A Paradoxical Increase of a Metabolite upon Increased Expression of Its Catabolic Enzyme: the Case of Diadenosine TetrAPHosphate (Ap₄A) and Ap₄A Phosphorylase I in Saccharomyces cerevisiae

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The APA1 gene in Saccharomyces cerevisiae encodes Ap₄A phosphorylase I, the catabolic enzyme for diadenosine 5′,5″-P₁,P₄-tetrAPHosphate (Ap₄A). 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 intracellular Ap₄A phosphorylase activity, respectively, as assayed in vitro. However, the intracellular concentration of Ap₄A exhibited increases of 2- and 15-fold, respectively, from the two different plasmids. Intracellular Ap₄A increased 3- to 20-fold during growth on galactose of a transformant with APA1 under the control of the GAL1 promoter. Intracellular adenosine 5′-P₁-tetrAPHospho-5″-guanosine (Ap₄G) and diguanosine 5′,5″-P₁,P₄-tetrAPHosphate (Gp₄G) also increased in the transformant under these conditions. The chromosomal locus of APA1 has been disrupted in a haploid strain. The Ap₄A phosphorylase activity decreased by 80% and the intracellular Ap₄A 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 Ap₄A 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″-P₁,P₄-tetrAPHosphate (Ap₄A) has been identified in a variety of prokaryotic and eukaryotic organisms (15, 45), but its physiological function(s) is unknown. Ap₄A 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 Ap₄A 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 Ap₄A and related dinucleoside polyaphosphates 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 Ap₄A hydrolase demonstrate that Ap₄A cannot be the signal for induction of the oxidative stress and heat shock responses in this bacterium (12, 28).

In Saccharomyces cerevisiae, Ap₄A is degraded by two similar phospholysases (29, 30). Phosphorylisis of Ap₄A in the presence of P₄ and a divalent cation yields ADP and ATP (18). About 80 to 85% of the endogenous Ap₄A is catalyzed by Ap₄A 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 Ap₄A phosphorylase I [22] but have now chosen to use APA1 [30].) Experiments applying molecular genetics to elucidate the function of Ap₄A 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 intracellular concentration of Ap₄A. However, enhanced expression of APA1 via a multicopy plasmid did not yield a decrease in intracellular Ap₄A (22).

Here we report a paradoxical increase, up to 20-fold, in intracellular Ap₄A upon enhanced expression of APA1 via a plasmid with a GAL1 promoter. Such expression of APA1 resulted in a 90-fold increase in Ap₄A phosphorylase I activity in crude extracts. Disruption of APA1 caused the expected increase, up to fivefold, in intracellular Ap₄A.

MATERIALS AND METHODS

Materials. Medium reagents were purchased from Difco Laboratories (Detroit, Mich.). Ap₄A was purchased from Sigma Chemical Co. (St. Louis, Mo.) and custom labeled with tritium (Amersham Corp., Arlington Heights, Ill.). [³H]Ap₄A was purified by chromatography on hydroxyboreonyl-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 KpnI-EcoRI 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

* Corresponding author.
| Strain          | Genotype                | Reference or source |
|----------------|-------------------------|---------------------|
| CGY339         | ura3-52 his3-29 pep4-3 GAL⁺ | G. Fink             |
| CGY339(YEp352) | Transformant with YEp352 | 21                  |
| CGY339(YEp352-APA1) | Transformant with YEp352 containing APA1 | 21                  |
| CGY339(pBDA⁺)  | Transformant with a URAl3 GAL promoter plasmid containing APA1 | This study          |
| CGY339(pBDA4)  | Transformant with pBDA containing APA1 | This study          |
| YPH252         | ura3-52 his3-Δ200 leu2-801 ade2-101 leu2-21 trp1-Δ1 | 37                  |
| YPH252-H       | YPH252apalA:URA3 | This study          |
| YPH252(pBDA)   | Transformant with pBDA containing APA1 | This study          |
| YPH252(pBDA4)  | Transformant with pBDA containing APA1 | This study          |

