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A Paradoxical Increase of a Metabolite upon Increased Expression of Its Catabolic Enzyme: the Case of Diadenosine Tetraphosphate (Ap_4A) and Ap_4A Phosphorylase I in *Saccharomyces cerevisiae*

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The *APA1* gene in *Saccharomyces cerevisiae* encodes Ap_4A phosphorylase I, the catabolic enzyme for diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A). *APA1* has been inserted into a multicopy plasmid and into a centromeric plasmid with a *GAL1* promoter. Enhanced expression of *APA1* via the plasmids resulted in 10- and 90-fold increases in Ap_4A phosphorylase activity, respectively, as assayed in vitro. However, the intracellular concentration of Ap_4A exhibited increases of 2- and 15-fold, respectively, from the two different plasmids. Intracellular Ap_4A increased 3- to 20-fold during growth on galactose of a transformant with *APA1* under the control of the *GAL1* promoter. Intracellular adenosine 5′-P^1-tetraphospho-P^4-5‴-guanosine (Ap_4G) and diguanosine 5′,5‴-P^1,P^4-tetraphosphate (Gp_4G) also increased in the transformant under these conditions. The chromosomal locus of *APA1* has been disrupted in a haploid strain. The Ap_4A phosphorylase activity decreased by 80% and the intracellular Ap_4A concentration increased by a factor of five in the null mutant. These results with the null mutant agree with previous results reported by Plateau et al. (P. Plateau, M. Fromant, J.-M. Schmitter, J.-M. Buhler, and S. Blancquet, J. Bacteriol. 171:6437–6445, 1989). The paradoxical increase in Ap_4A upon enhanced expression of *APA1* indicates that the metabolic consequences of altered gene expression may be more complex than indicated solely by assay of enzymatic activity of the gene product.

Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) has been identified in a variety of prokaryotic and eukaryotic organisms (15, 45), but its physiological function(s) is unknown. Ap_4A is synthesized in a side reaction catalyzed by some tRNA synthetases from several different organisms (4, 7, 17, 46). Rapaport and Zamecnik (31) initially proposed that Ap_4A is a regulatory nucleotide that couples protein synthesis and other processes of proliferation such as replication, but this hypothesis remains unproven. Ames and coworkers have proposed that Ap_4A and related dinucleoside polyphosphates are signal nucleotides or alarmones involved in the adaptive response to oxidative stress (5, 24). However, experiments with an *Escherichia coli* mutant in the gene encoding Ap_4A hydrolase demonstrate that Ap_4A cannot be the signal for induction of the oxidative stress and heat shock responses in this bacterium (12, 28).

In *Saccharomyces cerevisiae*, Ap_4A is degraded by two similar phosphorylases (29, 30). Phosphorolysis of Ap_4A in the presence of P_i and a divalent cation yields ADP and ATP (18). About 80 to 85% of the endogenous Ap_4A is catabolized by Ap_4A phosphorylase I (29, 30). The *APA1* gene encoding this enzyme has been cloned and sequenced (22, 30) and physically mapped on chromosome III (16, 22). (We had previously used the designation *DTP* for the gene encoding Ap_4A phosphorylase I [22] but have now chosen to use *APA1* [30].) Experiments applying molecular genetics to elucidate the function of Ap_4A in *S. cerevisiae* have been initiated. The basic approach is to analyze the physiological effects in *S. cerevisiae* of enhanced expression of *APA1* and disruption of *APA1* on the basis of presented concepts (6). We anticipated that disruption and enhanced expression of *APA1* would result in an increase and a decrease, respectively, in the in vivo concentration of Ap_4A. However, increased expression of *APA1* via a multicopy plasmid did not yield a decrease in intracellular Ap_4A (22).

Here we report a paradoxical increase, up to 20-fold, in intracellular Ap_4A upon enhanced expression of *APA1* via a plasmid with a *GAL1* promoter. Such expression of *APA1* resulted in a 90-fold increase in Ap_4A phosphorylase I activity in crude extracts. Disruption of *APA1* caused the expected increase, up to fivefold, in intracellular Ap_4A.

