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Protein Kinase A Mediates Growth-Regulated Expression of Yeast Ribosomal Protein Genes by Modulating RAP1 Transcriptional Activity

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Yeast ribosomal protein genes are coordinately regulated as a function of cell growth; RNA levels decrease during amino acid starvation but increase following a carbon source upshift. Binding sites for RAP1, a multifunctional transcription factor, are present in nearly all ribosomal protein genes and are associated with growth rate regulation. We show that ribosomal protein mRNA levels are increased twofold in strains that have constitutively high levels of cyclic AMP-dependent protein kinase (protein kinase A [PKA]) activity. The PKA-dependent induction requires RAP1 binding sites, and it reflects increased transcriptional activation by RAP1. Growth-regulated transcription of ribosomal protein genes strongly depends on the ability to regulate PKA activity. Cells with constitutively high PKA levels do not show the transcriptional decrease in response to amino acid starvation. Conversely, in cells with constitutively low PKA activity, ribosomal protein mRNAs levels are lower and largely uninducible upon carbon source upshift. We suggest that modulation of RAP1 transcriptional activity by PKA accounts for growth-regulated expression of ribosomal protein genes.

In the yeast *Saccharomyces cerevisiae*, the cyclic AMP (cAMP)-dependent protein kinase (protein kinase A [PKA]) is a key regulator of growth control (reviewed in reference 1). Yeast PKA consists of a regulatory subunit homodimer, encoded by *BCY1* (42), and a catalytic subunit dimer encoded by three redundant *TPK* genes (43). PKA activity is regulated by the *RAS* signal transduction pathway, which modulates intracellular cAMP levels. cAMP releases the BCY1 inhibitory subunits from the catalytic subunits, thereby resulting in phosphorylation of substrates. In general, high levels of cAMP and PKA activity are associated with cell growth. Cells grown in glucose have higher intracellular cAMP levels than do cells grown in less optimal carbon sources, and cAMP levels increase sharply upon a carbon source upshift (25, 41). Moreover, high PKA levels are associated with breakdown of stored carbohydrates and induction of the glycolytic pathway.

Very little is known about how signals transduced through the *RAS* pathway and PKA lead to changes in gene transcription that are relevant to growth control. Unlike the situation in mammalian cells (11, 16, 31), transcription dependent on ATF/CREB sites does not appear to be affected by PKA (46). The only yeast genes known to be affected by PKA are *ADH2* (6, 7), *UBI4* (39), and *CTT1* (24). However, PKA is not significantly involved in glucose repression of *ADH2* (7) or heat shock regulation of *UBI4* (39). PKA plays an important role in regulating *CTT1* transcription in response to stress conditions (24).

PKA is a candidate for connecting growth signals to the level of ribosome biosynthesis, which is regulated according to growth rate of yeast cells (47). Rapidly growing yeast cells require high rates of protein synthesis and hence contain two to five times more ribosomes than slowly growing or starved cells do. Growth rate control of ribosome synthesis results primarily from the coordinately regulated transcription of the more than 70 ribosomal protein genes that are scattered throughout the yeast genome.

Nearly all of the cloned ribosomal protein genes contain a RAP1 binding site(s) upstream of the TATA element (40). RAP1 is an abundant nuclear protein that binds specific DNA sequences (consensus RMACCCANNCAYY) in a wide variety of promoters, in the mating-type silencers, and at telomeres (2, 18, 21, 33). Depending on the promoter, RAP1 either stimulates or inhibits transcription, utilizing distinct activation and repression functions that are likely to be mediated through interactions with other proteins (38). Interaction with RIF1 is critical for silencing and for telomere length regulation (14), whereas interaction with GCR1 increases transcriptional activation (44).

RAP1 binding sites, and presumably RAP1, are involved in transcriptional regulation of ribosomal protein genes. Deletions of RAP1 binding sites reduce transcription, indicating that RAP1 acts as a transcriptional activator of ribosomal protein genes (32, 49). Moreover, RAP1 binding sites are associated with increased transcription in response to a carbon source upshift (15) as well as decreased transcription in response to histidine starvation (26). Thus, RAP1 appears to up- or down-regulate ribosomal protein mRNA levels in response to changes in growth rate. However, the steps between the environmental stimuli that alter growth rate and transcriptional regulation mediated through RAP1 binding sites are poorly understood.

In this report, we investigate the role of PKA in the transcription of ribosomal protein genes by comparing wild-type and *bcy1* deletion strains under equivalent growth conditions. *bcy1* deletion strains lack the regulatory subunit of PKA and therefore have high levels of PKA activity that are not affected by cAMP (42). We show that constitutively activated PKA increases transcription of ribosomal protein genes by increasing RAP1-dependent transcriptional activity. More importantly, we show that growth rate regulation of ribosomal protein genes strongly depends on the ability to regulate PKA activity in response to environmental conditions. We suggest

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TABLE 1. Genotypes of yeast strains used

Strain	Genotype	Source or reference
KY2002	*MAT***a** *ura3-52 leu2Δ::PET56 trp1-Δ63*	This work
KY2001	Isogenic to KY2002 but his3-*Δ200*	This work
KY2012	Isogenic to KY2002 but *bcy1::URA3*	This work
KY2005	Isogenic to KY2002 but *his3-Δ84*	This work
KY2015	Isogenic to KY2005 but *bcy1::URA3*	This work
KY2006	Isogenic to KY2002 but *his3-Δ84*RAP	This work
KY2016	Isogenic to KY2006 but *bcy1::URA3*	This work
KY2011	Isogenic to KY2002 but *rps13-*ΔR	This work
KY2021	Isogenic to KY2011 but *bcy1::URA3*	This work
SP1	*MAT***a** *his3 leu2 ura3 trp1 ade8 can1*	3
RS13-58A-1	Isogenic to SP1 but *tpk1*^{w1} *tpk2::HIS3 tpk3::TRP1 bcy1::LEU2*	3

that modulation of RAP1-dependent activity by PKA might account for the growth rate-regulated transcription of ribosomal protein genes.

