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# Isolation, Characterization, and Inactivation of the *APA1* Gene Encoding Yeast Diadenosine 5′,5‴-$P^1$,$P^4$-Tetraphosphate Phosphorylase

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**The gene encoding diadenosine 5′,5‴-$P^1$,$P^4$-tetraphosphate ($Ap_4A$) phosphorylase from yeast was isolated from a λgt11 library. The DNA sequence of the coding region was determined, and more than 90% of the deduced amino acid sequence was confirmed by peptide sequencing. The $Ap_4A$ phosphorylase gene (*APA1*) is unique in the yeast genome. Disruption experiments with this gene, first, supported the conclusion that, in vivo, $Ap_4A$ phosphorylase catabolizes the $Ap_4N$ nucleotides (where N is A, C, G, or U) and second, revealed the occurrence of a second $Ap_4A$ phosphorylase activity in yeast cells. Finally, evidence is provided that the *APA1* gene product is responsible for most of the ADP sulfurylase activity in yeast extracts.**

Bis(5′-nucleosidyl) tetraphosphates ($Ap_4N$, where N stands for A, C, G, or U) are unusual nucleotides occurring in a wide variety of procaryotic and eucaryotic cells (17). Although their role in the cell has not yet been precisely established, they are believed to be regulatory nucleotides, since their intracellular concentration greatly depends on the physiological state of the cell (4, 5, 11, 15, 30, 36, 58). Thus, the very strong $Ap_4N$ accumulation during heat shock led to the proposal that these nucleotides could be alarmones, signaling to the cell the onset of the stress (9).

Several enzymes involved in $Ap_4N$ metabolism have already been characterized. It is well established that some aminoacyl-tRNA synthetases can catalyze $Ap_4N$ formation in vitro from ATP and NTP (28, 45, 47, 57). Recently, their participation in in vivo $Ap_4N$ biosynthesis was evidenced by the demonstration that overproduction of several aminoacyl-tRNA synthetases in *Escherichia coli* is accompanied by an important increase in $Ap_4N$ concentration (A. Brevet, J. Chen, F. Lévêque, P. Plateau, and S. Blanquet, Proc. Natl. Acad. Sci. USA, in press). Various enzymes which catabolize $Ap_4N$ have also been identified. Three reaction patterns have already been described, depending on the origin of the enzyme. Nonspecific phosphodiesterases are present in various eucaryotic cells. They hydrolyze 5′,5‴-$P^1$,$P^4$-tetraphosphate ($Ap_4A$) into ATP plus AMP (7, 29, 33, 49). In crude extracts from higher eucaryotic cells, a specific $Ap_4A$ hydrolase converts $Ap_4A$ into ATP plus AMP (29, 31, 41). In *E. coli* and *Physarum polycephalum*, a specific $Ap_4N$ hydrolase converts $Ap_4N$ into ADP plus NDP, where N is A, C, G, or U (16, 21, 46). Finally, in the yeast *Saccharomyces cerevisiae* and in the protozoan *Euglena gracilis*, $Ap_4Ns$ are phosphorolytically cleaved into ATP plus NDP (or ADP plus NTP) (19, 23).

The enzyme which phosphorolyzes $Ap_4N$ in yeast extracts is intriguing for two reasons. First, as the number of energy-rich bonds is not modified by the reaction, $Ap_4N$ phosphorolysis is reversible, and indeed, $Ap_4N$ synthesis from ATP and NDP could be evidenced in vitro (10, 19). At present, it is not known whether the enzyme synthesizes or degrades $Ap_4N$ in vivo. Second, yeast $Ap_4A$ phosphorylase was shown to catalyze three other reactions: (i) the conversion of adenosine 5′-phosphosulfate (AMPS) plus $P_i$ into ADP plus sulfate (20); (ii) the exchange between NDP and phosphate (NDP + $^{32}P_i$↔[β-$^{32}$P]NDP + $P_i$) (20); and (iii) the synthesis of $Ap_4A$ from AMPS plus ATP (22). Thus, it was conceivable that $Ap_4A$ phosphorylase from yeast cells could be identical to the ADP sulfurylase already described for this organism (1, 12, 39, 48) and/or to the enzyme characterized by Grunberg-Manago et al. (18) by its ability to catalyze incorporation of $^{32}P_i$ into ADP.

One way to approach these questions is to modify $Ap_4A$ phosphorylase expression in yeast cells and to follow the consequent changes in $Ap_4N$ concentration and in the levels of ADP-sulfurylase and ADP-$P_i$ exchange activities. In this article, we report the cloning of the gene encoding $Ap_4A$ phosphorylase from *S. cerevisiae* and describe some properties of strains in which the $Ap_4A$ phosphorylase gene has been disrupted.

## MATERIALS AND METHODS

**Yeasts, bacteria, plasmids, and gene libraries.** Bacterial and yeast strains are listed in Table 1. The yeast genomic library from *S. cerevisiae* X2180 prepared in phage λgt11 was kindly provided by M. Snyder. Plasmid pBluescript KS was from Stratagene (San Diego, Calif.).

**Transformation and culture media.** Bacterial transformations were performed as described by Hanahan (24), while yeast transformations were done by the lithium acetate method of Ito et al. (27). YPD and minimal sporulation media were made as described before (53).

**Enzymes and reagents.** Homogeneous $Ap_4A$ phosphorylase was obtained as previously described (19). Alkaline phosphatase from calf intestine was purchased from Boehringer (Mannheim, Federal Republic of Germany). DNA-modifying and -restricting enzymes were from Boehringer, Bethesda Research Laboratories (Rockville, Md.), Pharmacia Fine Chemicals (Uppsala, Sweden), or Appligene (Strasbourg, France). Incubations for DNA cleavage were carried

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TABLE 1. Bacterial and yeast strains used in this study

| Strain | Relevant genotype | Reference |
|---|---|---|
| *E. coli* | | |
| JM101Tr | Δ(*lac pro*) *supE thi recA56 srl-300*::Tn*10* (F′ *traD36 proAB lacI*$^q$ *lacZ*ΔM15) | 25 |
| Y1089 | Δ*lacU169 proA*$^+$ Δ*lon araD39 strA thi hflA150* [chr::Tn*10*](pMC9 [=pBR322 *lac*I$^q$]) | 61 |
| IBPC111 | F$^-$ Δ(*lac pro*) *gyrA rpoB metB argE*(Am) *supE ara recA1* λ$^s$ λ$^-$ | 54 |
| *S. cerevisiae* CMY214 | a/α, *trp1-Δ1/trp1-Δ1 his3Δ200/his3Δ200 ura3-52/ura3-52 ade2-101/ade2-101 lys2-801/lys2-801 can1/CAN1* | 35 |

out as recommended by the suppliers. $^{32}P_i$ (carrier free), [α-$^{32}$P]dATP (29.6 TBq/mmol), and [$^3$H]Ap$_4$A (322 GBq/mmol) were from Amersham (Amersham, U.K.). Before use, [$^3$H]Ap$_4$A was purified through dihydroxyboryl-BioRex 70 chromatography (3).