MATERIALS AND METHODS

Materials. Medium reagents were purchased from Difco Laboratories (Detroit, Mich.). Ap_4A was purchased from Sigma Chemical Co. (St. Louis, Mo.) and custom labeled with tritium (Amersham Corp., Arlington Heights, Ill.). [^{3}H]Ap_4A was purified by chromatography on dihydroxyboronyl-RioRex 70 resin (2) and analyzed for purity by isocratic high-pressure liquid chromatography (HPLC) (14). Restriction endonucleases and DNA-utilizing enzymes were from New England Biolabs, Inc. (Beverly, Mass.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.).

Strains and growth. The strains of *S. cerevisiae* and transformants used in this study are listed in Table 1. Yeast cells were grown in YNB (0.67% yeast nitrogen base without amino acids and with ammonium sulfate) with 2% glucose or 2% galactose and supplements (each at 20 mg/liter) as required (36). Yeast cells were grown to specified cell densities as measured by cell counts on a hemacytometer.

Plasmid construction and transformation techniques. A 3.1-kb *Kpn*I-*Eco*RI fragment containing the *APA1* gene was subcloned into the multicopy vector YEp352 (20) as previously described (22). We constructed a plasmid for enhanced expression of the *APA1* gene under the control of the yeast *GAL1* promoter. Polymerase chain reaction (11) amplification was done by using primers based on the 5′ and 3′ flanking regions of the *APA1* gene to generate a 1.4-kb

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TABLE 1. *S. cerevisiae* strains and transformants used in this study

Strain	Genotype	Reference or source
CGY339	*ura3-52 his4-29 pep4-3 GAL*$^+$	G. Fink
CGY339(YEp352)[a]	Transformant with YEp352	21
CGY339(YEp352-*APA1*)	Transformant with YEp352 containing *APA1*	21
CGY339(pBDA)[b]	Transformant with a *URA3 GAL1* promoter plasmid	This study
CGY339(pBDA4)	Transformant with pBDA containing *APA1*	This study
CGY339(pBDA7)	Transformant with pBDA containing *APA1* in reverse orientation	This study
YPH252	*ura3-52 his3-Δ200 lys2-801 ade2-101 leu2-Δ1 trp1-Δ1*[c]	37
YPH252-H	YPH252*apa1Δ::HIS3*	This study
YPH252(pBDA)	Transformant with pBDA	This study
YPH252(pBDA4)	Transformant with pBDA containing *APA1*	This study

[a] YEp352, a multicopy plasmid, described by Hill et al. (20).
[b] pBDA, a single-copy, centromeric plasmid, prepared in this study from pBM150 (15).
[c] *trp1-Δ1* deletes the UAS elements required for expression of *GAL3*, so YPH252 is gal3 and requires adaptation on galactose (27, 37).

fragment encoding *APA1*. Appropriate restriction sites were added by 5′ extensions of the primers. This fragment was sequenced by using the dideoxynucleotide chain termination method (34) to confirm the absence of mutations induced by the polymerase chain reaction. The pBDA plasmid was constructed by the addition of a *Hin*dIII linker at the *Bam*HI site in pBM150 (21). The 1.4-kb sequence containing 11 bp of the 5′ flanking region, the 963-bp open reading frame of *APA1*, and 426 bp of the 3′ flanking region was inserted into pBDA at the *Hin*dIII site, which is 66 bp downstream of the *GAL1* transcription initiation site. The resulting plasmid was designated pBDA4. A control plasmid, pBDA7, was constructed by inserting the 1.4-kb *APA1* gene component into pBDA in the reverse orientation. Transformants with pBDA and pBDA7 served as controls for the pBDA4 transformant.