MATERIALS AND METHODS

DNA manipulations. A 3.5-kb fragment of *RPS13* was isolated from a partial *Sau*3A genomic library (provided by Martine Collart). *rps13-*ΔR, which is deleted for the RAP1 binding site in the *RPS13* promoter, was generated by PCR, using primers designed to replace the region between nucleotides −190 to −220 with a *Bam*HI site. *rps13-Δ1*, which lacks most of the *RPS13* structural gene, was obtained by removing a 310-bp region between the *Pfl*mI and *Hpa*I sites. DNAs of both *rps13* derivatives were cloned as 1.7-kb *Hin*dIII fragments into the *Hin*dIII site of pRS306 (34), a *URA3* integrating vector. To fuse a consensus RAP1 binding site upstream of the *HIS3* TATA region and structural gene, an *Eco*RI-flanked oligonucleotide containing ACACCCAGACATC was cloned into the *Eco*RI site of YIp55-Sc3386, which contains the *his3-Δ84* allele (37). The resulting DNA lacks *HIS3* sequences between −447 to −93 and hence does not contain any *HIS3* upstream promoter elements.

YCp91-LexA, which was generated by Dimitris Tzamarias, is a *TRP1* centromeric expression vector that contains the *ADH1* promoter and 5′ untranslated region (nucleotides −410 to +10), followed by the entire LexA coding region (residues 1 to 202), sequences encoding the simian virus 40 nuclear localization signal (27), and the HA1 epitope from influenza virus (8). To generate LexA-RAP1, pUC19-*RAP1* DNA (obtained from David Shore) was mutagenized by PCR to convert the RAP1 translational initiation codon to GGG. A fragment starting from this codon and extending beyond the 3′ end of the *RAP1* structural gene (nucleotides 768 to 3675) was fused in frame to the HA1 epitope. The reporter plasmid containing a single LexA operator upstream of the *CYC1* TATA element and *lacZ* structural gene was generated by inserting the *Sal*I-*Pst*I fragment of YEp21-Sc3423 (17) into *Sal*I-*Pst*I-cleaved YEplac181, a 2μm/*LEU2* vector (10).

Construction and propagation of yeast strains. The yeast strains used in these studies are listed in Table 1. With the exception of SP1 and RS13-58A-1, which were obtained from Michael Wigler (3), yeast strains were derived from KY2002 (**a** *ura3-52 trp1-Δ63 leu2Δ::PET56*). A strain lacking the RAP1 binding site in the *RPS13* promoter was obtained by successive gene replacement. The wild-type *RPS13* locus was first replaced by DNA containing *rps13-Δ1*, which lacks the structural gene. This *rps13* deletion allele was then replaced by DNA containing *rps13-*ΔR. The *his3-Δ84* derivatives that do or do not contain the RAP1 binding site upstream of the TATA region and structural gene were also introduced into yeast by gene replacement. The structures of all relevant alleles were confirmed by genomic hybridization. Strains containing LexA-RAP1 (or the LexA control) and the *lacZ* reporter containing a LexA operator were generated by transforming the relevant plasmids. *bcy1* derivatives of the above-mentioned strains were generated by transformation with a *Bam*HI fragment containing a *bcy1::URA3* disruption allele (obtained from Kunihiro Matsumoto). Because *bcy1* deletion strains grow poorly and often accumulate suppressor mutations during prolonged growth or storage, we were careful to minimize the time from strain construction to RNA analysis. For each experiment, three independent *bcy1::URA3* transformants were generated anew, colony purified, and immediately grown to mid-log phase for subsequent analysis.

For the experiments shown in Fig. 1 to 4, cells were grown in YPD. For the experiment shown in Fig. 6, cells were grown in glucose minimal medium in the presence or absence of 20 mM aminotriazole, a competitive inhibitor of the *HIS3* gene product (36) that elicits the stringent response on ribosomal protein genes (26). The carbon source upshift experiment (Fig. 7) was performed as described previously (49). Cells were grown in minimal medium containing 2% ethanol, 0.05% yeast extract, and 0.04% glucose until mid-log phase. Glucose was then added to 2%, and samples were taken after 0, 30, and 60 min. For the experiment in Fig. 5, strains were assayed for β-galactosidase activity as described previously (17).

RNA analysis. Total RNA was prepared from each sample and quantitated by A_{260}. Ten micrograms of each RNA sample was hybridized to completion with a 10- to 100-fold excess of ^{32}P-labeled oligonucleotides and treated with S1 nuclease as described previously (4, 5). With the exception of the experiments in Fig. 7, all hybridization reactions included the *DED1* probe, which served as the internal control. The oligonucleotides all contain four residues at their 3′ ends that are not complementary to the RNA, thereby permitting an easy distinction between bands due to appropriate RNA-DNA hybrids and undigested probe. Conditions of hybridization probe excess were verified by showing that the observed band intensities were unchanged upon decreasing the amount of probe by a factor of 5. The *HIS3* and *DED1* oligonucleotides have been previously described (5), and the sequences of others used in this study are as follows: *RPS13*, CACCGGCCAACTTCAAT TCTGCGTCCAAACGAGAAGATTCGTAAGGTCCTCC; *RPS4*, GTGTTTCTTGGTCCTCTTCTGTTTGGACGGCCT CTGTTACGGCCACCGAGGTT; and *RPL29*, TTCTGTG GTGATGTTGACCACCGGCCATACCTCTACCACCGG GGTGCTCCTCA.