**Antibodies and plaque screening.** Antibodies against Ap$_4$A phosphorylase were obtained through immunization of a white New Zealand rabbit. Homogeneous enzyme (50 μg) was dialyzed against potassium phosphate buffer (5 mM, pH 6.75) containing 1 mM 2-mercaptoethanol and 0.1 mM EDTA. Then, the enzyme volume was adjusted to 1 ml with dialysis buffer, supplemented with 1 ml of Freund complete adjuvant, and injected both intraperitoneally and subcutaneously into the rabbit. Booster injections were performed in the same way with incomplete adjuvant after 21, 28, 42, 56, 70, 84, and 98 days. About 40 ml of blood was withdrawn through heart punctures 35, 49, 63, 77, 91, and 105 days after the first injection.

The immunoglobulin fraction was recovered from the serum by chromatography onto DEAE-Trisacryl (IBF, Villeneuve-la-Garenne, France) (51). For immunoblotting experiments, antibodies were further purified by immunoadsorption on a column of Ap$_4$A phosphorylase coupled to CNBr-activated Sepharose 4B (Pharmacia). Column preparation and antibody chromatography were done by the procedure of Mirande et al. (37).

Screening of the λgt11 recombinant phage plaques was achieved with the immunoscreening system Protoblot (Promega Biotec, Madison, Wis.). Nitrocellulose filters (BA85; Schleicher & Schuell) saturated with IPTG (isopropylthio-β-D-galactopyranoside) were overlaid on plaques. After incubation at 37°C for 2 h, filters were successively incubated with 3% (wt/wt) bovine serum albumin, 0.1 μg of affinity-purified anti-Ap$_4$A phosphorylase antibodies per ml, 0.1 μg of alkaline phosphatase-conjugated second antibodies per ml, and the appropriate color development substrates. Positive clones were further purified by three additional rounds of immunological screening at a low plaque density.

**Analysis of proteins expressed by the phages.** Lysogens were prepared by infecting *E. coli* Y1089 at a multiplicity of 5. Lysates from induced lysogens were prepared as described before (60) and analyzed by electrophoresis on 12.5% polyacrylamide–sodium dodecyl sulfate (SDS) gels, followed by electrotransfer on nitrocellulose filters (56) and coloration with anti-Ap$_4$A phosphorylase antibodies, as described above for plaque screening.

**DNA sequencing.** Nucleotide DNA sequence was determined by the dideoxy chain termination method (52). First, restriction fragments were cloned into M13mp18 and M13mp19 phage vectors (59). Other fragments were obtained by Bal31 exonuclease digestion (34); DNA from plasmid pBS22-49 was cut by *Pvu*II, digested with Bal31 for various times, and cut by *Bam*HI. The resulting fragments were cloned into M13mp18 previously cleaved by *Hin*dII and *Bam*HI. Finally, a few DNA sequence regions were determined by using synthetic specific primers.

**Southern blot analysis.** Southern blot analysis was performed by the method of Maniatis et al. (34). DNA probes were labeled by synthesizing the complementary strand of a single-stranded M13 phage DNA in the presence of [α-$^{32}$P]dATP. Yeast genomic DNA, prepared as described by Nasmyth and Reed (38), was digested with various restriction enzymes, electrophoresed on a 0.8% agarose gel, and transferred to a nitrocellulose filter (BA85; Schleicher & Schuell). The filter was then hybridized with the 1.1-kilobase-pair(kbp) *Stu*I-*Pvu*II fragment located at the end of the Ap$_4$A phosphorylase gene. Hybridization was carried out at 37°C in a mixture containing 5× SSC (75 mM sodium citrate, 750 mM NaCl, pH 7.0), 5× Denhardt solution (34), 0.3% SDS, 50% formamide, and about $5 \times 10^7$ dpm of the labeled probe. After hybridization, the filter was washed with 1× SSC and 20% formamide at 37°C.

**Peptide sequencing.** Reductive pyridylethylation of Ap$_4$A phosphorylase (5 nmol) was performed by the method of Andrews and Dixon (2). The protein was desalted by chromatography over a reverse-phase column (20 by 2 mm; Nucleosil C4; Macherey and Nagel) (10-μm particles, 30-nm pore size). The column was washed with 0.1% trifluoroacetic acid, and the protein was eluted with 0.1% trifluoroacetic acid in isopropanol. Then, the protein was lyophilized and resuspended in 0.1 ml of 200 mM ammonium bicarbonate (pH 8.0). After treatment with trypsin at an enzyme-to-substrate ratio of 1:30 (wt/wt) during 4 h at 37°C, the digest was separated by reverse-phase chromatography (C18 Superspher [Merck], 250 by 4 mm, 4-μm particles, 10-nm pore size) with a linear gradient (from 0 to 90% eluant B in 120 min, with eluant A [0.1% trifluoroacetic acid] and eluant B [0.1% trifluoroacetic acid in acetonitrile]). Peptides were sequenced with an Applied Biosystems model 470A sequencer.

**Preparation of crude extracts.** For crude-extract preparation, cells were harvested by centrifugation (10 min, 5,000 × *g*). The pellet was suspended in 20 mM Tris hydrochloride (Tris-HCl) buffer (pH 7.8)–10 mM 2-mercaptoethanol–1 mM phenylmethylsulfonyl fluoride at a cell density of 0.5 g (wet weight) per ml of buffer and sonicated twice for 1 min at 0°C. Cell debris was removed by centrifugation at 10,000 × *g* for 10 min. Prior to DEAE-Sephacel chromatography, the extract was further centrifuged at 200,000 × *g* for 30 min.