We constructed an *APA1* disruption vector by inserting a 316-bp 5′ fragment of *APA1*, the *HIS3* gene (42), and a 498-bp 3′ fragment of *APA1* into pBluescript II KS (Stratagene). The 5′ and 3′ fragments were generated by polymerase chain reaction (11) amplification. The 5′ fragment extended from nucleotide −151 to nucleotide +165, and the 3′ fragment extended from nucleotide 894 to nucleotide 1392, with nucleotide 1 being A of the ATG start codon. For each fragment, appropriate restriction sites were added by 5′ extensions of the primers. A 729-bp segment of the open reading frame of *APA1* was deleted and replaced with the *HIS3* gene. The disrupted *APA1* gene with the *HIS3* insert was removed from the plasmid by treatment with *Nru*I and *Tth*111 I restriction endonucleases such that the integrity of the flanking sequences was maintained to facilitate homologous recombination (32). The resulting DNA fragment was purified by agarose gel electrophoresis (33). *S. cerevisiae* YPH252 was transformed with the purified DNA fragment to disrupt the endogenous *APA1* gene and generate the null mutant as described by Rothstein (32). In vivo disruption of *APA1* was confirmed by Southern blot analysis (33, 39) and a decrease in Ap_4A phosphorylase activity as assayed in the crude supernatant fraction. All yeast transformations were done by using single-stranded DNA as a carrier (35).

Collection and extraction of cells for nucleotide analyses. Portions (50 to 100 ml) of the yeast cultures were rapidly harvested (10 to 30 s) by filtration on Millipore HA filters (1.2-μm pore size, 47-mm diameter). Each filter was immediately immersed and vortexed in 6.5 ml of cold 5% (wt/vol) trichloroacetic acid containing 1 pmol of [^{3}H]Ap_4A. The dispersed cells were sonicated in trichloroacetic acid for two 30-s periods and kept on ice for 30 min. Preparations were centrifuged at 27,000 × *g* for 20 min at 4°C. Pellets were washed with 95% (vol/vol) ethanol and dissolved in 2 ml of 2 N NaOH for the determination of protein (26). A 100-μl portion of each supernatant was used for measurement of ADP and ATP. The remainder of each supernatant was subjected to boronate chromatography and treatment with alkaline phosphatase for the isolation of dinucleoside polyphosphates as previously described in detail (14).

Measurement of nucleotides. Cellular extracts purified by boronate chromatography and treated with alkaline phosphatase were quantitatively analyzed for Ap_4A by area integration of peaks on a Partisil SAX HPLC column (Whatman, Inc.). In some experiments, the dinucleoside tetraphosphates adenosine 5′-P^1-tetraphospho-P^4-5‴-guanosine (Ap_4G) and diguanosine 5′,5‴-P^1,P^4-tetraphosphate (Gp_4G) were also measured. The dinucleoside tetraphosphates were eluted isocratically with 0.30 M ammonium phosphate (pH 5.5) after a precolumn purification described in detail previously (14). Recovery was determined by counting the [^{3}H]Ap_4A radioactivity in a portion of the collected Ap_4A peak for each sample. Measured Ap_4A values were corrected for recoveries which averaged 64 ± 5%. The recoveries for Ap_4G and Gp_4G were assumed to be the same as for Ap_4A.

ATP and ADP in the crude extracts were quantitatively measured by HPLC in a manner similar to that described above except that a precolumn purification process was not used, and they were eluted isocratically with 0.27 M ammonium phosphate (pH 5.5). The recoveries of ATP and ADP were assumed to be 100%.

Preparation of crude supernatants. Crude supernatants were prepared for assay of Ap_4A phosphorylase activity and for Western blot (immunoblot) analysis. Cells were harvested from separate cultures grown under the same conditions and to the same cell density as cultures used for nucleotide analysis. Cells were harvested by centrifugation; suspended in 2 ml of a mixture containing 50 mM potassium phosphate (pH 6.8), 10% (vol/vol) glycerol, 5 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin per liter, and 0.5 μg of pepstatin per liter per g (wet weight) of cells; and broken by vortexing with glass beads. Homogenates were centrifuged at 27,000 × *g* for 20 min at 4°C to obtain crude supernatants.

Assay of enzymatic activity. Ap_4A phosphorylase in crude supernatants was assayed by formation of [^{3}H]ADP and [^{3}H]ATP from [^{3}H]Ap_4A and P_i. Activity was measured in a mixture of 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–KOH (pH 7.5), 0.5 mM $MnCl_2$, and 1 mM K_2HPO_4 at 37°C for 10 min in a volume of 100 μl. The

reaction products were separated from residual substrate by column chromatography on a boronate-derivatized resin (2). The activity was expressed as nanomoles of ADP + ATP formed per minute per milligram of protein.

