

APT1, but Not *APT2*, Codes for a Functional Adenine Phosphoribosyltransferase in *Saccharomyces cerevisiae*

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The yeast *Saccharomyces cerevisiae* has two separate genes (*APT1* and *APT2*) that encode two potentially different forms of adenine phosphoribosyltransferase (APRT). However, genetic analysis indicated that only *APT1* could code for a complementing activity. Cloning and expression of both the *APT1* and *APT2* genes in *Escherichia coli* showed that although discrete proteins (APRT1 and APRT2) were made by these genes, only APRT1 had detectable APRT activity. Northern and Western blot analyses demonstrated that only *APT1* was transcribed and translated under normal physiological conditions in yeast. Phylogenetic analysis revealed that APRT1 and APRT2 are evolutionary closely related and that they arise from a gene duplication event. We conclude that *APT1* is the functional gene in *S. cerevisiae* and that *APT2* is a pseudogene.

In most organisms, purine nucleotides can be synthesized de novo by a series of 10 enzymatic reactions that lead to the formation of IMP (4, 5, 20). IMP can be further converted to AMP or GMP to satisfy the purine nucleotide requirements of a growing cell. Alternatively, free-purine bases can be directly recycled from the growth medium and incorporated into purine nucleotide pools. Both sets of reactions involve the action of phosphoribosyltransferases (PRTases), a group of enzymes that have in common the chemistry of transferring the ribose 5-phosphate from α -D-5-phosphoribosyl 1-pyrophosphate (PRPP) onto a nitrogenous base to form an *N*-riboside monophosphate and pyrophosphate (20). PRTases are essential not only for purine biosynthesis but also for the biosynthesis of pyrimidines, pyridines, histidine, and tryptophan (20).

Adenine PRTase (APRT) is the key enzyme in the direct recycling of free adenine into purine nucleotide pools. APRT catalyzes the Mg^{2+} -dependent transfer of the phosphoribosyl group from PRPP to adenine to form the nucleotide AMP (2, 4, 8, 11, 14, 16, 19, 21, 28, 30). In humans, APRT deficiency causes the accumulation of a poorly soluble adenine derivative, 2,8-dihydroxy adenine, which leads to urolithiasis and renal failure (27).

Typically, APRTs are encoded by constitutively expressed single-copy genes (22). However, two genes encoding two forms of APRT in *Arabidopsis thaliana* that differ in their nucleotide specificities have recently been reported (18, 25). We have previously identified a gene coding for APRT in *Saccharomyces cerevisiae* (*APT1*) (2). In addition, Yuryev and Corden reported the sequence of another potential *APT* gene in yeast (*APT2*), based on sequence comparison analysis (32). The finding of two different genes that may encode APRT in yeast has led us to propose that more than one form of this enzyme may also occur in yeast.

To help understand the relationship between *APT1* and *APT2* and address the possibility that two forms of the enzyme

also occur in *S. cerevisiae*, both genes were used in complementation studies with yeast mutants deficient in APRT activity. Mutations disrupting *APT1* and *APT2* were made. Whereas disruption of *APT1* eliminated APRT activity, disruption of *APT2* had no effect on APRT activity or repression of de novo purine biosynthesis by adenine. Both genes were also individually expressed in *Escherichia coli* cells. Expression of a recombinant *APT2* showed that this gene is defective in that it does not encode a functional APRT enzyme. From the complementation and expression studies, evidence is presented to support the hypothesis that *APT1* is required and by itself sufficient to code for APRT in *S. cerevisiae*. In addition, the data presented here lead us to propose that *APT2* is in fact a pseudogene, derived from a gene duplication event.

Strains, plasmids, and culture conditions. *E. coli* NM522 (GIBCO-BRL) was grown on 2 \times YT medium supplemented with ampicillin (50 μ g/ml of culture) for routine growth of plasmids. *E. coli* B25 and B26, used in the high-level expression of the *APT1* and *APT2* genes, were grown on similar medium but also supplemented with kanamycin to select for the *lacZ*-repressor plasmid, as described in the Qiagen manual.