With the exception of the experiments in Fig. 7, RNA levels of the ribosomal protein genes were quantitated with respect to the *DED1* internal control, using PhosphorImager (Molecular Dynamics) analysis. Each determination represents the average of three RNA samples, which were prepared from three independent colonies (or transformants in the case of *bcy1* deletion strains). The values are accurate to ±15%.

In the carbon source upshift experiment (Fig. 7), *DED1* RNA was unsuitable as an internal control because its levels increased upon glucose addition. Moreover, glucose upshift is a complex physiological phenomenon that is largely uncharacterized except for its effect on ribosomal protein genes; hence, an internal control RNA that is not affected by glucose upshift cannot be chosen with confidence. Thus, for each time point, RNA from equal amounts of cells were hybridized to the ribosomal protein probes; for both the wild-type and *tpk1*^{w}

strains, the samples obtained prior to the shift contained only 70% as much total RNA (assayed by A_{260}) as did the samples obtained 30 and 60 min after the shift. Because of the lack of an internal control, RNA level determinations for this experiment are ±30%.

DNA binding assays. Whole cell extracts were prepared from 100-ml cultures of isogenic *BCY1* and *bcy1* strains grown in YPD to an A_{600} of 1. Cells were resuspended in 1 ml of 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5)–5 mM $MgCl_2$–0.1 mM EDTA–0.5 mM dithiothreitol–10% glycerol–1 mM phenylmethylsulfonyl fluoride–2 μM pepstatin–1 μM leupeptin–50 mM KCl–0.3 M ammonium sulfate. An equal volume of glass beads was added, and cells were lysed by vigorous vortexing, using 10 15-s bursts with cooling on ice between each cycle. Extracts were clarified by microcentrifugation at 4°C for 15 min and stored at −70°C. Extracts (0.8 mg/ml as determined by the Bradford assay) were qualitatively examined by electrophoresis on a sodium dodecyl sulfate–8% polyacrylamide gel followed by Coomassie blue staining. RAP1 protein levels were determined by Western blotting (immunoblotting), using polyclonal mouse antisera (provided by David Shore) at a 1:1,000 dilution followed by a 1:7,500 dilution of goat anti-mouse immunoglobulin G-specific antibody conjugated to alkaline phosphatase.

DNA binding assays were performed with a 23-bp oligonucleotide containing a consensus RAP1 binding site (AATTCA CACCCAGACATCGAATT) that was ^{32}P labeled at its 3′ end. A 34-bp oligonucleotide that contained a mutant AP-1 site (GATCCGGATGTCACATTTTTTTTGGACGGGAA TT; underlined bases represent deviations from the optimal site) served as a negative control. Binding reactions were carried out in 20 μl of extract buffer containing 1 μg of poly(dI-dC), 1 ng of ^{32}P-labeled oligonucleotide, and 0 to 18 μl of whole cell extract. Samples were incubated for 20 min at room temperature and then loaded directly onto 5% native acrylamide gels. The intensity of the RAP1 binding site-dependent complex was roughly proportional to the amount of extract added.

RESULTS

Disruption of BCY1 increases ribosomal protein RNA levels. In the course of experiments designed to identify target genes of ACR1, an ATF/CREB repressor (46), we cloned *RPS13* (*SUP46*), a gene encoding ribosomal protein S13 (45). Regulation of *RPS13* by ACR1 is marginal (transcription is increased by 25% in an *acr1* deletion strain) and probably not direct because the *RPS13* promoter appears to lack an ATF/CREB site. However, in our initial characterization, we investigated whether *RPS13* transcription was affected by the cAMP-dependent protein kinase (PKA), because mammalian ATF/CREB proteins mediate transcriptional induction in response to increased levels of cAMP (9, 11, 12, 31).

Removal of the regulatory subunit of PKA (Bcy1) results in constitutively high levels of PKA activity and increased phosphorylation of substrates. We used quantitative S1 analysis to measure levels of *RPS13* mRNA in isogenic wild-type (KY2002) and *bcy1* disruption (KY2012) strains. To ensure accuracy of these and subsequent determinations, values were normalized to the internal *DED1* control, *bcy1* strains were analyzed immediately after their construction, and at least three independent colonies for each strain were tested; errors were within ±15%. As shown in Fig. 1, *RPS13* transcription is increased 1.9-fold in the *bcy1* strain compared with the isogenic wild-type strain. A similar twofold increase is observed on RNA blots, using *TBP* RNA levels as an internal control (data not shown).

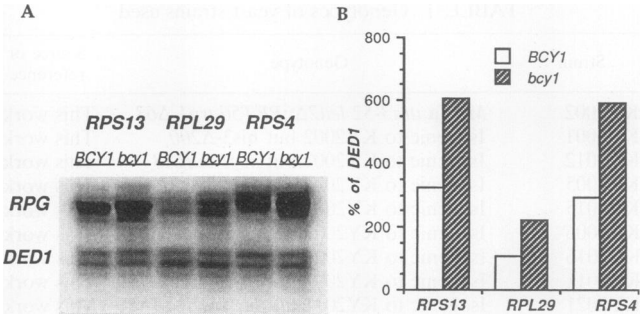

FIG. 1. Constitutively activated PKA increases transcription of ribosomal protein genes. (A) Total RNAs from *BCY1* (KY2002) and *bcy1* (KY2012) strains were hybridized to completion with an excess of *DED1* and the indicated ribosomal protein (*RPS13*, *RPL29*, *RPS4*) oligonucleotide probes, and the products were treated with S1 nuclease. The positions of the *DED1* and the ribosomal protein gene (RPG) transcripts are indicated. (B) RNA levels of the indicated ribosomal protein genes (expressed as the percentage of *DED1* mRNA levels) in *BCY1* and *bcy1* strains.