Electrophoresis and blotting. Crude supernatants were subjected to electrophoresis on 12.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) by using the discontinuous buffer system described by Laemmli (23) and modified by Studier (43). Proteins in gels were transferred electrophoretically to nitrocellulose (44) and probed immunologically with polyclonal antibodies to Ap_4A phosphorylase I (1). Goat anti-rabbit antibody coupled to horseradish peroxidase was used to detect immunopositive proteins.

Yeast genomic DNA was isolated as described by Spencer et al. (40) by using lyticase (Sigma) to prepare spheroplasts. Plasmid DNA was prepared on a large scale by adaptation of the procedure described by Birnboim and Doly (3). Various DNA samples, after treatment with appropriate restriction endonucleases, were analyzed electrophoretically on 0.8 to 1.2% agarose (33). Gels containing genomic DNA fragments were blotted to nylon membranes (39). Blots were probed with DNA fragments labeled with digoxigenin-11-dUTP and visualized on X-ray film by chemiluminescence by using the Genius system from Boehringer Mannheim.

Data analyses. In each assay of enzymatic activity, each crude supernatant was assayed in duplicate. Values are the mean and standard deviation ($\bar{x} \pm$ SD) for triplicate assays. For the measurement of nucleotides, triplicate or quadruplicate samples were analyzed at each cell density in each experiment. Values ($\bar{x} \pm$ SD) are expressed in picomoles of nucleotide per milligram of protein (measured in the pellets obtained by precipitation with trichloroacetic acid).

RESULTS

Enzymatic activity with enhanced expression or disruption of *APA1*. We previously observed a ninefold increase in Ap_4A phosphorylase activity in a crude supernatant from *S. cerevisiae* CGY339 transformed with YEp352 containing *APA1* (22). The transformant had been grown in a rich medium containing 3% glucose and 2% NZamineA to 6×10^7 to 7×10^7 cells per ml (late log phase). In the present study, enzymatic activities were assayed in crude supernatants from transformants grown in minimal medium to 3×10^7 to 4×10^7 cells per ml (early stationary phase in this medium). The 10-fold increase in Ap_4A phosphorylase activity in the experimental transformant grown in minimal medium (Table 2) was the same increase found when the transformant had been grown in rich medium.

To increase the Ap_4A phosphorylase activity further than obtainable via a multicopy plasmid, we subcloned *APA1* into pBDA under the control of the *GAL1* promoter and induced expression of *APA1* by growth of the transformant in the presence of galactose. *S. cerevisiae* CGY339 and YPH252 were both transformed with control and experimental plasmids, and enzymatic activities were assayed in crude supernatants prepared from the transformants. The results are shown in Table 2. The specific activities in the crude supernatants from the pBDA4 transformants were about 70 to 90 times larger than the specific activities in the crude supernatants from control transformants for both strains. The crude supernatants were subjected to SDS-gel electrophoresis and Western blot analysis by using polyclonal antibodies specific for Ap_4A phosphorylase I. Results of a Western blot of the crude supernatants indicated that there was a marked increase in the mass of Ap_4A phosphorylase I in the pBDA4 transformants after induction with galactose in comparison with the mass of enzyme in the control transformants (blot not shown).

TABLE 2. Specific activity of Ap_4A phosphorylase in crude supernatants from *S. cerevisiae*[a]

Strain	Plasmid in transformant	Sp act of Ap_4A phosphorylase (nmol of ADP + ATP/min/mg of protein)[b]
CGY339[c]	YEp352	24.5 ± 3.6
CGY339[c]	YEp352-*APA1*	256 ± 78
CGY339[d]	pBDA	18.3 ± 3.8
CGY339[d]	pBDA7	21.0 ± 4.0
CGY339[d]	pBDA4	1901 ± 76
YPH252[d]	pBDA	40.5 ± 10.7
YPH252[d]	pBDA4	2702 ± 583
YPH252[c]		18.4 ± 2.6
YPH252-H[c]		3.4 ± 0.3

[a] Strains and plasmids are described in Table 1.
[b] Values are $\bar{x} \pm$ SD for triplicate assays.
[c] Strains were grown in YNB plus 2% glucose to a cell density of 3×10^7 to 4×10^7 cells per ml.
[d] Strains were grown in YNB plus 2% galactose to a cell density of 3×10^7 to 4×10^7 cells per ml.