**Enzymatic assays.** Initial rates of Ap$_4$A degradation were assayed by a procedure resembling the radioisotopic assay previously established for *E. coli* Ap$_4$A hydrolase activity measurements (46). The incubation mixture (100 μl), buffered with 50 mM Tris-HCl (pH 7.8), contained 120 μM [$^3$H]Ap$_4$A (370 GBq/mol), 1 mM potassium phosphate, 5 mM $MgCl_2$, 50 μg of bovine serum albumin per ml, and 60 U of alkaline phosphatase from calf intestine per ml. The reaction was initiated by the addition of Ap$_4$A phosphorylase at a final concentration of about 0.003 U/ml. After incubation at 37°C for 5 to 20 min, the reaction was quenched by adding 400 μl of a solution containing 200 μl of DEAE-Sephadex A25 resin (from Pharmacia), 5 mM potassium phosphate (pH 6.75), and 1 mM EDTA. The solution was centrifuged (1 min, 12,000 × *g*), and 200 μl of the supernatant was mixed with 2.5 ml of Picofluor scintillation cocktail (Packard) and then counted for tritium in a Beckman LS 1801 counter. One

unit is defined as the enzyme activity capable of transforming 1 μmol of Ap$_4$A per min in the above conditions.

For AMPS phosphorolysis, the reaction mixture (100 μl), buffered with 50 mM Tris-HCl (pH 7.8), contained 0.1 mM AMPS, 1 mM potassium phosphate, 0.1 mM EDTA, and 50 μg of bovine serum albumin per ml. The reaction was initiated by the addition of Ap$_4$A phosphorylase at a final concentration of about 0.003 U/ml. After incubation at 37°C for 1 to 10 min, the solution was diluted 100-fold with 0.1 mM EDTA (pH 7.5). The ADP produced was immediately assayed by bioluminescence with a luciferin-luciferase mixture (Bioluminescence HS; Boehringer) supplemented with 0.5 mM phosphoenolpyruvate and 5 U of pyruvate kinase (200 U/mg; Boehringer) per ml.

ADP-$P_i$ exchange was assayed in a reaction mixture (100 μl) buffered with 50 mM Tris-HCl (pH 7.8) containing 1 mM ADP, 5 mM $^{32}P_i$ (690 GBq/mol), 0.01 mM EDTA, and 50 μg of bovine serum albumin per ml. The reaction was initiated by the addition of Ap$_4$A phosphorylase at a final concentration of about 0.003 U/ml. The reaction was quenched by adding 2.5 ml of a solution containing 50 mM sodium acetate, 100 mM potassium phosphate, 0.35% (wt/wt) perchloric acid, and 4 g of activated charcoal (Sigma) per liter. Samples were filtered on Whatman no. 1 filter paper disks, washed twice with water, and counted on an IDL crystal scintillation counter.

**DEAE-Sephacel chromatography.** Crude extracts from 0.4 g of yeast cells (wet weight) were diluted 30-fold in potassium phosphate buffer (20 mM potassium phosphate [pH 6.75], 10 mM 2-mercaptoethanol, 0.1 mM EDTA) and applied to a DEAE-Sephacel (Pharmacia) column (1.1 by 7.5 cm) equilibrated in the same buffer. The column was washed with 7 ml of equilibration buffer, and elution was achieved with a linear gradient from 0 to 500 mM KCl in the same buffer, delivered by a Pharmacia fast protein liquid chromatography system at a flow rate of 0.10 ml/min (total volume, 90 ml). The $OD_{280}$ of the column effluent was followed on a Pharmacia UV-2 monitor.

**Ap$_4$N measurements.** For Ap$_4$N measurements, nucleotides were extracted as described previously (14), with the following slight modifications. Portions (30 ml) of the culture were quickly filtered on Sartorius SM 11303 membrane filters (pore size, 1.2 μm; 25-mm diameter). The filters were then immediately placed cell side down onto a petri dish containing 0.5 ml of $HClO_4$ (10%, wt/wt). After 2 min, the extracts were removed and the filters were rinsed with 0.5 ml of 10% $HClO_4$. The extract solutions were pooled, centrifuged, neutralized with 5 M $K_2CO_3$, and digested with alkaline phosphatase (20 mM Tris-HCl buffer [pH 7.8], 1 mM $MgCl_2$, 40 U of alkaline phosphatase from calf intestine per ml, 30 min, 37°C) to remove residual ATP. Then, Ap$_4$N was quantified in the luciferin-luciferase phosphodiesterase assay described previously (10, 40). The cellular concentration of nucleotides was calculated with the assumption that an $OD_{650}$ of 1 corresponded to 0.4 μl of intracellular volume (14).

## RESULTS AND DISCUSSION

**Cloning of the gene encoding Ap$_4$A phosphorylase from yeast cells.** The gene encoding Ap$_4$A phosphorylase from *S. cerevisiae* was isolated by screening the products in *E. coli* of a λgt11 library containing chromosomal DNA inserts from *S. cerevisiae* X2180 (61). Positive clones were detected by immunodetection with affinity-purified anti-Ap$_4$A phosphorylase antibodies, as described in Materials and Methods.

TABLE 2. AMPS phosphorolysis activity in *E. coli* strains transformed by various plasmids[a]

| Strain | AMPS phosphorolysis rate (pmol of ADP/s per mg of protein) |
|---|---|
| Y1089(λgt11) | <5 |
| Y1089(λ22-4) | 300 |
| JM101Tr | <25 |
| JM101Tr(pBS22-49) | 260 |
| JM101Tr(pBS22-49 Δ*Cla*I-*Xba*I) | 2,900 |
| JM101Tr(pBS22-49 Δ*Cla*I-*Bam*HI) | <25 |
| JM101Tr(pBS22-49 Δ*Cla*I-*Stu*I) | <25 |

[a] AMPS phosphorolysis was measured in crude extracts obtained by sonication of cells grown in LB medium (supplemented with 60 μg of ampicillin per ml when needed). JM101Tr derivatives were grown overnight at 37°C. Y1089 lysogens were grown at 30°C to an $OD_{650}$ of 0.4. They were incubated first at 42°C for 15 min in order to induce the lytic cycle of the phage and then at 37°C for 2 h. The total amount of protein was calculated from the $OD_{280}$ of the extract, assuming that 1 $OD_{280}$ unit corresponded to 1 mg of protein. Conditions of AMPS phosphorolysis measurements are given in Materials and Methods.

The analysis of 200,000 plaques at a density of 14,000 per 90-mm plate led to the isolation of 41 positive clones. To characterize further the proteins recognized by the antibodies in these clones, lysogens of 15 of the 41 clones were prepared in *E. coli* Y1089. Cellular extracts from the lysogenic clones were analyzed by SDS-polyacrylamide gel electrophoresis, followed by transfer on nitrocellulose and immunodetection with anti-Ap$_4$A phosphorylase antibodies. In most extracts, the molecular weight of the polypeptide recognized by the antibodies was greater than 110,000. These polypeptides probably corresponded to fusion proteins between the β-galactosidase encoded by λgt11 and the Ap$_4$A phosphorylase. In two extracts, however, antibodies revealed a polypeptide with an $M_r$ of about 40,000, identical to that of the Ap$_4$A phosphorylase (19). One of them, λ22-4, was chosen for further investigation.