To determine whether this PKA effect was specific to *RPS13*, we analyzed the same RNA preparations with probes to *RPS4* and *RPL29*, which encode small-subunit protein S4 and large-subunit protein L29, respectively (Fig. 1). In *bcy1* strains, *RPS4* and *RPL29* RNA levels are 2.0-fold higher than in the wild-type strain. Thus, constitutively activated PKA increases transcription of at least three ribosomal protein genes.

RAP1 binding sites are necessary and sufficient for PKA-dependent induction. RAP1 binding sites in ribosomal protein promoters are associated with increased transcription in response to a carbon source upshift (15) and decreased transcription in response to histidine starvation (26). The *RPS13* promoter contains two sites (−220 to −209 and −207 to −193) that strongly resemble the consensus RAP1 binding site (45). To determine whether the RAP1 binding sites mediate the PKA effect, we replaced the wild-type *RPS13* gene by a derivative containing a 30-bp deletion that removes both RAP1 binding sites. Quantitative RNA analysis (Fig. 2) shows that the PKA-dependent transcriptional increase is not observed in strains containing the deleted *RPS13* promoter; in fact, RNA levels in the *bcy1* strain (KY2021) are slightly decreased (by 15%) in comparison with the wild-type strain (KY2011). As expected from analyses of other ribosomal protein genes (32, 49), deletion of the RAP1 binding sites in the *RPS13* promoter reduces transcription to 60% of the wild-type level. Thus, the RAP1 binding sites act positively in *RPS13* transcription, and they are necessary for increased transcription mediated by constitutively activated PKA.

We analyzed transcription from a *HIS3* promoter derivative containing a consensus RAP1 binding site upstream of the *HIS3* T_C and T_R TATA elements at the position normally occupied by a GCN4 binding site (Fig. 3). In a wild-type strain (KY2006), transcription from the artificial promoter is initiated equally from the +1 and +13 sites at a level comparable to that of the native *HIS3* promoter under noninducing conditions. However, in a *bcy1* disruption strain (KY2016), the level of the +13 transcript was increased by a factor of 2, whereas the level of the +1 transcript was slightly decreased (by 20%). Transcription depends on the RAP1 binding site, because a control promoter lacking this site shows very low

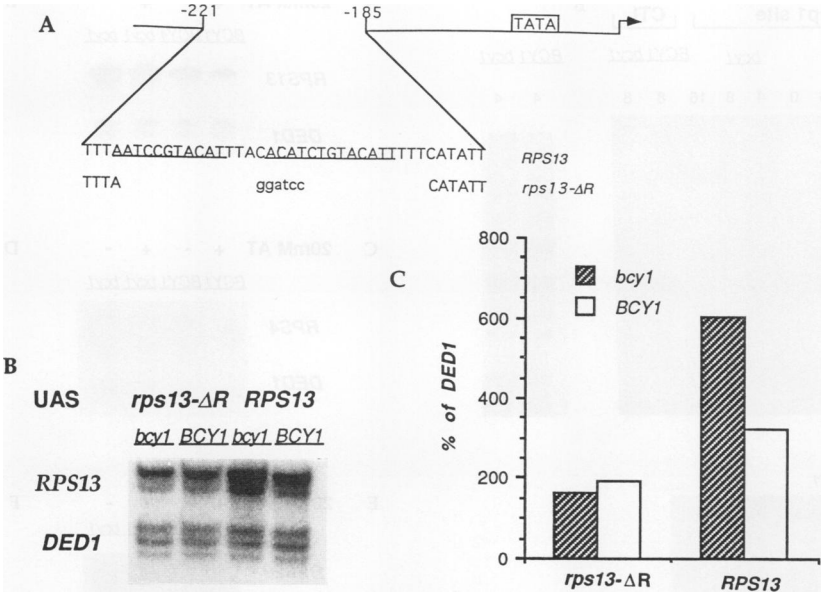

FIG. 2. RAP1 binding sites in the *RPS13* promoter are necessary for the PKA-induced transcriptional increase. (A) Structure of the *RPS13* promoter and the deleted derivative (*rps13*-ΔR) lacking both RAP1 binding sites. (B) Total RNAs from *BCY1* (KY2011) and *bcy1* (KY2021) strains containing the wild-type or deleted *RPS13* promoter were hybridized to completion with an excess of *DED1* and *RPS13* oligonucleotide probes, and the products were treated with S1 nuclease; the positions of the *DED1* and *RPS13* transcripts are indicated. UAS, upstream activation sequence. (C) Quantitation of *RPS13* RNA levels (expressed as the percentage of *DED1* mRNA levels) in *BCY1* and *bcy1* strains.

levels of *HIS3* RNA (strains KY2005 and KY2015). Thus, a single RAP1 binding site can confer PKA inducibility to a heterologous promoter, and the magnitude of the transcriptional effect is comparable to that of native ribosomal protein genes. Explanations for the different *HIS3* initiation patterns in wild-type and *bcy1* deletion strains will be considered in Discussion.

Constitutively activated PKA increases RAP1 transcriptional activation. The PKA-dependent increase in transcription of ribosomal protein genes could be due to increased expression, DNA-binding activity, or transcriptional activity of RAP1. To address these possibilities, we quantitated RAP1 DNA-binding activity in cell extracts from wild-type (KY2002) and *bcy1* disruption (KY2012) strains (Fig. 4). Although *bcy1* strains show increased transcription through RAP1 binding sites, RAP1 DNA-binding activity appears to be approximately threefold lower than that observed in a wild-type strain. It is likely that this slight reduction in RAP1 DNA-binding activity reflects decreased RAP1 protein levels (assayed by Western blotting). Consistent with this observation, a *RAP1-lacZ* protein fusion containing the intact *RAP1* promoter region (−1200 to +50) shows threefold less β-galactosidase activity in a *bcy1* deletion strain than in an isogenic *BCY1* strain. Thus, the PKA effect on ribosomal protein genes is unlikely to be due to increased RAP1 binding to the promoters.