* YEp352, a multicopy plasmid, described by Hill et al. (20).
* pBDA, a single-copy, centromeric plasmid, prepared in this study from pBMY13 (15).
* trp1-Δ1 deletes the USAS 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 HindIII linker to the BamHI site in pBAM130 (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 HindIII site, which is 66 bp downstream of the GAL3 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 +1 to nucleotide +151, 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 NruI and ThrIII 1 restriction endonucleases such that the integrity of the flanking sequences was maintained to facilitate homology-gous 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,D 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 μmol of [3H]Ap4A. 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 polynucleotides 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,D by area integration of peaks on a Partisil SAX HPLC column (Whatman, Inc.). In some experiments, the dinucleoside tetraphosphates adenosine 5'-P-tetraphospho-5'-guanosine (Ap, G) and diguanosine 5',5'-P₄-tetraphosphate (Gp, G) were also measured. The dinucleoside tetraphosphates were eluted isocratically with 0.3 M ammonium phosphate (pH 5.5) after a precolumn purification described in detail previously (14). Recovery was determined by counting the [3H]Ap4A radioactivity in a portion of the collected Ap,D peak for each sample. Measured Ap,D values were corrected for recoveries which averaged 64 ± 5%. The recoveries for Ap,G and Gp,G were assumed to be the same as for Ap,D.

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,D 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 30 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,D phosphorylase in crude supernatants was assayed by formation of [3H]ADP and [3H]ATP from [3H]Ap4A and P₄. Activity was measured in a mixture of 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-KOH (pH 7.5), 0.5 mM MnCl₂, and 1 mM K₂HPO₄ at 37°C for 10 min in a volume of 100 μl. The

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

| Strain          | Genotype                | Reference or source |
|-----------------|-------------------------|---------------------|
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| CGY339(YEp352)  | Transformant with YEp352 | 21                  |
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| CGY339(pBDA⁺)   | Transformant with a URAl3 GAL promoter plasmid containing APA1 | This study          |
| CGY339(pBDA4)   | Transformant with pBDA containing APA1 | This study          |
| YPH252          | ura3-52 his3-Δ200 leu2-801 ade2-101 leu2-21 trp1-Δ1 | 37                  |
| YPH252-H        | YPH252apalA:URA3 | This study          |
| YPH252(pBDA)    | Transformant with pBDA containing APA1 | This study          |
| YPH252(pBDA4)   | Transformant with pBDA containing APA1 | This study          |
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 Ap₄A phosphorolase 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 (± SD) for triplicate assays. For the measurement of nucleotides, triplicate or quadruplicate samples were analyzed at each cell density in each experiment. Values (± 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 APAI.** We previously observed a ninefold increase in Ap₄A phosphorolase activity in a crude supernatant from *S. cerevisiae* CGY339 transformed with YEp352 containing APAI (22). The transformant had been grown in a rich medium containing 3% glucose and 2% NZamine A to 6 x 10⁷ to 7 x 10⁷ cells/ml (late log phase). In the present study, enzymatic activities were assayed in crude supernatants from transformants grown in minimal medium to 3 x 10⁷ to 4 x 10⁷ cells/ml (early stationary phase in this medium). The 10-fold increase in Ap₄A phosphorolase 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 Ap₄A phosphorolase activity further than obtainable via a multicopy plasmid, we subcloned APAI into pBDA under the control of the GAL1 promoter and induced expression of APAI 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 Ap₄A phosphorolase I. Results of a Western blot of the crude supernatants indicated that there was a marked increase in the mass of Ap₄A phosphorolase 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 Ap₄A phosphorolase in crude supernatants from S. cerevisiae**

| Strain   | Plasmid in transformant | Sp act of Ap₄A phosphorolase (nmol of ADP + ATP/min/mg of protein) |
|----------|-------------------------|------------------------------------------------------------------|
| CGY339   | YEp352                 | 24.5 ± 3.6                                                        |
| CGY339   | YEp352-APA1            | 256 ± 78                                                         |
| CGY339   | pBDA                   | 18.3 ± 3.8                                                       |
| CGY339   | pBDA7                  | 21.0 ± 4.0                                                       |
| CGY339   | pBDA4                  | 1901 ± 76                                                        |
| YPH252   | pBDA                   | 40.5 ± 10.7                                                      |
| YPH252   | pBDA4                  | 2702 ± 583                                                      |
| YPH252-H²|                       | 18.4 ± 2.6                                                      |
| YPH252-H²|                       | 3.4 ± 0.3                                                       |