To confirm that λ22-4 carried the gene for Ap$_4$A phosphorylase, we used the ability of Ap$_4$A phosphorylase to catalyze the conversion of AMPS plus phosphate into ADP plus sulfate. Table 2 shows that, under our experimental conditions, formation of ADP from AMPS could not be detected in extracts of *E. coli* Y1089 lysogenized by λgt11, whereas ADP appeared at a rate of 300 pmol/s per mg of protein in extracts of strain Y1089 lysogenized by λ22-4. This result strongly indicated that λ22-4 carried the structural gene for Ap$_4$A phosphorylase and that this gene was not interrupted by introns. This gene will now be called *APA1*.

**Restriction analysis, subcloning of the λ22-4 DNA, and mapping of the *APA1* gene.** *Eco*RI and *Sac*I restriction sites were mapped on the λ22-4 DNA. As shown in Fig. 1, one of the two *Eco*RI sites expected on each side of the insert was missing. For subcloning, the λ DNA was submitted to partial *Sac*I digestion, and the 5.5-kb *Eco*RI-*Sac*I fragment was cloned into plasmid pBluescript KS to give pBS22-49 (Fig. 1). To localize *APA1* on this plasmid, several deletions were constructed. pBS22-49 DNA was cut by *Cla*I and by either *Xba*I, *Bam*HI, or *Stu*I. After circularization, the deleted plasmids were used to transform *E. coli* IBPC111. Then, conversion of AMPS into ADP was assayed in the crude extracts of the various transformants. The smallest deleted plasmid still expressing AMPS phosphorylase activity corresponded to deletion of the 2.5-kb *Cla*I-*Xba*I fragment (Table 2). This indicated that *APA1* was included in the *Xba*I-*Sac*I fragment. It may also be noted that AMPS phosphorylase activity was increased about 10-fold by dele-

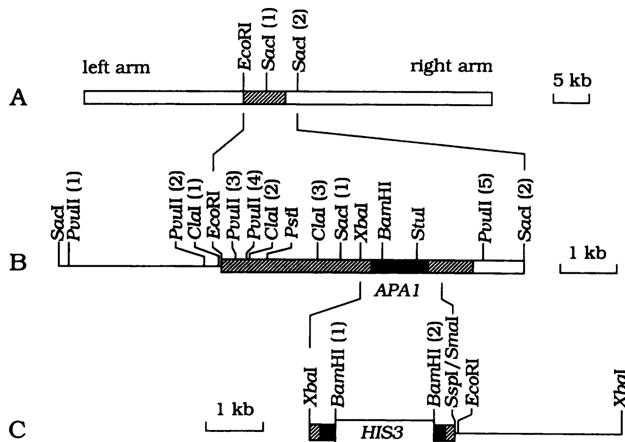


FIG. 1. Restriction map of the DNAs from λ22-4 (A), pBS2249 (B), and pBS2249-HYS (C). (A) Structure of λ22-4 DNA. The yeast DNA is represented by a hatched box. One of the two *Eco*RI sites expected on each side of the insert was missing. The right boundary between λgt11 and yeast DNA was deduced from sequencing data (not shown). (B) λ22-4 DNA was submitted to partial *Sac*I digestion, and the resulting 5.5-kb *Eco*RI-*Sac*I(2) fragment was cloned into plasmid pBluescript KS to give pBS22-49. The *APA1* coding region is represented by a black box. (C) DNA from plasmid pBS22-49 was cut by *Bam*HI and *Stu*I, filled in, and recircularized. This construction deleted the 595-bp *Bam*HI-*Stu*I fragment but conserved the *Bam*HI site. DNA sequence revealed the presence of an *Ssp*I site 210 bp after the end of *APA1*. This site was used to subclone a 778-bp *Xba*I-*Ssp*I fragment containing the deleted *APA1* gene into pBluescript KS digested by *Xba*I and *Sma*I. Finally, this plasmid was cleaved by *Bam*HI, and a ligation reaction was performed in the presence of a 1.77-kbp *HIS3* fragment (42). This *HIS3* fragment is represented by an open box. The resulting recombinant plasmid, pBS22-49HYS, was cut by *Eco*RI and *Xba*I and used to disrupt the chromosomal *APA1* gene.

tion of the *Cla*I-*Xba*I fragment of pBS22-49 (Table 2). This increase probably reflects more efficient transcription of *APA1* when the lactose promoter carried by the plasmid is nearer the start of *APA1*. Localization of *APA1* was made more precise by taking advantage of the presence on this fragment of a *Bam*HI site, unique in the pBS22-49 DNA. Plasmid pBS22-49 was cut by *Bam*HI and circularized after the 3′-recessed termini were filled in. The resulting four-nucleotide insertion caused the complete loss of AMPS phosphorylase activity. This result suggested that the *Bam*HI site was internal to the *APA1* gene, and DNA sequencing was therefore undertaken on both strands around the *Bam*HI site.

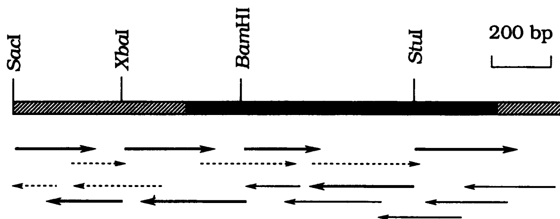


FIG. 2. Sequencing strategy for the *APA1* gene. The black box represents the coding region for $Ap_4A$ phosphorylase. Arrows represent length and direction of sequences obtained by the dideoxy chain termination method. Thick arrows indicate regions determined by subcloning restriction fragments from pBS22-49, thin solid arrows show regions obtained after Bal31 digestions, and broken arrows show regions obtained by using specific primers.

**Sequencing of the *APA1* gene.** The sequencing strategy is summarized in Fig. 2. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 3. One open reading frame of 321 codons, initiated by an ATG codon, was found. It corresponded to a protein product with a molecular ratio of 36,451, close to 40,000, the $M_r$ previously assigned to $Ap_4A$ phosphorylase from *S. cerevisiae* (19). At position +1, the ATG contained the necessary environment for efficient translational initiation in yeast cells, ANNAUGNNU, with the absence of U at position −1 (32). The 5′-flanking region contained a T-rich region, 26 T's from nucleotide −189 to nucleotide −157. Such T-rich clusters have already been observed in several other yeast genes and were suggested to be a signal for constitutive transcription (55). At the 3′ end of the open reading frame, the sequence TATG....TATGT....TTT (Fig. 3) conformed to the tripartite consensus sequence for termination and polyadenylation of yeast transcripts (62).