To directly test the hypothesis that PKA increases RAP1 transcriptional activity, we examined a LexA-RAP1 hybrid protein for its ability to stimulate transcription from an artificial promoter containing a LexA operator upstream of a TATA element (Fig. 5). In a *BCY1* strain, LexA-RAP1 behaves as a weak activator protein in that it stimulates transcription only 2.4-fold more efficiently than the LexA control. Importantly, transcriptional activation by LexA-RAP1 is increased by a factor of 8 in a *bcy1* strain, whereas activation by LexA is only twofold higher. Thus, PKA increases RAP1-dependent transcriptional activity.

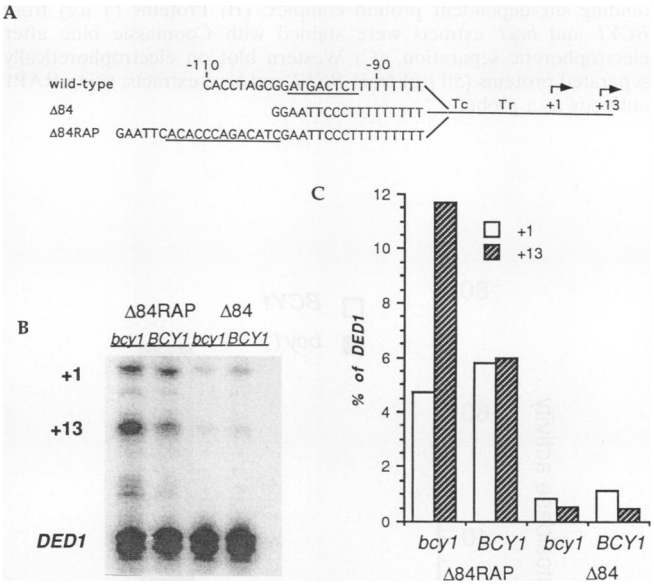

FIG. 3. A RAP1 binding site confers PKA inducibility to a heterologous promoter. (A) Structures of *his3* promoters, with relevant sequences shown upstream of the T_C and T_R TATA elements and +1 and +13 initiation sites (23, 30, 35). The wild-type *HIS3* promoter (only residues −85 to −110 are shown) contains a GCN4 binding site (underlined); *his3-Δ84* contains an *Eco*RI octanucleotide linker in place of the region between −95 to −447 (37); *his3-Δ84*RAP contains a RAP1 binding site (underlined) cloned at the *Eco*RI site of *his3-Δ84*. (B) Total RNAs from *BCY1* and *bcy1* strains containing the *his3-Δ84* promoters with (KY2006 and KY2016, respectively) or without (KY2005 and KY2015, respectively) a RAP1 binding site were hybridized to completion with an excess of *DED1* and *HIS3* oligonucleotide probes, and the products were treated with S1 nuclease; the positions of the *DED1* and *HIS3* transcripts are indicated. (C) Quantitation of the *HIS3* +1 and +13 transcripts (expressed as the percentage of *DED1* mRNA levels) in the indicated strains.

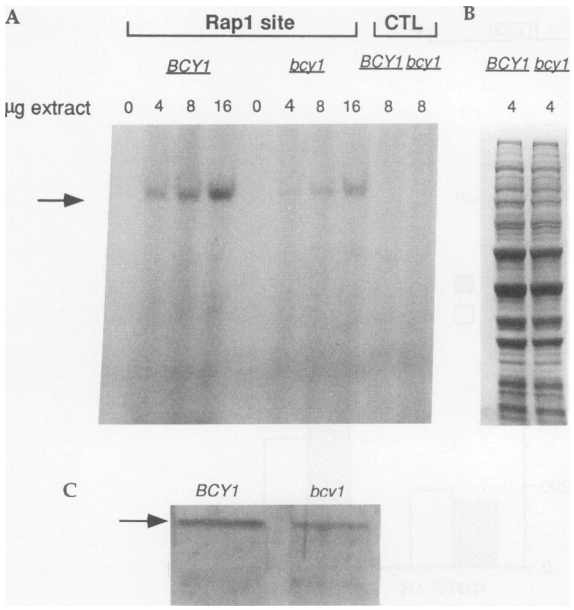

FIG. 4. RAP1 DNA-binding activity in *BCY1* and *bcy1* strains. (A) Cell extracts (micrograms of total protein are indicated) from *BCY1* (KY2002) and *bcy1* (KY2012) strains were incubated with oligonucleotides containing a RAP1 binding site or a mutated AP-1 site (CTL); the resulting products were electrophoretically separated on a 5% native acrylamide gel. The arrow indicates the position of the RAP1 binding site-dependent protein complex. (B) Proteins (4 µg) from *BCY1* and *bcy1* extracts were stained with Coomassie blue after electrophoretic separation. (C) Western blot on electrophoretically separated proteins (50 µg) from *BCY1* and *bcy1* extracts, using RAP1 antibody as a probe.