* Strains and plasmids are described in Table 1.
* Values are ± SD for triplicate assays.
* Strains were grown in YNB plus 2% glucose to a cell density of 3 x 10⁷ to 4 x 10⁷ cells/ml.
* Strains were grown in YNB plus 2% galactose to a cell density of 3 x 10⁷ to 4 x 10⁷ cells/ml.

Induced expression of APAI in strain CGY339 was examined as a function of growth by measuring enzymatic activity in crude supernatants. Ap₄A phosphorolase 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 (± 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 APAI gene in strain YPH252 resulted in an 80% loss of Ap₄A phosphorolase activity in the crude supernatant (Table 2). The residual activity is due to Ap₄A phosphorolase II (29, 30). Ap₄A phosphorolase 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 Ap₄A did not decrease in this transformant even though the in vitro Ap₄A phosphorolase I activity increased ninefold (22). The yeast cells had been grown in a rich medium containing 3% glucose and 2% NZamine A to 6 x 10⁷ to 7 x 10⁷ cells/ml (late log phase). In the present study, the in vivo concentration of Ap₄A was measured in transformants grown in minimal medium with 2% glucose to a density of 3 x 10⁷ to 4 x 10⁷ cells/ml (early stationary phase). Under these conditions, the intracellular Ap₄A increased twofold in the YEp352-APA1 transformant in comparison with the control transformant (Table 3). Intracellular Ap₄A in pBDA4 transformants of strains CGY339 and YPH252 grown in the presence of galactose increased about 10- to 15-fold in comparison with the Ap₄A concentration in pBDA transformants when the cells were harvested in early stationary phase (Table 3). Intracellular Ap₄G also increased
TABLE 3. In vivo Ap4A and Ap4G concentrations in *S. cerevisiae*

| Strain       | Plasmid in transformant | Conc (pmol/mg of protein) of: |
|--------------|-------------------------|-----------------------------|
|              |                         | Ap4A                        | Ap4G                        |
| CGY339*      | YEp352                  | 0.58 ± 0.04                 | 0.35 ± 0.06                 |
| CGY339*      | YEp352-APA1             | 1.16 ± 0.28                 | 1.61 ± 0.33                 |
| CGY339d      | pBDA                    | 0.80 ± 0.17                 | 0.46 ± 0.16                 |
| CGY339d      | pBDA4                   | 12.4 ± 0.8                  | 18.7 ± 1.4                  |
| YPH252d      | pBDA                    | 0.58 ± 0.21                 | ND                         |
| YPH252d      | pBDA4                   | 5.4 ± 0.99                  | 10.1 ± 2.1                  |
| YPH252d      |                         | 0.22 ± 0.04                 | 0.12 ± 0.03                 |
| YPH252-Hc    |                         | 1.09 ± 0.15                 | 1.63 ± 0.23                 |

* Strains and plasmids are described in Table 1.
* Values are ± SD for three to four measurements.
* Strains were grown in YNB plus 2% glucose to a cell density of 3 × 10⁷ to 4 × 10⁷ cells per ml.
* Strains were grown in YNB plus 2% galactose to a cell density of 3 × 10⁷ to 4 × 10⁷ cells per ml.
* ND, not determined.

