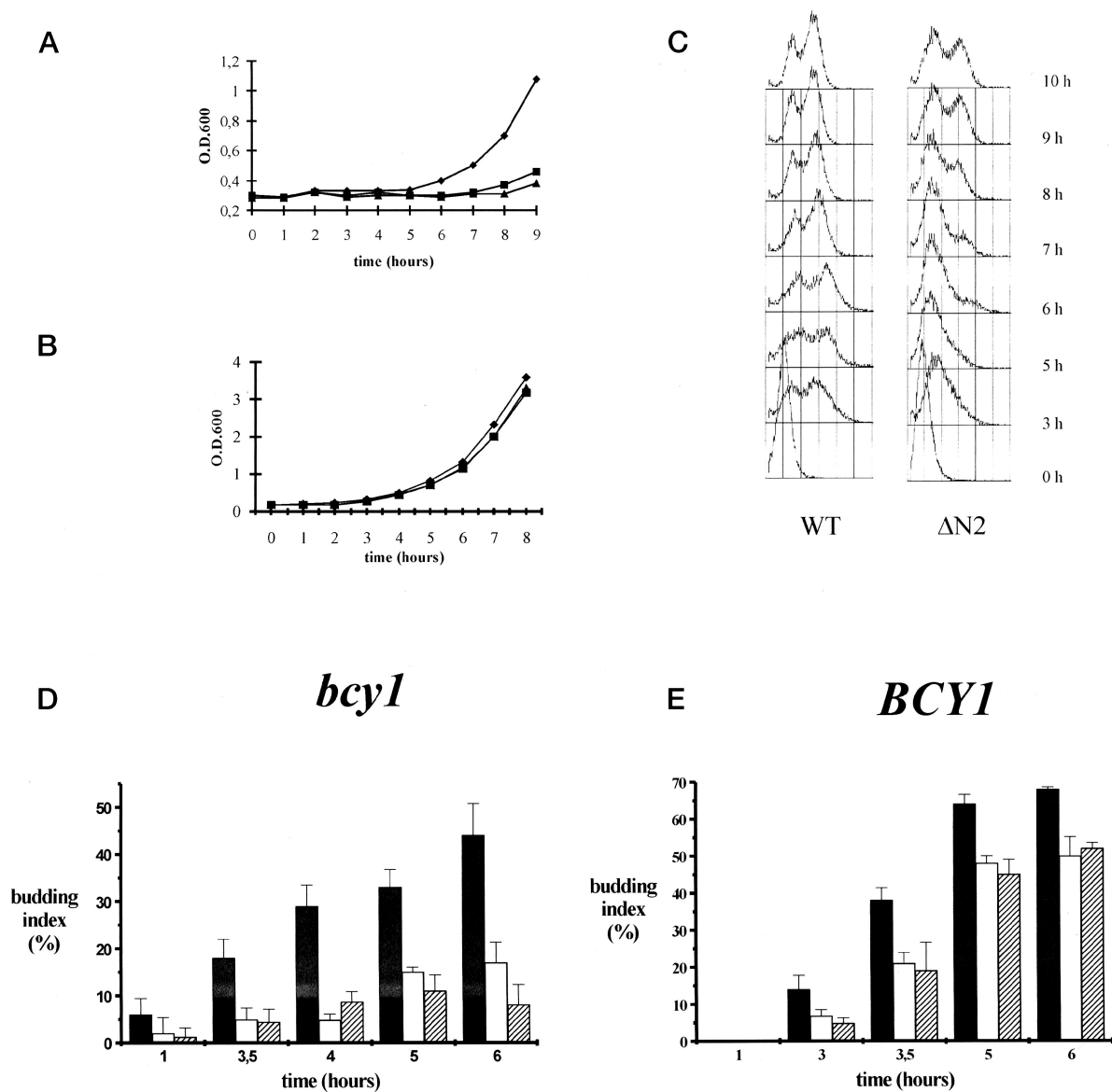
FIG. 5. **Growth curve of stationary phase (A) or logarithmic ($A_{600} = 1$) MR1 (*bcy1*) cells (B) transformed with 313HBwt (WT; *diamonds*), 313HBΔN1 (ΔN1; *squares*), or 313HBΔN2 (ΔN2; *triangles*).** Optical density at 600-nm wavelength (A_{600}) was determined at the indicated times after transfer to fresh growth medium. *C*, FACS analysis of MR1 (*bcy1*) cells transformed with 313HBwt (WT) and 313HBΔN2 (ΔN2). Stationary phase cells were resuspended in fresh growth medium. Samples were taken and fixed in 70% ethanol at the times indicated. *D*, budding index of living stationary phase MR1 (*bcy1*) cells transformed with 313HBwt (WT; *black bars*), 313HBΔN2 (ΔN2; *open bars*), and 313NES-HBwt (NES-Bcy1pWT; *striped bars*) after resuspension in fresh growth medium. Values shown by the *bars* represent the mean budding index (S.D. is indicated by *error bars*). Samples were taken 1 h before the times indicated and were incubated for 1 h at 30 °C in the presence of 20 μM FUN-1 and 20 μM Calcofluor White M2R (Molecular Probes, Inc., Eugene, OR). Stained cells were detected and quantified by fluorescence microscopy according to the instructions of the supplier. The budding index is the percentage of living budded cells relative to the total number of living cells. *E*, budding index of living W303-1A (*BCY1*) cells transformed with the same plasmids as described for *D*; *bars* are assigned correspondingly, and the budding index was determined as described for *D*.