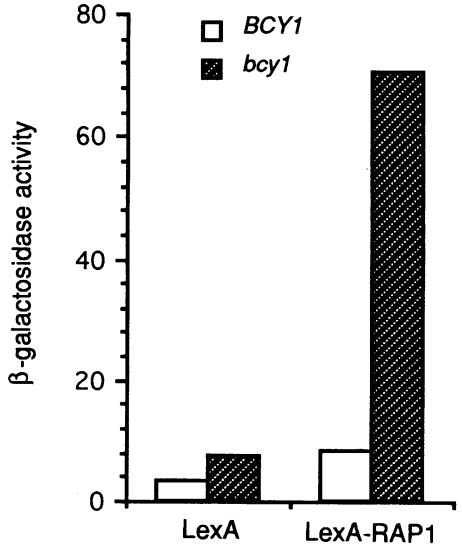

FIG. 5. RAP1 transcriptional activity is increased in *bcy1* strains. β-Galactosidase activities (standard units normalized to number of cells) were averaged from three independent transformants of *BCY1* and *bcy1* strains containing either LexA or LexA-RAP1 and a *lacZ* reporter plasmid carrying a LexA operator; the error is ±30%.

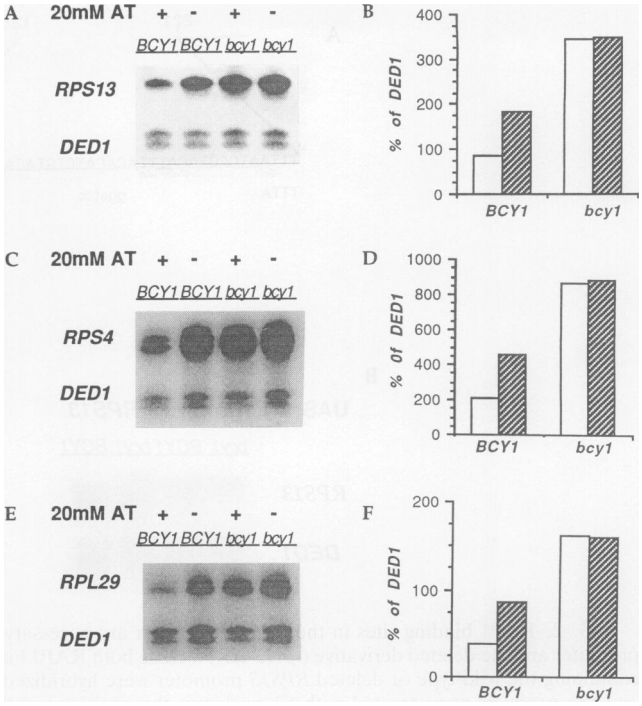

FIG. 6. Constitutively activated PKA overrides the stringent response. (A, C, and E) Total RNAs from *BCY1* (KY2002) and *bcy1* (KY2012) strains grown in the presence or absence of 20 mM aminotriazole (AT) were hybridized to completion with an excess of *DED1* and the indicated ribosomal protein oligonucleotide probes, and the products were treated with S1 nuclease; the positions of the *DED1* and ribosomal protein RNAs are indicated. (B, D, and F) Quantitation of ribosomal protein RNA levels (expressed as the percentage of *DED1* mRNA levels) in cells grown in the presence (open boxes) or absence (shaded boxes) of 20 mM aminotriazole.

Constitutively activated PKA overrides stringent control of ribosomal protein genes. Although the preceding experiments indicate that PKA affects the transcription of ribosomal protein genes, they do not address the more important issue of whether PKA is a physiologically relevant regulator. We therefore examined the role of PKA in the two known situations in which transcription of ribosomal protein genes is affected by changes in growth conditions.

Yeast cells have a stringent response in which expression of ribosomal protein genes is reduced upon amino acid deprivation (48). When cells are starved for histidine by exposure to 20 mM aminotriazole, transcription of ribosomal protein genes is reduced in a RAP1 binding site-dependent manner (26). Given the importance of PKA in RAP1-dependent transcription (Fig. 2, 3, and 5), we investigated whether stringent control of ribosomal protein genes involves the down-regulation of PKA activity during conditions of starvation. Such a hypothesis would predict that strains with constitutively activated PKA would not show a stringent response. Indeed, exposure of a *bcy1* disruption strain (KY2012) to 20 mM aminotriazole does not affect *RPS13*, *RPS4*, and *RPL29* RNA levels, whereas similar treatment of a wild-type strain (KY2002) results in the expected twofold decreases (Fig. 6). Thus, the inability to regulate PKA activity leads to the inability to regulate ribosomal protein genes in response to starvation.

Cells with unregulated low PKA levels do not significantly increase transcription of ribosomal protein genes upon carbon