Purified $Ap_4A$ phosphorylase was not amenable to Edman degradation due to a blocked N-terminus. Therefore, to confirm that we had sequenced the $Ap_4A$ phosphorylase gene, peptides were generated from purified $Ap_4A$ phosphorylase and sequenced. The analysis of 23 peptides, starting from the tyrosine at nucleotides 43 to 45 and ending at the C-terminus, corroborated the DNA sequence, leaving only two small gaps (Fig. 3). The N-terminus of the protein may be an acetylserine, resulting from methionine excision followed by $N^{\alpha}$-acetylation of the serine, as expected from the work of Huang et al. (26).

The amino acid sequence deduced from the *APA1* sequence showed no strong identities with any of the known sequences stored in GenBank 58 and National Biomedical Research Foundation 17 sequence data banks.

***APA1* gene is unique and not essential.** To analyze the function of $Ap_4A$ phosphorylase in yeast cells, a plausible strategy is to search for the effect of inactivation of its gene. To do this, we first verified the uniqueness of the *APA1* gene and then inactivated it by the one-step disruption method of Rothstein (50).

The copy number of *APA1* was determined by Southern analysis. A digest of yeast DNA by eight distinct restriction endonucleases was hybridized with the 1.1-kbp *Stu*I-*Pvu*II DNA probe located at one end of the *APA1* gene. With each of the seven enzymes which did not cut within the probe, a unique hybridizing DNA fragment was observed (Fig. 4). This result demonstrated that the $Ap_4A$ phosphorylase gene was unique.

To inactivate *APA1*, plasmid pBS22-49HYS was constructed by replacing the 595-bp *Bam*HI-*Stu*I DNA portion of *APA1* with the selectable marker *HIS3* (Fig. 1). This plasmid, digested by *Eco*RI and *Xba*I, was used to transform a diploid yeast strain, CMY214. One of the recombinants obtained was sporulated, and haploids were randomly selected as canavanine-resistant cells (CMY214 is $can^r/CAN^s$). Among them, 58% proved to be $his^+$. Southern analysis of chromosomal DNA from one of these $his^+$ haploid cells confirmed disruption of the *APA1* gene (Fig. 4). Consequently, it can be immediately concluded that the product of *APA1* is not required for the viability of yeast cells. No difference in the growth rates of $APA1^+$ and Δ*apa1*::*HIS3* strains could be noted in rich as well as in minimal medium at 30°C.

**$Ap_4A$ phosphorolysis in Δ*apa1*::*HIS3* strains.** As expected, $Ap_4A$ degradation activity in extracts of the Δ*apa1*::*HIS3* strain was lower than that in extracts of the corresponding $APA1^+$ strain (Table 3). However, residual $Ap_4A$ degrada-

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                                                                                  -484
GAGCTCCAAAGAAGGCTCTGAAATGCTTATTTTTTTCAAGCGATACAAAGTAAACAGTTACATACCTGAATGATACTACCACAACTAT

                                                                                  -394
TTACATTATCGTCCTGTCAAAAGGCGAATAATCAGAACTGTTTGTTAACATGTCATTTTATAAGAGCGGCCTGAAATACTGTCATAAGTG

                                                                                  -304
GTCTGTCGAAAGTAGTTATTACGGCGTCGGGCGTCGCGAATAGCTCAAAAGTCACCCCAGCTTACACGATATTCCCATAACCTACAGCAA

                                                                                  -214
GAAGCTATTCTTGGATACAAGCACCAGTTACAAGCACTGCATTCATATATTGCCTTTTACTTTCCTATTCTGTATTTGTGTGCTAAGTGT

                                                                                  -124
CCTGTTTCGGAACGTTCTAGAAGATTTTCTTATTTTTCTCTTTTTCAGTTTTTTTTTCATCACGCAAAGGAAATACAATACAAGGTTAAA

                                                                                   -34
ATGAAGGAACTAACATTACTATTATTTCCAATTTAAAGTTTACCTTAAAAGACAATAGATTGATCTTGAAAGCCAGTTTAGATAGATTTT

                                                                                    57
AGAACAAACAGAAAAGTAAAAATTAAAATAAAAATGAGTATCCCCGCTGACATTGCATCTTTAATTAGTGACAAGTACAAAAGTGCCTTC
                                   MetSerIleProAlaAspIleAlaSerLeuIleSerAspLysTyrLysSerAlaPhe

                                                                                   147
GATAATGGTAACTTAAAATTTATCCAGACTGAAACAACGAAAACAAAGGACCCAAAAACCAGCATGCCATACTTGATTAGTCACATGCCA
AspAsnGlyAsnLeuLysPheIleGlnThrGluThrThrLysThrLysAspProLysThrSerMetProTyrLeuIleSerHisMetPro

                                                                                   237
AGTCTGATCGAAAAGCCAGAGCGTGGCCAAACTCCAGAAGGAGAGGATCCACTAGGCAAACCTGAGGAAGAATTAACGGTTATCCCAGAA
SerLeuIleGluLysProGluArgGlyGlnThrProGluGlyGluAspProLeuGlyLysProGluGluGluLeuThrValIleProGlu

                                                                                   327
TTTGGTGGTGCCGATAACAAAGCGTATAAATTGCTATTAAACAAATTCCCTGTAATCCCTGAACACACTTTATTGGTAACTAACGAATAC
PheGlyGlyAlaAspAsnLysAlaTyrLysLeuLeuLeuAsnLysPheProValIleProGluHisThrLeuLeuValThrAsnGluTyr

                                                                                   417
CAACATCAAACTGATGCCTTGACCCCAACCGATTTATTGACTGCTTATAAGTTGCTGTGTGCCTTGGACAATGAAGAATCCGACAAGAGA
GlnHisGlnThrAspAlaLeuThrProThrAspLeuLeuThrAlaTyrLysLeuLeuCysAlaLeuAspAsnGluGluSerAspLysArg

                                                                                   507
CACATGGTCTTTTACAATTCTGGTCCAGCCAGTGGTTCTTCATTGGACCACAAACATTTGCAAATTCTGCAAATGCCTGAAAAGTTCGTC
HisMetValPheTyrAsnSerGlyProAlaSerGlySerSerLeuAspHisLysHisLeuGlnIleLeuGlnMetProGluLysPheVal

                                                                                   597
ACTTTCCAAGATAGACTATGTAATGGTAAAGAACATTTCCTACCAACTTTCAATACTGAACCTTTGCAAGATGCTAAAGTCTCGTTCGCT
ThrPheGlnAspArgLeuCysAsnGlyLysGluHisPheLeuProThrPheAsnThrGluProLeuGlnAspAlaLysValSerPheAla