tant cells with G_2/M DNA content, suggesting a delay in entering the mitotic cell cycle (Fig. 5*C*). To rule out any artifacts of the FACS analysis due to differences in viability, the budding index of only living stationary phase cells was determined after resuspension in fresh growth medium. We found that living cells with mislocalized Bcy1p display a delay in forming the first bud after resuspension in fresh medium (Fig. 5*D*), a result consistent with that of the FACS analysis. Considering these results, we conclude that the lower increase in cell number observed in Fig. 5*A*, although partly due to a decrease in cell viability, is also caused to a significant extent by a delay of living cells in entering S phase. Moreover, we found that diploids with mislocalized Bcy1p displayed reduced sporulation efficiency (Table II). A more detailed interpretation of this

observation requires further analysis.

In order to clarify whether the phenotypes observed in Bcy1p mislocalization mutants are indeed a consequence of this mislocalization or, alternatively, of some other effect of the N-terminal deletion of Bcy1p, a fusion was made between the NES of mammalian cAMP-dependent protein kinase inhibitor (33) and full-length Bcy1p. To check whether this NES sequence is functional, we also made a fusion between NES-Bcy1p with GFP. Fluorescence microscopy revealed that this fusion protein was excluded from the nucleus of cells growing logarithmically, and Western analysis demonstrated that NES-Bcy1p and Bcy1p wild type proteins are produced at comparable levels (results not shown). Characterization of strain MR1 producing NES-Bcy1p showed a normal growth behavior on

both glucose and ethanol but reduced sporulation efficiency, decreased viability in stationary phase (Table II), and a delay in the budding index of viable stationary phase cells resuspended in fresh growth medium (Fig. 5D). Thus, constitutive cytoplasmic localization of Bcy1p as a consequence of either fusing full-length Bcy1p to an NES or of removing the N-terminal targeting region results in very similar phenotypes. Wild type (W303-1A) cells producing Bcy1ΔN2 or NES-Bcy1p from an additional plasmid-borne gene also display a delay in budding index (Fig. 5E) and exhibit reduced viability in stationary phase (not shown). This demonstrates that aberrant cellular distribution of PKA activity and its phenotypic consequences occur irrespective of the presence of additional wild type Bcy1p. Dominance of the phenotypes shown in this way suggests that these are not due to decreased activity of the Bcy1p mutant proteins as negative regulators of PKA activity.