The following yeast strains were used in this study: Y642 (*MAT α ura3-52 leu2-3,112 lys2 Δ 201 his3 Δ 200 trp1::hisG*) (this study), Y643 (*MAT α ura3-52 leu2-3,112 lys2 Δ 201 his3 Δ 200 trp1::hisG*) (this study), L3852 (*MAT α ade2 his3 Δ 200 leu2-3,112 lys2 Δ 201 ura3-52*) (G. Fink), L4364 (*MAT α ade2 his3 Δ 200 leu2-3,112 lys2 Δ 201 ura3-52*) (G. Fink), Y810 (*MAT α ura3-52 leu2-3,112 lys2 Δ 201 his3 Δ 200 trp1::hisG apt2::HIS3*) (this study), Y812 (*MAT α ura3-52 leu2-3,112 lys2 Δ 201 his3 Δ 200 trp1::hisG apt2::HIS3*) (this study), Y814 (*MAT α ade2 his3 Δ 200 leu2-3,112 lys2 Δ 201 ura3-52 apt2::HIS3*) (this study), Y808 (*MAT α ade2 his3 Δ 200 leu2-3,112 lys2 Δ 201 ura3-52 apt2::HIS3*) (this study), W109-9C (*MAT α ade2 trp1 ura3 his3 hpt1-27*) (R. Woods), DS1.2B/1 (*MAT α ade2 apt1 aah1 ura3*) (R. Woods), Y839 (*MAT α ura3-52 leu2-3,112 lys2 Δ 201 his3 Δ 200 apt2::HIS3 hpt1::URA3*) (this study), Y511 (*MAT α ura3 lys2 leu2 apt1::URA3*) (B. Daignan-Fornier), Y350 (*MAT α ura3 lys3 leu2*) (B. Daignan-Fornier), Y842 (*MAT α ura3-52 leu2-3,112 lys2 Δ 201 his3 Δ 200 trp1::hisG apt2::HIS3 aah1::URA3*) (this study), X79 (*MAT α leu2-1 trp1-1*) (R. Woods), Y382 (*MAT α ade2 ade3 ura3 leu2 trp1*) (A. Bender), Y388 (*MAT α ade2 ade3 ura3 leu3 lys1*) (A. Bender), Y839 (*MAT α*

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TABLE 1. Complementation analysis of an *APT*⁻ mutant transformed with the *APT1* and *APT2* genes^a

Strain	Growth on adenine as purine source	APRT sp act (μmol/min/mg)	% of wild-type activity
X79 (<i>MATa leu2-1 trp1 ade3</i>)	+	6.62	100.0
DS1.2B (<i>MATα ura3 apt1 aah1 ade2</i>)	-	0.005	0.07
DS1.2B (pRS416 transformed)	-	0.004	0.04
DS1.2B (pYEPAPT1 transformed)	+	7.5	113.0
DS1.2B (pRSAPT1 transformed)	+	5.8	89.0
DS1.2B (pRSAPT2 transformed)	-	0.004	0

^a The APRT-deficient DS1.2B strain which carries an adenine mutation that blocks the de novo pathway was transformed with various plasmids carrying the *APT1* or *APT2* gene. This strain is unable to grow on adenine as the sole purine source. The APRT activities in cell extracts from the DS1.2B mutants as well as their parent strain (X79) are given in micromoles of [³H]AMP produced per minute per milligram of protein. Different constructs used to transform DS1.2B are as described in "Complementation and genetic analysis of APRT." The specific APRT activity of the X79 (wild-type) strain was considered 100% and used for comparison with the specific APRT activities of extracts from the various transformants.

ura3 trp1 leu2 his3 pep::His3 prb1 can1 GAL) (A. Bender), Y847 (*MATa ura3-52 leu2-3,112 lys2Δ201 his3Δ200 apt2::HIS3 apt1::URA3*) (this study), and MWT32 (*MATa leu2 ade2 ade3 apt1*) (M. Taylor).

Yeast cells were transformed by the lithium chloride-triacetate method (17). Bacterial transformations were carried out by the MnCl₂ method (24).