                                                                                   687
CATTTTGTCTTGCCAATGCCGGAGTCTGAAGAAACTGTTGATGAAGACCTATTAGCTATGTGTTACATCTCCATATTGCAAAGAGCTTTG
HisPheValLeuProMetProGluSerGluGluThrValAspGluAspLeuLeuAlaMetCysTyrIleSerIleLeuGlnArgAlaLeu

                                                                                   777
ACCTTTTTCCAGGACTGGTTGAACGAAAATCCAGAACTAAAGAAATCCTACAATCTTATGTTAACCAAGGAATGGATCTGTGTCGTTCCA
ThrPhePheGlnAspTrpLeuAsnGluAsnProGluLeuLysLysSerTyrAsnLeuMetLeuThrLysGluTrpIleCysValValPro

                                                                                   867
CGCTCGAAGGCCTTTTCTGATGAAATGAAGATAGGTTTCAACTCCACAGGTTATTGTGGTATGATCTTAACCAAAAATGATGAAGTTTTC
ArgSerLysAlaPheSerAspGluMetLysIleGlyPheAsnSerThrGlyTyrCysGlyMetIleLeuThrLysAsnAspGluValPhe

                                                                                   957
TCCAAGATTACTGAAAAACCTGAATTGATTAACGATATCTTATTGGAATGTGGTTTCCCAAACACTTCTGGTCAAAAACCAAACGAATAC
SerLysIleThrGluLysProGluLeuIleAsnAspIleLeuLeuGluCysGlyPheProAsnThrSerGlyGlnLysProAsnGluTyr