DISCUSSION

Cellular Protein Kinase A Localization in Budding Yeast Differs from That in Mammalian Cells—In mammalian cells, PKA regulatory RII subunits are predominantly found in the cytoplasm, and upon stimulation by cAMP, the released catalytic subunits diffuse to the nucleus (34–36). Also, most AKAPs identified in mammalian cells, which anchor regulatory subunits, have been reported to be extranuclear, although at least one type (AKAP95) has been found to be nuclear. However, in this case, interaction of AKAP95 with RII takes place only during mitosis, a condition when the nuclear envelope of mammalian cells is broken down (37).

This study shows that in yeast, localization of PKA is clearly different from that observed in mammalian cells. Deprivation of cAMP in rapidly growing yeast cells resulted in a pronounced nuclear localization of GFP-Tpk1p, and the subsequent addition of 3 mM cAMP to such cells, presumably causing dissociation of the Bcy1p-Tpk1p complex, leads to rapid entry of the catalytic subunits into the cytoplasm, while GFP-Bcy1p remains nuclear. Cytoplasmic entry of GFP-Tpk1p is rapid (in about 10 min, GFP-Tpk1p is almost equally distributed over nucleus and cytoplasm) and independent of *de novo* protein synthesis, suggesting that the effects observed are quite direct and not mere indirect physiological consequences of changing cAMP levels. These results allow the conclusion that the PKA holoenzyme is nuclear in rapidly growing cells. Consistent with such a model, GFP-Bcy1p, the kinase-inhibitory subunit, is predominantly accumulated in the nucleus of rapidly growing cells. In such cells, part of GFP-Tpk1p is found accumulated in the nucleus, and this accumulation is dependent on nuclear localization of Bcy1p (Fig. 4). However, also a significant part of the GFP-Tpk1p fusion protein is found to be cytoplasmic, indicating that, although the cAMP levels are supposed to be relatively high in rapidly growing cells, still a portion of Tpk1p is bound to the regulatory subunit, whereas liberated Tpk1p is able to enter the cytoplasm.

In contrast to cells growing on glucose, GFP-Bcy1p is partly cytoplasmic during growth on a nonfermentable carbon source or in stationary phase. Also, the nuclear concentration of the GFP-Tpk1p catalytic subunit, as observed in cells growing rapidly on glucose, is absent in stationary phase cells, a condition where cAMP levels are low. Altogether, our results are in agreement with a model in which Bcy1p determines the localization of Tpk1p associated with it. Since synthesis of cAMP, which affects localization of Tpk1p, is activated by glucose and the localization of Bcy1p depends on the carbon source, this demonstrates that nutritional signals control the localization of both types of yeast PKA subunits by at least two different molecular mechanisms.

The Bcy1p N Terminus, Which Is Not Conserved in Mamma-

lian RII Subunits, Is Involved in Localization of Bcy1p—Here we show that the Bcy1p N terminus plays a crucial role in Bcy1p localization, being both necessary and sufficient for localizing GFP-Bcy1p to the nucleus or cytoplasm in a growth phase- and carbon source-dependent manner. Inspection of this N-terminal region did not reveal an obvious cluster of basic amino acids characteristic for typical nuclear localization signals (38). Since the N-terminal part of Bcy1p and mammalian RII subunits are both determinants of subcellular localization, it is an attractive working hypothesis that, as in the mammalian case where the N terminus interacts with AKAPs, Bcy1p localization might be determined via anchoring proteins.

The change in localization of Bcy1p may involve relocalization of preexisting protein or be the consequence of the production of a cytoplasmic form. Bcy1p levels increase considerably during approach to stationary phase (39), hence not excluding a mechanism of preferential cytoplasmic accumulation of Bcy1p synthesized *de novo*.