Complementation and genetic analysis of APRT. Transformants were selected by complementation of the *URA* mutation to the *URA*⁺ phenotype (by the plasmid-derived *URA* marker) in strain DS1.2B and scored for their ability to use adenine as the sole source of purine. Both the *APT1* (pRSAPT1, single copy) and *APT2* (pRSAPT2, single copy) genes were individually transformed into the DS1.2B mutant and tested for complementation. The *APT1* gene complemented the *APT* mutant phenotype, allowing the APRT-deficient mutant DS1.2B to grow on defined media containing adenine as the sole purine source. The *APT2* gene (12) failed to complement the DS1.2B mutant (Table 1). Also, as shown in Table 1, only *APT1*-transformed cells had wild-type levels of APRT activity.

The *APT2* gene was disrupted by replacing its entire coding region with the *HIS3* gene (6). The resulting plasmid, named P878, carrying the *apt2::HIS3* construct was amplified with the following synthetic oligonucleotides: APT23, 5'-GCTACTGTGCATACCGC-3', and APT24, 5'-GAGGCACCTTTGAACG GC-3'. The resulting PCR product was used to transform the yeast strains Y642, Y643, L3852, and L4364. Transformants were selected for histidine prototrophy. Correct integration was verified by PCR. Disruption of the *APT2* gene in a wild-type strain does not lead to any obvious growth phenotype. Since *apt1* mutants were previously shown to be resistant to 8-azaadenine (23), we tested the resistance of the *apt2*-disrupted strains to this base analog. We found that the *apt2* mutant is as sensitive to 8-azaadenine as the isogenic wild-type strain. Furthermore, the *ade2 apt2* double mutant (where the *ade2* mutation blocks de novo purine biosynthesis) can use adenine or hypoxanthine as a purine source.

Mutations affecting purine salvage also inhibit adenine repression of the genes encoding enzymes of the purine de novo pathway (7). We have therefore tested whether the *apt2*-disrupted strain was affected in this regulation process. Expression of an *ADE1* (*N*-succinyl-5-aminoimidazole-4-carboxamide ribotide synthetase, a purine de novo enzyme)-*lacZ* fusion was assayed in the *apt2* mutant and isogenic wild-type strains. The *apt2* mutation has no effect on adenine repression of the *ADE1-lacZ* fusion.

apt2 aah1, *apt2 hpt1*, and *apt1 apt2* double mutants were constructed. All the double mutants grew normally and were phenotypically indistinguishable from the isogenic single mu-

tant in a wild-type *APT2* background. *apt1 apt2* double mutants salvaged adenine through adenine aminohydrolase. All together, these results suggest that *apt2* disruption does not severely affect purine utilization during vegetative growth.

To test whether *APT2* encodes a minor isoform of APRT, we introduced the *APT2* gene on a multicopy vector (P552) (12, 29) into an *apt1 aah1 ade2* triple mutant. This strain is unable to use adenine as a purine source but grows normally through conversion of hypoxanthine into IMP by hypoxanthine-guanine phosphoribosyl transferase. The multicopy vector carrying the *APT2* gene is unable to restore adenine utilization to the triple mutant strain, showing that even when overexpressed, *APT2* is unable to compensate for the lack of *APT1*. Similarly, overexpression of *APT2* does not restore utilization of hypoxanthine in an *hpt1 ade2* double mutant, thus indicating that APRT2 has no significant hypoxanthine PRase activity.

Our finding that *APT1*, but not *APT2*, could complement an APRT-deficient mutant suggests that either *APT2* does not encode a functional APRT or that *APT2* is indeed required for APRT activity but that it is not by itself sufficient to encode a functional enzyme. However, the fact that disruption of the *APT2* gene had no effect on adenine utilization, adenine analog resistance, or regulation of de novo purine biosynthesis further supports the view that APRT2 serves no function in purine recycling in *S. cerevisiae*.