Induced expression of *APA1* in strain CGY339 was examined as a function of growth by measuring enzymatic activity in crude supernatants. Ap_4A phosphorylase activity from the pBDA4 transformant was 58-fold larger than the activity from the pBDA control transformant at early log phase and was 33-fold larger at late stationary phase. For five points in the growth curve from mid-log phase to mid-stationary phase, the increase in enzymatic activity for the pBDA4 transformant was 68 ± 7 ($\bar{x} \pm$ SD, $n = 5$)-fold larger than that for the pBDA transformant. The diminished increase in late stationary phase was probably due to depletion of the inducer, galactose, in the medium. The growth rate in galactose was not measurably different between the two transformants.

Disruption of the *APA1* gene in strain YPH252 resulted in an 80% loss of Ap_4A phosphorylase activity in the crude supernatant (Table 2). The residual activity is due to Ap_4A phosphorylase II (29, 30). Ap_4A phosphorylase I was undetectable on a Western blot of 75 μg of crude supernatant from YPH252-H (blot not shown). (We can readily detect the enzyme on a Western blot with this mass of crude supernatant protein from a wild-type strain [1].)

Measurement of nucleotides. Previous results with strain CGY339 transformed with YEp352-*APA1* indicated that the intracellular Ap_4A did not decrease in this transformant even though the in vitro Ap_4A phosphorylase I activity increased ninefold (22). The yeast cells had been grown in a rich medium containing 3% glucose and 2% NZamineA to 6×10^7 to 7×10^7 cells per ml (late log phase). In the present study, the in vivo concentration of Ap_4A was measured in transformants grown in minimal medium with 2% glucose to a density of 3×10^7 to 4×10^7 cells per ml (early stationary phase). Under these conditions, the intracellular Ap_4A increased twofold in the YEp352-*APA1* transformant in comparison with the control transformant (Table 3). Intracellular Ap_4A in pBDA4 transformants of strains CGY339 and YPH252 grown in the presence of galactose increased about 10- to 15-fold in comparison with the Ap_4A concentration in pBDA transformants when the cells were harvested in early stationary phase (Table 3). Intracellular Ap_4G also increased

TABLE 3. In vivo Ap_4A and Ap_4G concentrations in *S. cerevisiae*[a]

Strain	Plasmid in transformant	Concn (pmol/mg of protein) of[b]:	
		Ap_4A	Ap_4G
CGY339[c]	YEp352	0.58 ±0.04	0.35 ±0.06
CGY339[c]	YEp352-APA1	1.16 ± 0.28	1.61 ± 0.33
CGY339[d]	pBDA	0.80 ± 0.17	0.46 ± 0.16
CGY339[d]	pBDA4	12.4 ± 0.8	18.7 ± 1.4
YPH252[d]	pBDA	0.58 ± 0.21	ND[e]
YPH252[d]	pBDA4	5.4 ± 0.99	10.1 ± 2.1
YPH252[c]		0.22 ± 0.04	0.12 ± 0.03
YPH252-H[c]		1.09 ± 0.15	1.63 ± 0.23

[a] Strains and plasmids are described in Table 1.
[b] Values are $\bar{x}$ ± SD for three to four measurements.
[c] Strains were grown in YNB plus 2% glucose to a cell density of 3×10^7 to 4×10^7 cells per ml.
[d] Strains were grown in YNB plus 2% galactose to a cell density of 3×10^7 to 4×10^7 cells per ml.
[e] ND, not determined.

significantly in the pBDA4 transformants grown in the presence of galactose (Table 3).