Physiological Consequences of PKA Mislocalization—In budding yeast, PKA plays an important role in reconfiguring metabolic activities and expression patterns involved in carbon source metabolism. Recently, it has been shown that activation of PKA by glucose facilitates the transition from nonfermentative to fermentative growth (15). Consistent with this, cells growing on glucose display higher basal levels of intracellular cAMP (40), and mutant cells with constitutive high PKA activity are unable to grow on carbon sources other than glucose (7). Therefore, in cells growing on a nonfermentable carbon source, it may be advantageous to increase the cytoplasmic level of the kinase-inhibitory subunit, Bcy1p, in order to adjust metabolism properly for gluconeogenic growth.

The nuclear accumulation of Bcy1p in rapidly growing cells and the cytoplasmic accumulation in slowly growing cells implies that some aspects of PKA-controlled nuclear processes, with the free catalytic PKA subunits not having to enter the nucleus upon activation as would have been the case with a cytoplasmic PKA holoenzyme, might be important for rapid cell proliferation. This could be most important under changing growth conditions, since stationary phase cells with mislocalized Bcy1p display a delay in reentry into the mitotic cell cycle. Although the underlying mechanism for this phenotype is elusive, there might be a connection to the observations made by Hubler *et al*. (41) that reentry of stationary phase cells into the cell cycle requires a short and transient activation of PKA by cAMP. The yeast cells used in this study (41) appeared responsive to cAMP only after preincubation in fresh growth medium. In terms of models, nuclear accumulation of Bcy1p upon transfer to fresh growth medium might be required for efficient reentry into mitotic growth. Alternatively, it might be important to remove Bcy1p from the cytoplasm, since it could interfere there with metabolic processes required for resumption of rapid growth. Our observation that the delay in reproliferation of mutant cells that are unable to concentrate Bcy1p into the nucleus is a dominant phenotype might be an argument for the second explanation.

Altogether, the data presented in this study suggest that proper PKA localization provides a selective advantage for yeast cells in their natural environment. There they are frequently in stationary phase and have to reproliferate rapidly when the growth conditions suddenly improve for a limited period of time.