Expression of recombinant APRT1 and APRT2 proteins. The *APT1* and *APT2* genes were individually ligated into His-tag expression vectors (Qiagen, Hilden, Germany), and the pQEAPT1 and pQEAPT2 expression constructs, respectively, were generated. These constructs allow for the expression of the recombinant APRT1 and APRT2 proteins in *E. coli* with an N-terminal hexahistidine tag. To express the APRT1 and APRT2 proteins, *E. coli* B25 (Qiagen) was individually transformed with each construct (pQEAPT1 or pQEAPT2) and cells were grown as described in the Qiagen manual. Cells (1L) were routinely grown to mid-log phase (*A*₆₀₀ ~0.5) at 37°C, the point at which IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM. The cells were grown for five additional hours after IPTG addition.

E. coli cells expressing *APT1* and *APT2* were harvested by centrifugation at 5,000 × *g* in a Sorvall centrifuge at 4°C for 15 min. The cells were suspended in 30 ml of sonication buffer (50 mM Tris-Cl [pH 7.4], 5 mM MgCl₂, 20 mM KCl) and broken by sonication at maximum power with a microtip for cycles of 1 min with a 1-min rest for a total of 10 min. The resulting extract was spun at 20,000 × *g* for 30 min, and the resulting cell extract was gravity loaded onto a 2-ml Ni²⁺-nitrilotriacetic acid column. The column was washed with 10 column volumes of

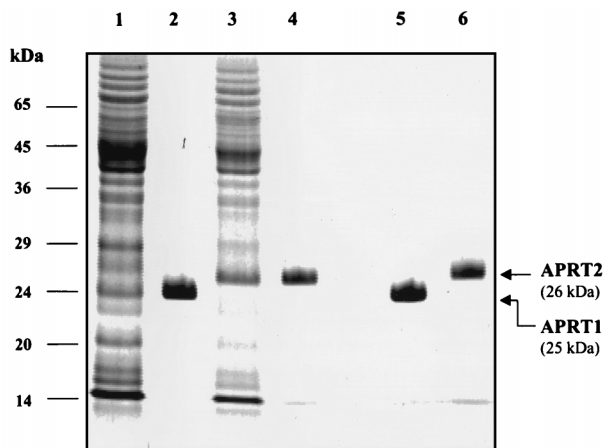


FIG. 1. Expression of hexahistidine-tagged APRT1 and APRT2 proteins in *E. coli* cells. Lane 1, total cell extract from *E. coli* cells transformed with the His-tagged *APT1* gene; lane 2, Ni²⁺-purified recombinant APRT1 protein; lane 3, total cell extract from *E. coli* cells transformed with the His-tagged *APT2* gene; lane 4, Ni²⁺-purified recombinant APRT2 protein; lanes 5 and 6, purified recombinant APRT1 and APRT2 proteins (respectively) run next to each other to emphasize their relative size difference.

sonication buffer (20 ml) containing 50 mM imidazole and eluted with 4 column volumes of the same buffer containing 150 mM imidazole, as described in the Qiagen protein expression manual. The *APT1* and *APT2* genes were inserted in plasmid vectors under the control of an IPTG-inducible promoter and expressed in *E. coli* cells (Qiagen). The *APT1* gene product (APRT1) migrated as a 25-kDa band, and the *APT2* gene product (APRT2) migrated as 26-kDa band in a sodium dodecyl sulfate–12.5% polyacrylamide gel (Fig. 1). These sizes are in agreement with the sizes expected from the deduced coding sequences of the two genes with the His tag at their N termini.

In order to assess the sizes of APRT1 and APRT2 in solution, both recombinant proteins were separately loaded onto a Sephadex G-200 column, calibrated with protein standards, and developed as previously described (1a) (Fig. 2). The purified APRT1 and APRT2 proteins were individually loaded, and the column was developed with the equilibration buffer. Five-milliliter fractions were collected, and their relative protein contents were estimated by measurements of optical density at 280 nm with a model 160 UV spectrophotometer (Shimadzu). Protein-containing fractions were assayed for APRT activity by measuring the incorporation of ³H-labeled adenine into AMP, after the enzyme fraction to be tested was incubated with PRPP and [³H]adenine in the appropriate buffer (15). The fraction with the highest APRT activity (100% relative activity) was used as the peak fraction for determining the native size of the enzyme in solution.