                                                                                  1047
AACTATTGAGTCCTTCACAAGTACAAGTATACACGTATACATGTATGTATATATATGTATTTAAATGATAGAAGTAATTTCTATATGTAT
AsnTyr

                                                                                  1137
ATGTCTATTCAATTTTTATTTCTGATGACTTTGAAATTTTATATTTATTATTCTACACTTTATTATTAAA
```

FIG. 3. Nucleotide and deduced amino acid sequences of the *APA1* gene. A stretch of 1,688 nucleotides encompassing the coding region of the *APA1* gene is shown. The sequence extends from *Sac*I(1) site to 330 bp beyond the *Stu*I site (Fig. 1). Sequence determination was carried out by the dideoxy chain termination method (52). Tryptic peptides resolved by Edman degradation are indicated by solid arrows. The underlined DNA sequence located at nucleotides 1001 to 1037 is homologous to the tripartite consensus sequence for transcriptional termination and polyadenylation of yeast transcripts (62).

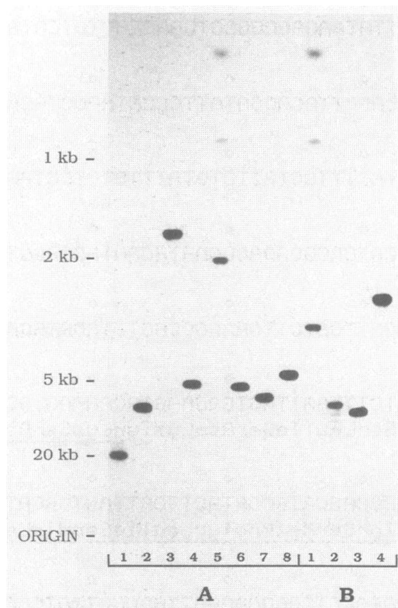


FIG. 4. Southern blot analysis demonstrating the uniqueness of the *APA1* gene in the yeast genome and the disruption of the *APA1* gene in the Δ*apa1::HIS3* strain. Genomic DNA from either an *APA1*$^+$ strain (lanes A1 to A8) or a Δ*apa1::HIS3* strain (lanes B1 to B4) was digested with the following restriction enzymes: *Pvu*II (lane A1), *Bam*HI (A2), *Stu*I (A3), *Cla*I (A4), *Ssp*I (A5 and B1), *Xba*I (A6 and B2), *Eco*RI (A7 and B3), or *Pst*I (A8 and B4). DNA fragments produced by these digestions were electrophoresed on a 0.8% agarose gel and transferred to a nitrocellulose filter. The filter was then hybridized with the $^{32}$P-labeled *Stu*I-*Pvu*II(5) probe from the 3' end of the *APA1* gene (Fig. 1). The enzyme *Ssp*I has two recognition sites within the probe. Other enzymes do not cut the probe and produce a single band. The sizes of the bands obtained with the DNA from strain CMY214 Δ*apa1::HIS3* agree with the disruption of the *APA1* gene in this strain; the DNA fragments recognized by the probe were systematically 1.2 kbp larger than those of the *APA1*$^+$ strain, except for *Pst*I, which has one recognition site within *HIS3*.

tion activity clearly remained in the Δ*apa1::HIS3* cells, corresponding to about 16% of the activity measured in the control *APA1*$^+$ cells. To account for this surprising result, one had to posit the occurrence in *S. cerevisiae* of another enzyme, distinct from the already described $Ap_4A$ phosphorylase, also capable of catabolizing $Ap_4A$.

To explore this idea, anti-$Ap_4A$ phosphorylase antibodies were added to crude extracts of *APA1*$^+$ and Δ*apa1::HIS3* strains, as well as to a solution of homogeneous $Ap_4A$ phosphorylase, and the activities of $Ap_4A$ degradation were compared. Table 3 shows that antibodies had similar effects on the activities in the *APA1*$^+$ extract and on the pure enzyme. Inhibition greater than 85% was observed in the presence of 0.8 mg of antibodies per ml. On the other hand, $Ap_4A$ degradation in the extract of the Δ*apa1::HIS3* extract appeared to be insensitive to the addition of the antibodies. This observation therefore establishes that the enzyme responsible for $Ap_4A$ degradation in the Δ*apa1::HIS3* strain is actually different from the enzyme previously described as $Ap_4A$ phosphorylase.

To further characterize this second $Ap_4A$-catabolizing enzyme, crude extracts from *APA1*$^+$ and Δ*apa1::HIS3* strains were chromatographed on DEAE-Sephacel columns, and the catabolic activity was followed in each fraction. Figure 5 shows the presence of two peaks of activity on the elution profile obtained with the extract from *APA1*$^+$ cells,

TABLE 3. Effect of the addition of anti-$Ap_4A$ phosphorylase I antibodies on the initial rates of $Ap_4A$ degradation, AMPS phosphorolysis, and ADP-$P_i$ exchange, catalyzed by crude extracts or by pure $Ap_4A$ phosphorylase[a]

| Catalyst | Antibodies added | Initial rate (pmol of ADP/s per mg of protein) | | |
|---|---|---|---|---|
| | | $Ap_4A$ degradation | AMPS phosphorolysis | ADP-$P_i$ exchange |
| Extract of *APA1*$^+$ strain | − | 32 | 88 | 17 |
| | + | 4.8 | 6.7 | 1.5 |
| Extract of Δ*apa1::HIS3* strain | − | 5.1 | 6.0 | 1.2 |
| | + | 4.7 | 6.0 | 1.2 |
| Pure $Ap_4A$ phosphorylase I[b] | − | 350,000 | 780,000 | 600,000 |
| | + | <30,000 | 40,000 | 40,000 |

[a] Initial rates were measured in the absence or presence of 0.8 mg of anti-$Ap_4A$ phosphorylase I antibodies per ml. Crude extracts were obtained by sonication of yeast cells grown at 30°C in YPD medium. In all assays, $Ap_4A$ degradation activity was adjusted to 0.004 U/ml. Total amounts of protein in the extracts were calculated from the $OD_{280}$ of the extract, assuming that 1 $OD_{280}$ unit corresponded to 1 mg of protein.

[b] For pure $Ap_4A$ phosphorylase I, initial rates of $Ap_4A$ degradation, AMPS phosphorolysis, and ADP-$P_i$ exchange without added antibodies corresponded to 13, 28, and 22 $s^{-1}$, respectively. This set of values is in agreement with the values obtained under slightly different experimental conditions (20).

whereas only one peak was observed from the *apa1* cell extract. On the profile corresponding to the *APA1*$^+$ extract, the smaller activity peak eluted at an ionic strength identical to that of the unique activity peak observed on the profile of

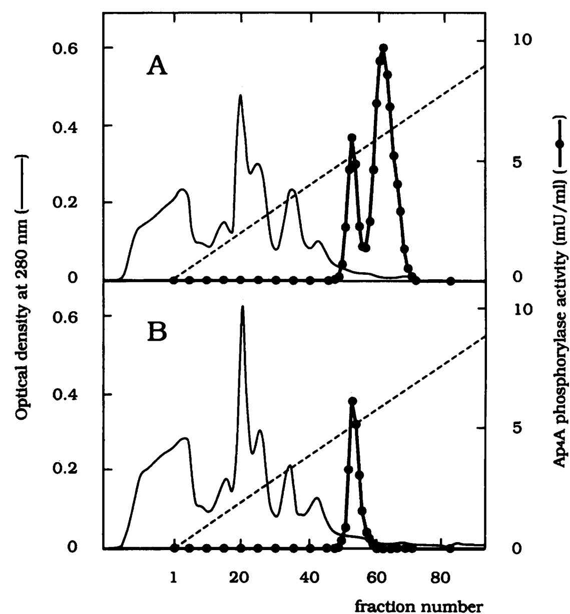


FIG. 5. DEAE-Sephacel chromatography of crude extracts of *APA1*$^+$ (A) and Δ*apa1::HIS3* (B) strains. Extracts were obtained by sonication of 0.4 g of yeast cells (wet weight) followed by centrifugation at 200,000 × *g* for 30 min. The supernatants were diluted 30-fold with column equilibration buffer (20 mM potassium phosphate [pH 6.75], 10 mM 2-mercaptoethanol, 0.1 mM EDTA) and applied to the column at a flow rate of 0.10 ml/min. The column was washed with 7 ml of equilibration buffer, and elution was achieved by increasing the KCl concentration from 0 to 500 mM (dashed line). Fractions of 1 ml were collected. The $A_{280}$ of the column effluent was monitored; $Ap_4A$ phosphorolysis was measured by the assay described in Materials and Methods.

the $\Delta$*apa1*::*HIS3* extract. Therefore, it was tempting to assume that this peak corresponded to the second $Ap_4A$-degrading activity. This was confirmed by pooling the corresponding fractions and testing the effect of the addition of anti-$Ap_4A$ phosphorylase antibodies on the activity. No inhibition could be detected. From this result, we concluded that the second $Ap_4A$-degrading activity detected in the $\Delta$*apa1*::*HIS3* extract was also expressed in *APA1*$^+$ cells. This activity had not been previously detected, probably because the DEAE-Sephacel resin was used batchwise (19).

To determine whether the second $Ap_4A$ phosphorylase activity hydrolyzed or phosphorolyzed $Ap_4A$, active fractions obtained from the DEAE-Sephacel chromatography of the $\Delta$*apa1*::*HIS3* extract were pooled, and the effect of phosphate on $Ap_4A$ degradation was tested. The rate of $Ap_4A$ degradation was at least 10-fold lower in the absence of added phosphate. This dependence of the activity on the presence of phosphate suggested phosphorolytic degradation of $Ap_4A$. In addition, high-pressure liquid chromatography analysis of the products of the reaction was performed. It established that $Ap_4A$ degradation by this new enzyme activity led to formation of ADP and ATP in equal amounts. All these results demonstrated that the second $Ap_4A$-catabolizing activity found in this study was again an $Ap_4A$ phosphorylase catalyzing the reaction $Ap_4A + P_i \leftrightarrow ATP + ADP$. In the rest of this article, the product of *APA1* will be called $Ap_4A$ phosphorylase I and the second form will be called $Ap_4A$ phosphorylase II.