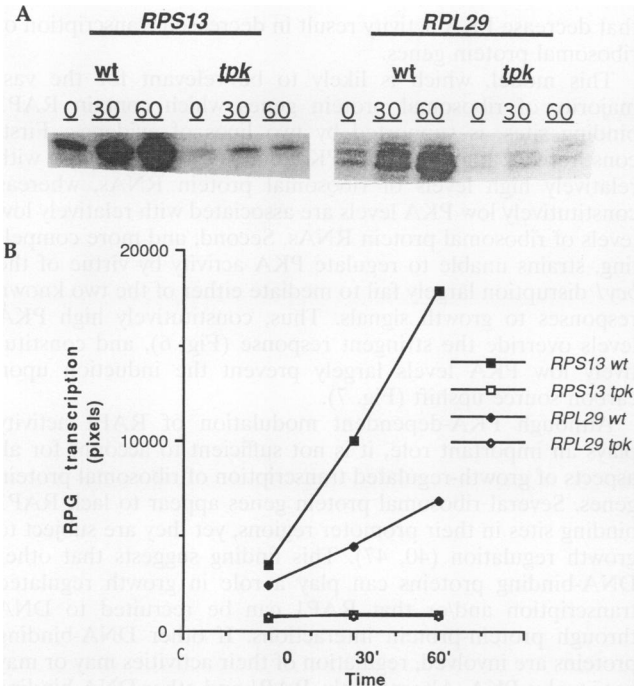

FIG. 7. Constitutive low levels of PKA largely abolish the response to carbon source upshift. (A) Total RNAs from *BCY1* (SP1) and *bcy1 tpk1*^{w} (RS13-58A-1) strains grown in ethanol medium and subjected to a glucose shift for 0, 30, and 60 min were hybridized to completion with an excess of the indicated oligonucleotide probes, and the products were treated with S1 nuclease; the positions of the ribosomal protein RNAs are indicated. Each hybridization reaction contained RNA from equal amounts of cells; for both strains, the samples obtained prior to the shift contained only 70% of the total RNA (assayed by A_{260}) as the samples obtained 30 and 60 min after the shift. (B) Quantitation of ribosomal protein RNA levels (expressed in pixels obtained from Phosphorimager analysis and normalized to amount of total RNA in each sample). Because of the lack of a suitable internal control (see Materials and Methods), determinations of RNA levels are somewhat less accurate than in other experiments (±30% instead of ±15%). wt, wild type; RPG, ribosomal protein gene.

source upshift. When glucose is added to yeast cells growing in medium containing ethanol as a sole carbon source, there is a rapid increase in ribosomal protein gene transcription (15) and in intracellular levels of cAMP (25, 41). Since cAMP regulates the catalytic activity of PKA, induction of ribosomal protein genes upon carbon source upshift could be explained by a simple mechanism in which elevated cAMP levels increase PKA activity, thereby leading to RAP1-dependent transcriptional activation. If this is correct, a prediction of this hypothesis is that *bcy1* strains, which do not regulate PKA activity, would be unable to induce ribosomal gene transcription upon a carbon source upshift.

To test this model, we carried out a standard carbon source upshift experiment (15) in isogenic wild-type and *bcy1* strains (Fig. 7). However, *bcy1* strains containing the three *TPK* genes, which encode the catalytic subunit of PKA, are unable to grow in ethanol medium (42) and cannot be tested. Instead, we examined a *bcy1* strain (RS13-58A-1, kindly sent by Michael Wigler) containing disrupted *TPK2* and *TPK3* genes and the *tpk1*^{w} allele that encodes a protein with low PKA activity (3). In this strain, *RPS13* and *RPL29* RNA levels in ethanol medium are about fourfold lower than in the isogenic wild-type strain (SP1, also obtained from Michael Wigler). Upon glucose addition, the wild-type strain shows the expected transcriptional increase (about fivefold for *RPS13* and threefold for *RPL29* at 60 min). In contrast, the strain with constitutively low PKA activity shows a minimal increase in *RPS13* RNA levels (30%, which is at the edge of the experimental error) and no detectable effect on *RPL29* RNA levels. Although the lack of a suitable internal control (see Materials and Methods) makes these RNA measurements somewhat less accurate than determinations in the other experiments in this study, it is clear that low, unregulated PKA levels largely abolish transcriptional induction upon carbon source upshift.

DISCUSSION

RAP1 mediates PKA-induced transcription of ribosomal protein genes. In this report, we demonstrate that ribosomal protein mRNA levels are increased twofold in strains that have constitutively high levels of PKA. In the case of *RPS13*, this effect is eliminated upon deleting a small region of the promoter that contains two RAP1 binding sites. Furthermore, a similar twofold induction is observed when a consensus RAP1 binding site is placed upstream of a heterologous promoter. Thus, increased expression of ribosomal protein genes by constitutively activated PKA is mediated primarily, and perhaps exclusively, through RAP1 binding sites. The increased levels of ribosomal protein mRNAs almost certainly reflect increased transcriptional initiation because it is extremely unlikely that RAP1 binding sites affect mRNA stability.

RAP1 is an abundant nuclear protein, and it is the only protein known to interact efficiently with RAP1 binding sites (2, 18, 21, 33). Moreover, in all cases examined, mutations in RAP1 can affect functions that depend on RAP1 binding sites, e.g., transcriptional activation, mating-type silencing, and telomere length regulation (13, 20, 22, 38). Although the involvement of an unknown DNA-binding protein that interacts with RAP1 binding sites cannot be rigorously excluded, we conclude that the PKA effect on ribosomal protein genes is mediated by RAP1 itself. This conclusion is strongly and independently supported by the increased transcriptional activity of the LexA-RAP1 hybrid protein in *bcy1* strains.

Constitutively activated PKA affects RAP1 transcriptional activity. In principle, the increase in RAP1-dependent transcription in *bcy1* deletion strains could reflect increased expression, DNA-binding activity, or transcriptional activity of RAP1. However, RAP1 DNA-binding activity in a *bcy1* deletion strain is slightly reduced from that observed in a wild-type strain. This result is consistent with the observation that RAP1 DNA-binding activity is not correlated with changes in the expression of ribosomal protein genes (19). Expression of RAP1, assayed by Western blotting or by a RAP1-LacZ protein fusion, is also slightly lower. In contrast, RAP1 transcriptional activity, assayed by a LexA-RAP1 hybrid protein, is clearly more efficient in a *bcy1* strain than in a *BCY1* strain. These results indicate that the PKA effect on ribosomal protein genes is due to increased transcriptional activity of RAP1.