APRT1 eluted between the bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa) standards. The peak of activity observed coincided with the molecular mass of a 50-kDa protein (Fig. 2A). This molecular mass agrees with that of a homodimer of 25-kDa subunits. When APRT2 was chromatographed through the same column, it eluted in a fraction lacking any detectable APRT activity. The peak of absorbance (*A*₂₈₀) corresponded to a 25-kDa protein (Fig. 2B); thus, APRT2 failed to dimerize. The finding that the APRT1 protein had enzymatic activity comparable to that of the native enzyme previously purified in our laboratory suggests that indeed the *APT1* gene is sufficient to code for a functional enzyme. Our

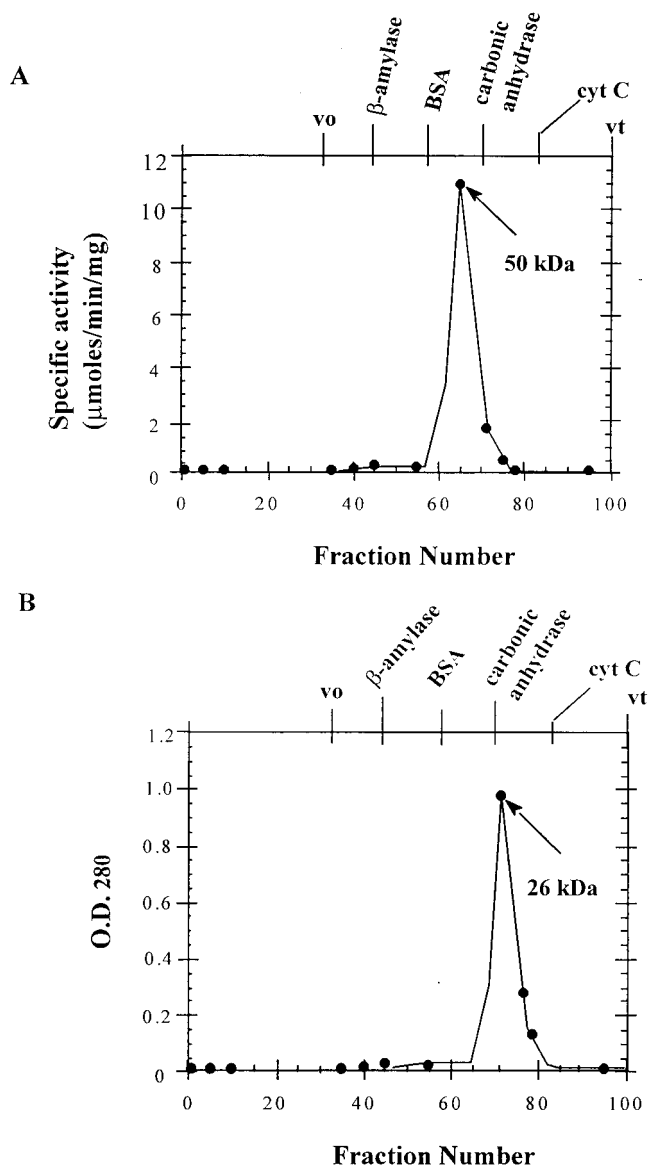


FIG. 2. Sizing of the APRT1 and APRT2 recombinant proteins. (A) His-tagged APRT1 was chromatographed on a Sephadex G-200 column as described in Materials and Methods. The peak of activity corresponds to that of a 50-kDa protein. (B) The His-tagged APRT2 protein was chromatographed through the same Sephadex column. As no APRT activity was detected with this protein, the peak of absorbance (optical density at 280 nm [O.D.280]) is given and corresponds to that of a 26-kDa protein. β -Amylase (200 kDa), bovine serum albumin (BSA; 66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (cyt C; 13 kDa) were used as standards to calibrate the column. vo, void volume; vt, total volume.