REFERENCES

1. Toone, W. M., and Jones, N. (1998) *Genes Cell* **3,** 485–498
2. Waskiewicz, A. J., and Cooper, J. A. (1995) *Curr. Opin. Cell Biol.* **7,** 798–805
3. Elion, A. (1995) *Trends Cell Biol.* **5,** 322–327
4. Dell'Acqua, M. L., and Scott, J. D. (1997) *J. Biol. Chem.* **272,** 12881–12884
5. Pawson, T., and Scott, J. D. (1997) *Science* **278,** 2075–2080
6. Klauck, T. M., Faux, M. C., Labudda, K., Langeberg, L. K., Jaken, S., and Scott, J. D. (1996) *Science* **271,** 1589–1592
7. Cannon, J. F., and Tatchell, K. (1987) *Mol. Cell. Biol.* **7,** 2653–2663
8. Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J. D., McMullen, B., Hurwitz, M., Krebs, E. G., and Wigler, M. (1987) *Mol. Cell. Biol.* **7,** 1371–1377
9. Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. (1987) *Cell* **50,** 277–287
10. Johnson, K. E., Cameron, S., Toda, T., Wigler, M., and Zoller, M. J. (1987) *J. Biol. Chem.* **262,** 8636–8642
11. Thevelein, J. M. (1992) *Antonie Leeuwenhoek* **62,** 109–130
12. Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., and Wigler, M. (1985) *Cell* **40,** 27–36
13. Francois, J., Van Schaftingen, E., and Hers, H. G. (1984) *Eur. J. Biochem.* **145,** 187–193
14. Thevelein, J. M. (1991) *Mol. Microbiol.* **5,** 1301–1307
15. Jiang, Y., Davis, C., and Broach, J. R. (1998) *EMBO J.* **17,** 6942–6951
16. Thevelein, J. M. (1994) *Yeast* **10,** 1753–1790
17. Marchler, G., Schüller, C., Adam, G., and Ruis, H. (1993) *EMBO J.* **12,** 1997–2003
18. Schüller, C., Brewster, J. L., Alexander, M. R., Gustin, M. C., and Ruis, H. (1994) *EMBO J.* **13,** 4382–4389
19. Martinez-Pastor, M. T., Marchler, G., Schüller, C., Marchler-Bauer, A., Ruis, H., and Estruch, F. (1996) *EMBO J.* **15,** 2227–2235
20. Gorner, W., Durchschlag, E., Martinez Pastor, M. T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H., and Schüller, C. (1998) *Genes Dev.* **12,** 586–597
21. Hall, D. D., Markwardt, D. D., Parviz, F., and Heideman, W. (1998) *EMBO J.* **17,** 4370–4378
22. Rose, M., Winston, F., and Hieter, P. (1990) *Methods in Yeast Genetics: A Laboratory Course Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
23. Gietz, R. D., and Sugino, A. (1988) *Gene* (*Amst.*) **74,** 527–534
24. Tyers, M., Tokiwa, G., and Futcher, B. (1993) *EMBO J.* **12,** 1955–1968
25. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122,** 19–27
26. Nasmyth, K., Adolf, G., Lydall, D., and Seddon, A. (1990) *Cell* **62,** 631–647
27. Tornow, J., and Santangelo, G. M. (1990) *Gene* (*Amst.*) **90,** 79–85
28. Hausken, Z. E., Coghlan, V. M., Hastings, C. A., Reimann, E. M., and Scott, J. D. (1994) *J. Biol. Chem.* **269,** 24245–24251
29. Boorstein, W. R., and Craig, E. A. (1990) *EMBO J.* **9,** 2543–2553
30. Moskvina, E., Schüller, C., Maurer, C. T. C., Mager, W. H., and Ruis, H. (1998) *Yeast* **14,** 1041–1050
31. Klein, C., and Struhl, K. (1994) *Mol. Cell. Biol.* **14,** 1920–1928
32. Griffioen, G., Mager, W. H., and Planta, R. J. (1994) *FEMS Microbiol. Lett.* **123,** 137–144
33. Wen, W., Meinkoth, J. L., Tsien, R. Y., and Taylor, S. S. (1995) *Cell* **82,** 463–473
34. Harootunian, A. T., Adams, S. R., Wen, W., Meinkoth, J. L., Taylor, S. S., and Tsien, R. Y. (1993) *Mol. Biol. Cell* **4,** 993–1002
35. Meinkoth, J. L., Ji, Y., Taylor, S. S., and Feramisco, J. R. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9595–9599
36. Nigg, E. A., Hilz, H., Eppenberger, H. M., and Dutly, F. (1985) *EMBO J.* **4,** 2801–2806
37. Eide, T., Coghlan, V., Orstavik, S., Holsve, C., Solberg, R., Skalhegg, B. S., Lamb, N. J., Langeberg, L., Fernandez, A., Scott, J. D., Jahnsen, T., and Tasken, K. (1998) *Exp. Cell Res.* **238,** 305–316
38. Dingwall, C., and Laskey, R. A. (1991) *Trends Biochem. Sci.* **16,** 478–481
39. Werner-Washburne, M., Brown, D., and Braun, E. (1991) *J. Biol. Chem.* **266,** 19704–19709
40. Francois, J., Eraso, P., and Gancedo, C. (1987) *Eur. J. Biochem.* **164,** 369–373
41. Hubler, L., Bradshaw-Rouse, J., and Heideman, W. (1993) *Mol. Cell. Biol.* **13,** 6274–6282

Nutritional Control of Nucleocytoplasmic Localization of cAMP-dependent Protein Kinase Catalytic and Regulatory Subunits in *Saccharomyces cerevisiae*

Gerard Griffioen, Paola Anghileri, Esther Imre, Maurizio D. Baroni and Helmut Ruis