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

The in vivo concentrations of Ap4A, Ap4G, and Gp4G 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 APAI gene in the pBDA4 transformant resulted in a 3- to 20-fold increase, depending upon the stage of growth, in intracellular Ap4A in comparison with the pBDA control transformant (Fig. 1A). In the pBDA transformant, Ap4A 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 Ap4G and Gp4G were also measured in these transformants as a function of growth (Fig. 1B). The Ap4G concentration was about 40% of the Ap4A concentration in the pBDA transformant throughout the growth curve (Fig. 1A and B). The concentration of Ap4G relative to that of Ap4A was similar to the value reported previously (14). Gp4G was undetectable in early-log-phase samples of the pBDA transformant (Fig. 1B). The basal concentration of Gp4G 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 Ap4G and Gp4G exceeded the concentrations of Ap4A by about 20% during log phase, but both decreased more rapidly than Ap4A 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 Ap4A reported in Fig. 1 except for ADP in cells harvested at 4 × 10⁷ 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 Ap4A increased by a factor of five in the YPH252 transformant with the apdA::HIS3 disruption in comparison with the YPH252 strain (Table 3).

DISCUSSION

The data demonstrate a paradoxical increase in intracellular Ap4A upon increased expression of APAI, the gene encoding the catabolic enzyme Ap4A phosphorlyase I. Ap4A phosphorlyase activity increased as expected with increased expression of APAI, but the observed increase in intracellular Ap4A was the opposite of the expected result. This phenomenon occurred in two different strains of *S. cerevisiae* and with two different plasmids containing APAI. The level of increase in intracellular Ap4A was proportional to the level of increased expression of APAI as measured by the in vitro Ap4A phosphorlyase activity. Thus, when the Ap4A phosphorlyase activity in strain CGY339 was increased by about 10-fold and 100-fold as measured in vitro, the intracellular Ap4A 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 Ap4A is dependent upon enzymatic activity per se or some other property of Ap4A phosphorlyase I.

Disruption of APAI resulted in a decrease in Ap4A phos-
phorylase activity and an increase in intracellular Ap4A. 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 Ap4A 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 Ap4A observed upon enhanced expression of APA1 is not an artifact of measuring Ap4A.

The molecular mechanism for this paradoxical increase in Ap4A is unknown. The increase in GpG comitochond with the increases in Ap4A and ApGpG 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 GpG. Ap4A phosphorylase I can synthesize Ap4A and other dinucleoside tetraphosphates, including GpG, 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 P1 as stated by Brevet et al. (8), and the equilibrium constant of the reaction (8), the equilibrium concentration of Ap4A under intracellular conditions would be about 0.09 μM. Both the basal intracellular concentrations of Ap4A (0.3 to 0.55 μM [9, 14]) and the elevated levels reported here are significantly higher than this. This indicates that the Ap4A 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 Pi) or by stimulation of dinucleotide synthesis by some unknown enzyme. Ap4A phosphorylase I also can synthesize Ap4A and ApGpG by using adenosine 5′-phosphosulfate with ATP and GTP, respectively, as substrates (19). However, GpG 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 in the cases of Ap4A phosphorylase I and Ap4A.