**Effect of inactivation of *APA1* on the $Ap_4N$ concentration in yeast cells.** In order to investigate the role of $Ap_4A$ phosphorylase I in $Ap_4N$ metabolism, $Ap_4N$ concentrations were compared in *APA1*$^+$ and $\Delta$*apa1*::*HIS3* cells. For this purpose, yeast cells were grown in rich medium, and cell samples were withdrawn during the exponential phase of growth. Then, $Ap_4N$s were extracted and quantitated as described in Materials and Methods. Cellular $Ap_4N$ concentrations of 0.83 and 2.4 μM were calculated for the *APA1*$^+$ and $\Delta$*apa1*::*HIS3* strains, respectively. Therefore, the absence of the $Ap_4A$ phosphorylase I product caused a threefold increase in the cellular $Ap_4N$ concentration.

Several stresses, including cadmium addition, are known to trigger strong accumulation of $Ap_4N$ in yeast cells (6, 14). To determine the role of $Ap_4A$ phosphorylase I during this accumulation, $Ap_4N$ concentration was followed within *APA1*$^+$ and $\Delta$*apa1*::*HIS3* strains exposed to cadmium ions. Cells were grown in YPD medium, and when the $OD_{650}$ of the culture reached 1.2, $CdSO_4$ was added to a final concentration of 5 mM. Again, $Ap_4N$ concentrations were higher in the $\Delta$*apa1*::*HIS3* strain than in the control strain, whatever the time after cadmium addition (Fig. 6). Thus, $Ap_4A$ phosphorylase I seems to catabolize $Ap_4N$ when yeast cells grow exponentially, as well as when they are stressed by cadmium.

**$Ap_4A$ phosphorylase I and ADP sulfurylase are probably identical.** As mentioned in the Introduction, $Ap_4A$ phosphorylase I shares common properties with ADP sulfurylase (1, 12, 39, 48) and with the enzyme characterized by Grunberg-Manago et al. (18) through its ability to incorporate $^{32}P_i$ into ADP. However, the identity between $Ap_4A$ phosphorylase I and ADP sulfurylase could not be unambiguously established (20). The $M_r$ of ADP sulfurylase was estimated at ca. 150,000 to 200,000 by sucrose gradient centrifugation (18). Such an $M_r$ appears to be significantly different from that calculated for $Ap_4A$ phosphorylase I (ca. 40,000). In addition, the sulfate-dependent ADP-$P_i$ exchange activity in partially purified yeast fractions was reported to be inhibited by the addition of KF (12), while the ADP-$P_i$ exchange activity of homogeneous $Ap_4A$ phosphorylase, measured in the absence of sulfate, was not sensitive to fluoride (20).

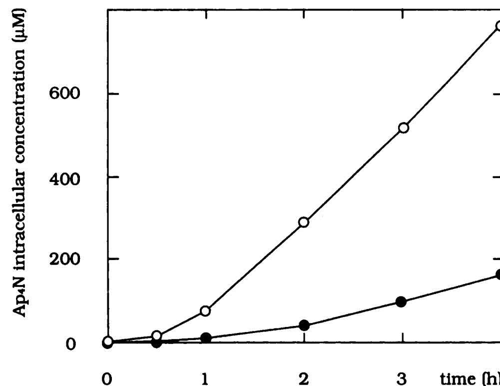


FIG. 6. Effect of cadmium on $Ap_4N$ concentration in *APA1* (●) and $\Delta$*apa1*::*HIS3* (○) yeast cells. Cells were grown in YPD rich medium. When the $OD_{650}$ of the culture reached 1.2, $CdSO_4$ was added to a final concentration of 5 mM, and $Ap_4N$ concentrations were followed as a function of time.

To examine further the possible identity between $Ap_4A$ phosphorylase I and ADP sulfurylase, the following experiments were conducted. AMPS phosphorolysis and ADP-$P_i$ exchange rates were compared in *APA1*$^+$ and $\Delta$*apa1*::*HIS3* crude extracts. Inactivation of the *APA1* gene resulted in a 13-fold decrease in AMPS phosphorolysis activity and an 11-fold decrease in ADP-$P_i$ exchange activity (Table 3). Thus, despite the discrepancies between $M_r$, it could be suspected that ADP sulfurylase and the enzyme studied by Grunberg-Manago et al. were, in fact, the same enzyme as $Ap_4A$ phosphorylase I.

However, an indirect effect of *APA1* gene disruption on the expression of ADP sulfurylase activity also had to be considered. To examine this point, the effects of anti-$Ap_4A$ phosphorylase I antibodies on AMPS phosphorolysis and ADP-$P_i$ exchange activities in the *APA1*$^+$ and $\Delta$*apa1*::*HIS3* crude extracts were examined. The addition of antibodies to the crude extract of the *APA1*$^+$ strain inhibited AMPS phosphorolysis and ADP-$P_i$ exchange reactions by more than 85% (Table 3). This result clearly indicates that AMPS phosphorolysis and ADP-$P_i$ exchange activities mainly originate from the $Ap_4A$ phosphorylase I present in this crude extract.

On the other hand, anti-$Ap_4A$ phosphorylase I antibodies had no effect on the residual AMPS phosphorolysis and ADP-$P_i$ exchange activities catalyzed by the extract of the $\Delta$*apa1*::*HIS3* strain. It is possible that these activities proceed from $Ap_4A$ phosphorylase II, which was shown above to be resistant to anti-$Ap_4A$ phosphorylase I antibodies.

**Conclusion.** The cloning and inactivation of the gene encoding $Ap_4A$ phosphorylase I from *S. cerevisiae* revealed several features of $Ap_4N$ metabolism in this organism. (i) Suppression of $Ap_4A$ phosphorylase I expression by inactivation of *APA1* is accompanied by a threefold increase in $Ap_4N$ concentrations. This indicates that, under the growth conditions used here, $Ap_4A$ phosphorylase I is an $Ap_4N$ catabolic enzyme rather than an anabolic one. Therefore, the ability of this enzyme to produce $Ap_4A$ from ATP plus ADP (10, 19) or from AMPS plus ATP in vitro (22) does not seem to play a significant role in vivo.

At this stage, however, it cannot be excluded that disruption of the *APA1* gene indirectly modifies the cellular $Ap_4N$

pool by changing the efficiency of the enzymes capable of synthesizing the $Ap_4N$ family. Among these enzymes are the aminoacyl-tRNA synthetases (Brevet et al., in press), the $Ap_4N$-synthetase activity of which has been shown in vitro to be very sensitive to ionic conditions (8). Therefore, it is conceivable that in the $\Delta$*apa1*::*HIS3* context, the aminoacyl-tRNA synthetase environment or expression is slightly changed, accounting for the observed threefold increase in cellular $Ap_4N$ concentration.

(ii) A second $Ap_4A$ phosphorylase was discovered. At present, it is not known whether the two yeast $Ap_4A$ phosphorylases have distinguishable catalytic properties or are located in distinct cellular compartments. In this context, it should be remembered that, in higher eucaryotes, a diadenosine 5′,5‴-$P^1$,$P^3$-triphosphate-catabolizing activity occurring in the mitochondria has been reported (13). The strain devoid of $Ap_4A$ phosphorylase I will be useful in addressing the question of the subcellular localization of the two forms of $Ap_4A$ phosphorylase and to prepare homogeneous $Ap_4A$ phosphorylase II for comparison with $Ap_4A$ phosphorylase I.

(iii) Finally, this study favors the idea that $Ap_4A$ phosphorylase I and the previously described ADP sulfurylase could be a same enzyme, reinforcing the hypothesis of a connection between $Ap_4A$ and AMPS metabolism (22). ADP sulfurylase activity has already been detected in various microorganisms, such as *Thiobacillus thioparus* (43) and *Desulfovibrio desulfuricans* (44), and in the chloroplasts of higher plants (12). It may be that the responsible enzymes are also $Ap_4A$-metabolizing enzymes.

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