The in vivo concentrations of Ap_4A, Ap_4G, and Gp_4G were measured in *S. cerevisiae* CGY339(pBDA) and *S. cerevisiae* CGY339 (pBDA4) transformants as a function of growth in the presence of galactose. Enhanced expression of the *APA1* gene in the pBDA4 transformant resulted in a 3- to 20-fold increase, depending upon the stage of growth, in intracellular Ap_4A in comparison with the pBDA control transformant (Fig. 1A). In the pBDA transformant, Ap_4A increased from about 1 pmol/mg of protein in the early log phase to about 5 pmol/mg of protein in the stationary phase. The in vivo concentrations of Ap_4G and Gp_4G were also measured in these transformants as a function of growth (Fig. 1B). The Ap_4G concentration was about 40% of the Ap_4A concentration in the pBDA transformant throughout the growth curve (Fig. 1A and B). The concentration of Ap_4G relative to that of Ap_4A was similar to the value reported previously (14). Gp_4G was undetectable in early-log-phase samples of the pBDA transformant (Fig. 1B). The basal concentration of Gp_4G was probably less than 0.2 pmol/mg of protein on the basis of the sensitivity of detection. In the pBDA4 transformant, the concentrations of both Ap_4G and Gp_4G exceeded the concentrations of Ap_4A by about 20% during log phase, but both decreased more rapidly than Ap_4A during stationary phase (Fig. 1A and B).

There were no significant differences ($P > 0.05$ in a two-tailed group t test) in the intracellular ATP or ADP between the experimental and control transformants for the experiments on measurement of Ap_4A reported in Fig. 1 except for ADP in cells harvested at 4×10^7 cells per ml. Although the concentrations of ADP in the transformants were statistically different, the ADP values differed only by 40%.

The in vivo concentration of Ap_4A increased by a factor of five in the YPH252 transformant with the *apa*Δ::*HIS3* disruption in comparison with the YPH252 strain (Table 3).

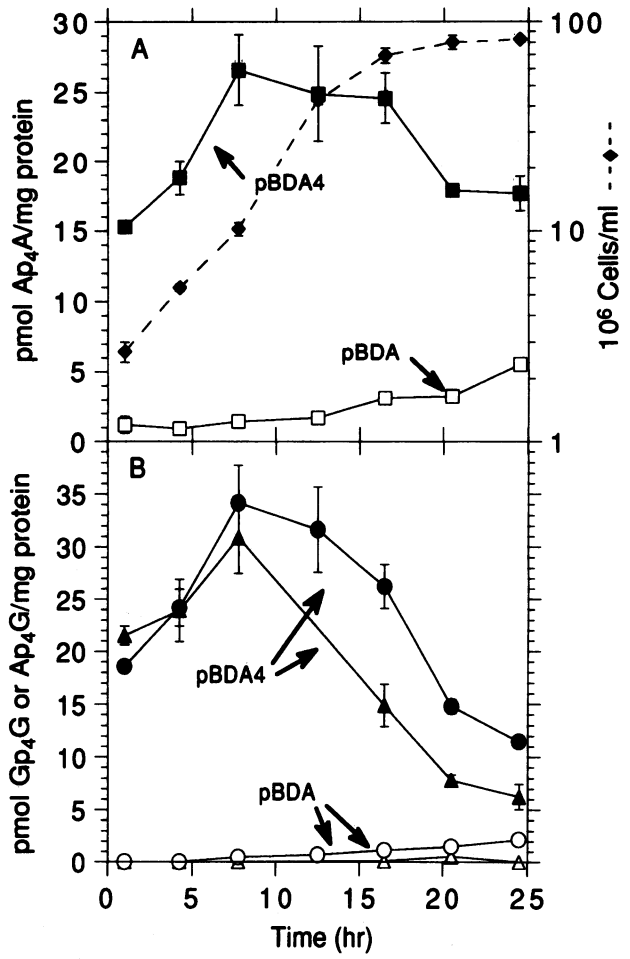

FIG. 1. In vivo concentrations of Ap_4A, Ap_4G, and Gp_4G in *S. cerevisiae* CGY339 transformed with pBDA and pBDA4 and grown in YNB plus 2% galactose. Cells were collected at the indicated cell densities by filtration, and the intracellular Ap_4A, Ap_4G, and Gp_4G were measured in trichloroacetic acid extracts by anion-exchange HPLC as described in Materials and Methods. The open and closed symbols represent the values for the pBDA and pBDA4 transformants, respectively. (A) Symbols: □ and ■, Ap_4A; ◆, cell density. There was no significant difference in the growth rate for the two transformants. The error bars on the growth curve represent the range of cell densities for the cultures of the pBDA and pBDA4 transformants. (B) Symbols: ○ and ●, Ap_4G; △ and ▲, Gp_4G. Values are $\bar{x}$ ± SD for triplicate measurements of Ap_4A, Ap_4G, and Gp_4G.