The conclusion of increased RAP1 transcriptional activity is further supported by the initiation pattern of the artificial promoter containing a consensus RAP1 binding site upstream of the *HIS3* T_C and T_R TATA elements. In wild-type strains, RAP1 confers equal transcription from the +1 and +13 sites, whereas the increase in the *bcy1* deletion strain is restricted to the +13 site (Fig. 3). Extensive analysis of the *HIS3* promoter indicates that a preferential increase in +13 transcription is associated with increased transcriptional activation mediated through T_R, a conventional TATA element (23, 28–30, 35). In

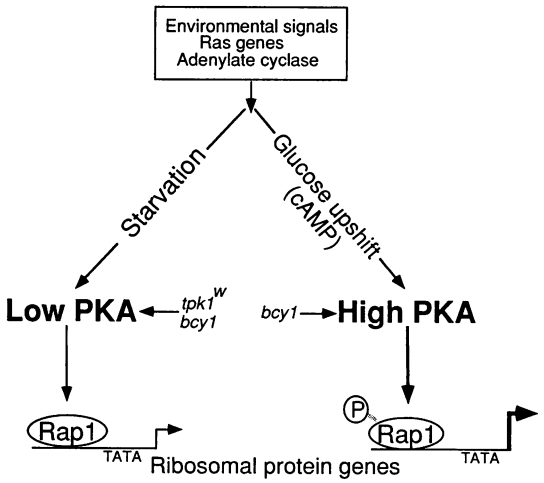

FIG. 8. Model for transcriptional regulation of ribosomal protein genes in response to growth signals. High levels of PKA increase ribosomal protein RNA levels by stimulating RAP1 transcriptional activity. The increased transcriptional activity of RAP1 reflects phosphorylation (P) of RAP1 (or an associated protein) by PKA (or a kinase affected by PKA). Conditions of amino acid starvation or glucose upshift result in low or high PKA activity, respectively. Strains that have constitutively high (*bcy1*) or low (*bcy1 tpk1*^{w}) levels of PKA activity do not respond to growth signals. Environmental signals, *ras* genes, adenylate cyclase, and other factors are involved in regulation of PKA activity. Increased PKA activity during glucose upshift involves increased cAMP levels, whereas the mechanism for decreased PKA activity during starvation is unknown. See text for details and limitations of the model.

an equivalent promoter containing an optimal GCN4 binding site upstream of T_C and T_R, GCN4 derivatives with weak activation domains stimulate transcription equally from +1 and +13, whereas derivatives with stronger activation domains preferentially stimulate +13 transcription (28, 29). Conversely, a full-length GCN4 derivative with slightly reduced DNA-binding activity shows the initiation pattern conferred by wild-type GCN4 even though the overall transcriptional level is decreased (29). Thus, the fact that increased RAP1-dependent transcription in *bcy1* strains is preferentially initiated at the +13 site suggests that PKA increases transcriptional activation, not DNA binding, of RAP1.

Increased transcription of ribosomal protein genes must involve phosphorylation of some substrate(s) by PKA. The simplest hypothesis is that PKA phosphorylates RAP1 and increases its transcriptional activity. Given the functional complexities of RAP1 (14, 44), it is also possible that PKA phosphorylates a RAP1-associated protein that affects transcriptional activation. Even if increased transcriptional activity is due to RAP1 phosphorylation, PKA might function indirectly through a protein kinase cascade. Our results do not distinguish between these hypotheses.

PKA-dependent modulation of RAP1 activity plays a major role in growth-regulated transcription of ribosomal protein genes. RAP1 is responsible for the regulation of ribosomal protein genes in response to growth conditions (15, 26) and to constitutively high levels of PKA. Our results strongly suggest that modulation of RAP1 transcriptional activity by PKA largely accounts for this growth-regulated transcription (Fig. 8). In this view, conditions that increase PKA activity result in increased transcription of ribosomal genes, whereas conditions that decrease PKA activity result in decreased transcription of ribosomal protein genes.

This model, which is likely to be relevant for the vast majority of ribosomal protein genes which contain RAP1 binding sites, is supported by two lines of evidence. First, constitutively high levels of PKA activity are associated with relatively high levels of ribosomal protein RNAs, whereas constitutively low PKA levels are associated with relatively low levels of ribosomal protein RNAs. Second, and more compelling, strains unable to regulate PKA activity by virtue of the *bcy1* disruption largely fail to mediate either of the two known responses to growth signals. Thus, constitutively high PKA levels override the stringent response (Fig. 6), and constitutively low PKA levels largely prevent the induction upon carbon source upshift (Fig. 7).

Although PKA-dependent modulation of RAP1 activity plays an important role, it is not sufficient to account for all aspects of growth-regulated transcription of ribosomal protein genes. Several ribosomal protein genes appear to lack RAP1 binding sites in their promoter regions, yet they are subject to growth regulation (40, 47). This finding suggests that other DNA-binding proteins can play a role in growth regulated transcription and/or that RAP1 can be recruited to DNA through protein-protein interactions. If other DNA-binding proteins are involved, regulation of their activities may or may not involve PKA. Alternatively, RAP1 and other DNA-binding proteins may recruit a common cofactor that is regulated by PKA. Conversely, RAP1 binding sites are found in genes that are unlikely to be regulated as a function of growth rate. This observation is likely to reflect the ability of RAP1 to interact with multiple proteins that differentially affect promoter function (14, 38, 44).

Our results do not address the question of how growth signals alter PKA activity. However, in the case of carbon source upshift, this is probably due to the increased cAMP levels that occur under these conditions (25, 41) and are mediated by the *ras*-dependent signal transduction pathway (reviewed in reference 1) (Fig. 8). Higher cAMP levels would certainly increase PKA activity and hence ribosomal protein gene transcription. In the case of the stringent response, the nature of the signal and the signalling pathway is less clear. The putative decrease in PKA activity might be due to lowered cAMP levels or to an alternative mechanism. Although mechanistic details remain to be elucidated, our results clearly implicate PKA in transducing growth signals into RAP1-dependent transcriptional regulation of ribosomal protein genes.

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