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REFERENCES

1. Avila, D. M., V. Kaushal, and L. D. Barnes. 1990. Immunoaffinity chromatography of diadenosine 5′,5″-P1,P1-tetraphosphate phosphorylase from Saccharomyces cerevisiae. Biotechnol. Appl. Biochem. 12:276–283.
2. Barnes, L. D., A. K. Robinson, C. H. Mumford, and P. N. Garrison. 1985. Assay of diadenosine tetraphosphate hydrolytic enzymes by boronate chromatography. Anal. Biochem. 144:296–304.
3. Birballo, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513–1523.
4. Blanquet, S., P. Plateau, and A. Brevet. 1989. The role of zinc in 5′,5″-diadenosine tetraphosphate production by aminoacyl transfer RNA synthetases. Mol. Cell. Biochem. 25:3–11.
5. Bochner, B. R., P. C. Lee, S. W. Wilson, and B. N. Ames. 1984. Ap4A and related adenylylated nucleotides are synthesized as a consequence of oxidation stress. Cell 37:225–232.
6. Botstein, D., and R. W. Davis. 1982. Principles and practice of recombinant DNA research with yeast, p. 607–636. In J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast Saccharomyces: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. Brevet, A., J. Chen, F. Lévêque, P. Plateau, and S. Blanquet. 1989. In vivo synthesis of adenylylated bis(5′-nucleosidyl) tetraphosphates (Ap4N) by Escherichia coli aminoacyl-tRNA synthetases. Proc. Natl. Acad. Sci. USA 86:8275–8279.
8. Brevet, A., H. Coste, M. Fromant, P. Plateau, and S. Blanquet. 1987. Yeast diadenosine 5′,5″-P1,P1-tetraphosphate α,β-phosphorylase behaves as a dinucleotide tetraphosphate synthetase. Biochemistry 26:4763–4768.
9. Coste, H., A. Brevet, P. Plateau, and S. Blanquet. 1987. Non-adenylated bis(5′-nucleosidyl) tetraphosphates occur in Saccharomyces cerevisiae and in Escherichia coli and accumulate upon temperature shift or exposure to cadmium. J. Biol. Chem. 262:12096–12103.
10. den Hollander, J. A., K. Uguribil, T. R. Brown, and R. G. Shulman. 1981. Phosphorus-31 nuclear magnetic resonance studies of the effect of oxygen upon glycolysis in yeast. Biochemistry 20:5870–5880.
11. Erlich, H. A. 1989. PCR technology: principles and applications for DNA amplification. Stockton Press, New York.
12. Farr, S. B., D. N. Arnosti, M. J. Chamberlin, and B. N. Ames. 1989. An apalI mutation causes ApppA to accumulate and affects motility and catabolite repression in Escherichia coli. Proc. Natl. Acad. Sci. USA 86:5010–5014.
13. Freist, W., H. Sternbach, and F. Cramer. 1981. Survey on substrate specificity with regard to ATP analogs of aminoacyl-tRNA synthetases from E. coli and baker’s yeast. Hoppe-Seyler’s Z. Physiol. Chem. 362:1247–1254.
14. Garrison, P. N., and L. D. Barnes. 1984. Assay of adenosine 5′-P1-tetraphosphate-5″-adenosine and adenosine 5′-P1-tetraphosphate-5″-guanosine in Physarum polycephalum and other eukaryotes. An isotopic high-pressure liquid chromatography method. Biochem. J. 217:805–811.
15. Garrison, P. N., and L. D. Barnes. Determination of dinucleoside polyphosphates. In A. G. McNelenn (ed.), Dinucleoside polyphosphates, in press. CRC Press, Inc., Boca Raton, Fla.
16. Garrison, P. N., M. Koob, and L. D. Barnes. Physical mapping of the Saccharomyces cerevisiae Ap4A phosphorylase I-encoding gene by the Achilles’ cleavage method. Gene, in press.
17. Goerlich, O., R. Foeckler, and E. Hoffer, 1982. Mechanism of synthesis of adenosine 5′-tetraphospho-5″-adenosine (AppppA) by aminoacyl-tRNA synthetases. Eur. J. Biochem. 126:135–142.
18. Gurawnowski, A., and S. Blanquet. 1985. Phosphorolytic cleavage of diadenosine 5′,5″-P1,P1-tetraphosphate. J. Biol. Chem. 260:3542–3547.
19. Gurawnowski, A., G. Just, E. Hoffer, and H. Jakubowski, 1988. Synthesis of diadenosine 5′,5″-P1,P1-tetraphosphate (AppppA) from adenosine 5′-phosphosulfate and adenosine 5′-triphosphate catalyzed by yeast AppppA phosphorylase. Biochemistry 27:2959–2964.
20. Hill, J. E., A. M. Myers, T. J. Koerner, and A. Tzagoloff. 1986. Yeast E. coli shuttle vectors with multiple unique restriction sites. Yeast 2:163–167.
21. Johnston, M., and R. W. Davis. 1984. Sequences that regulate the divergent GAL1-GAL10 promoter in Saccharomyces cerevisiae. Mol. Cell. Biol. 4:1440–1448.
22. Kaushal, V., D. M. Avila, S. C. Hardies, and L. D. Barnes. 1990. Sequencing and enhanced expression of the gene encoding diadenosine 5',5''-P',P'-tetraphosphate (Ap4A) phosphorylase in Saccharomyces cerevisiae. Gene 95:75-84.

23. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.

24. Lee, P. C., B. R. Bochner, and B. N. Ames. 1983. AppppA, heat-shock stress, and cell oxidation. Proc. Natl. Acad. Sci. USA 80:7496–7500.

25. Loffeld, R. B. 1972. The mechanism of aminoclaylation of transfer RNA. Prog. Nucleic Acid Res. Mol. Biol. 12:87–128.

26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.

27. Oshima, Y. 1982. Regulatory circuits for gene expression: the metabolism of galactose and phosphate, p. 159–180. In J. N. Strathern, E. W. Jones, and J. R. Broach (ed.). The molecular biology of the yeast Saccharomyces: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

28. Plateau, P., M. Fromant, and S. Blanquet. 1987. Heat shock and hydrogen peroxide responses of Escherichia coli are not changed by dinucleoside tetraphosphate hydrolase overproduction. J. Bacteriol. 169:3817–3820.

29. Plateau, P., M. Fromant, J.-M. Schmitter, and S. Blanquet. 1990. Catabolism of bis(5'-nucleosidyl) tetraphosphates in Saccharomyces cerevisiae. J. Bacteriol. 172:6892–6899.

30. Plateau, P., M. Fromant, J.-M. Schmitter, J.-M. Buhler, and S. Blanquet. 1989. Isolation, characterization, and inactivation of the APAl gene encoding yeast diadenosine 5',5''-P',P'-tetraphosphate phosphorylase. J. Bacteriol. 171:6437–6445.

31. Rapaport, E., and P. C. Zamecnik. 1976. Presence of diadenosine 5',5''-P',P'-tetraphosphate (Ap4A) in mammalian cells in levels varying widely with proliferative activity of the tissue: a possible positive "pleiotropic activator." Proc. Natl. Acad. Sci. USA 73:3984–3988.

32. Rothstein, R. J. 1983. One-step gene disruption in yeast. Methods Enzymol. 101:202–211.

33. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

34. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.

35. Schiestl, R. H., and R. D. Gietz. 1989. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr. Genet. 16:339–346.

36. Sherman, F., G. R. Fink, and J. B. Hicks. 1986. Methods in yeast genetics: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

37. Sikorski, R. S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122:19–27.

38. Soll, D., and P. R. Schimmel. 1974. Aminoacyl-tRNA synthetases, p. 489–538. In P. D. Boyer (ed.), The enzymes, vol. 10. Academic Press, Inc., New York.

39. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.

40. Spencer, J. F. T., D. M. Spencer, and I. J. Bruce. 1989. Yeast genetics. A manual of methods. Springer-Verlag, Berlin.

41. Struhl, K. 1983. The new yeast genetics. Nature (London) 305:391–397.

42. Struhl, K., and R. W. Davis. 1980. A physical, genetic and transcriptional map of the cloned his3 gene region of Saccharomyces cerevisiae. J. Mol. Biol. 136:309–332.

43. Studier, F. W. 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. 79:237–248.

44. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350–4354.

45. Zamecnik, P. C. 1983. Diadenosine 5',5''-P',P'-tetraphosphate (Ap4A): its role in cellular metabolism. Anal. Biochem. 134:1–10.

46. Zamecnik, P. C., M. L. Stephenson, C. M. Janeway, and K. Randerath. 1966. Enzymatic synthesis of diadenosine tetraphosphate and diadenosine triphosphate with a purified lysyl-tRNA synthetase. Biochem. Biophys. Res. Commun. 24:91–97.