DISCUSSION

The data demonstrate a paradoxical increase in intracellular Ap_4A upon increased expression of *APA1*, the gene encoding the catabolic enzyme Ap_4A phosphorylase I. Ap_4A phosphorylase activity increased as expected with increased expression of *APA1*, but the observed increase in intracellular Ap_4A was the opposite of the expected result. This phenomenon occurred in two different strains of *S. cerevisiae* and with two different plasmids containing *APA1*. The level of increase in intracellular Ap_4A was proportional to the level of increased expression of *APA1* as measured by the in vitro Ap_4A phosphorylase activity. Thus, when the Ap_4A phosphorylase activity in strain CGY339 was increased by about 10-fold and 100-fold as measured in vitro, the intracellular Ap_4A increased by about 2-fold and 15-fold, respectively, as indicated by the data in Tables 2 and 3. However, it is unknown whether the increase in intracellular Ap_4A is dependent upon enzymatic activity per se or some other property of Ap_4A phosphorylase I.

Disruption of *APA1* resulted in a decrease in Ap_4A phos-

phorylase activity and an increase in intracellular Ap_4A. Our data reproduce both qualitatively and quantitatively the results of Plateau et al. (30) for disruption of this gene in yeast cells. The changes that occurred in enzymatic activity and intracellular Ap_4A upon disruption of *APA1* are those expected for a gene encoding a catabolic enzyme (6, 30). Attainment of the expected data for the disruption of *APA1* suggests that the paradoxical increase in intracellular Ap_4A observed upon enhanced expression of *APA1* is not an artifact of measuring Ap_4A.

The molecular mechanism for this paradoxical increase in Ap_4A is unknown. The increase in Gp_4G concomitant with the increases in Ap_4A and Ap_4G suggests that the high levels of dinucleotides are not due to stimulation of synthesis by the tRNA synthetases, since these enzymes are generally specific for ATP for formation of the aminoacyl adenylate intermediate (13, 25, 38) and thus cannot synthesize Gp_4G. Ap_4A phosphorylase I can synthesize Ap_4A and other dinucleoside tetraphosphates, including Gp_4G, at a low pH in vitro (8). However, on the bases of the intracellular pH in growing yeast cells (about 7.2) (10), the intracellular concentrations of ATP, ADP, and P_i as stated by Brevet et al. (8), and the measured equilibrium constant of the reaction (8), the equilibrium concentration of Ap_4A under intracellular conditions would be about 0.09 μM. Both the basal intracellular concentrations of Ap_4A (0.3 to 0.55 μM [9, 14]) and the elevated levels reported here are significantly higher than this. This indicates that the Ap_4A phosphorylase I reaction should run in the catabolic direction in vivo, and the results of the disruption of *APA1* indicate that, under normal conditions, it does. The anomalous increase in the dinucleotides with overexpression of *APA1* might result from the excess enzyme being sequestered in a microenvironment in which the reaction equilibrium is shifted toward synthesis (by low pH and/or low P_i) or by stimulation of dinucleotide synthesis by some unknown enzyme. Ap_4A phosphorylase I also can synthesize Ap_4A and Ap_4G by using adenosine 5′-phosphosulfate with ATP and GTP, respectively, as substrates (19). However, Gp_4G cannot be synthesized in an analogous reaction, so usage of adenosine 5′-phosphosulfate as a substrate cannot explain the results.

We can conclude that the metabolic consequences of increased gene expression may be more complex than generally expected. Measurement of the gene product alone may be inadequate in elucidating the eventual physiological effect, and measurement of metabolites associated with the gene product may be necessary. One cannot assume that increased expression of a catabolic enzyme will always yield a decrease in the concentration of the substrate as demonstrated here in the cases of Ap_4A phosphorylase I and Ap_4A.

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