failure to detect any activity associated with the recombinant APRT2 protein led us to conclude that either the *APT2* gene does not encode a functional enzyme or the recombinant APRT2 expressed in *E. coli* does not fold into a catalytically active conformation. The failure of APRT2 to dimerize when it was separated through a sizing column suggested that the lack of APRT2 activity is due, in part, to its failure to dimerize. In fact, all of the previously characterized APRTs (from mouse, hamster, human, *E. coli*, and *Arabidopsis* cells), with the exception of *Leishmania donovani* APRT (30), are functional as homodimers in solution (20). This failure to dimerize may also be due to improper folding of *E. coli*-expressed

APRT2. However, given the degree of sequence similarity between the two genes (1a), the latter explanation seems unlikely. In addition, high-level expression of *APT2* in the APRT-deficient Y847 strain showed no APRT activity (6b).

Analysis of APRT expression. Total RNA was isolated from various yeast strains by the acid-phenol method (13). For Northern blot analysis, poly(A)⁺ RNA (5 µg) from strain Y382 or X79 was separated by electrophoresis in a 18% formaldehyde–1.2% agarose gel (Seakem). The gel was run at 100 V for 4 h. The RNA was transferred by means of capillarity to a Zeta-probe membrane as described in the Bio-Rad manual. The membrane was hybridized to the radiolabeled *APT1* and/or *APT2* probe as described in the Zeta-probe manual. The membrane was exposed to X-ray film (Kodak) for 16 h and developed in an X-Omat machine (Kodak).

Western blot analyses were carried out with cell extracts from various yeast strains, prepared by disruption with glass beads. Samples were electrophoretically separated in a sodium dodecyl sulfate–12% polyacrylamide gel. The proteins were transferred to Trans-Blot transfer membranes by electrophoresis as described in the Bio-Rad manual. Membranes were blocked with 5% nonfat milk (to prevent nonspecific binding of the antibody), and the blots were separately probed with rabbit anti-APRT1 or rabbit anti-APRT2 polyclonal antibodies. For detection, a horseradish peroxidase-linked secondary antibody (Bio-Rad) was hybridized to the same membrane. Bands were visualized by chemiluminescence with an Immuno-Star kit (Bio-Rad). The membrane was exposed to X-ray film, and the film was developed in an X-Omat machine (Kodak).

We observed that both the *APT1* and *APT2* genes were actively transcribed in wild-type yeast cells. However, we failed to detect any APRT2 protein in Western blot experiments using extracts from wild-type yeast and polyclonal antibodies against the recombinant APRT2 protein (Fig. 3B). When similar experiments were carried out with anti-APRT1 antibodies, we could easily detect an APRT1 signal (Fig. 3A). As previously mentioned, the APRT2 protein could still be made but quickly degraded. Regardless, the failure to detect activity and/or the presence of a protein corresponding to APRT2 argues that the bulk of the APRT activity in vivo is provided by *APT1* gene expression. These data also suggest that the *APT2* gene product either lacks elements important for efficient translation or lacks elements important for proper protein folding and/or stability in vivo. However, overproduction of APRT2 in *E. coli* and yeast results in a stable nonactive protein (6a).

We have hypothesized (1) that the role of the APRT2 protein is to interact with APRT1, thus down-regulating its activity and/or specificity. High levels of purine nucleotides can lead to repression of the de novo purine biosynthetic pathway. We hypothesized that the APRT1-APRT2 interaction would be particularly important during conditions of starvation (i.e., depletion of free adenine), where APRT activity becomes less important as the cell is forced to synthesize purine bases de novo. This hypothesis predicts that the levels of APRT2 transcripts increase as cells deplete free adenine from the medium (i.e., as cells enter stationary phase). However, when Northern blot analysis was performed with radiolabeled DNA probes specific for *APT1* or *APT2*, we detected comparable levels of *APT1* and *APT2* transcription in wild-type cells regardless of the growth phase of the culture (Fig. 4). *APT2* mRNA migrates slightly more slowly than *APT1* mRNA. These experiments demonstrate that even if the *APT2* gene is actively transcribed, at least under the culture conditions described, it is not actively translated (Fig. 3).

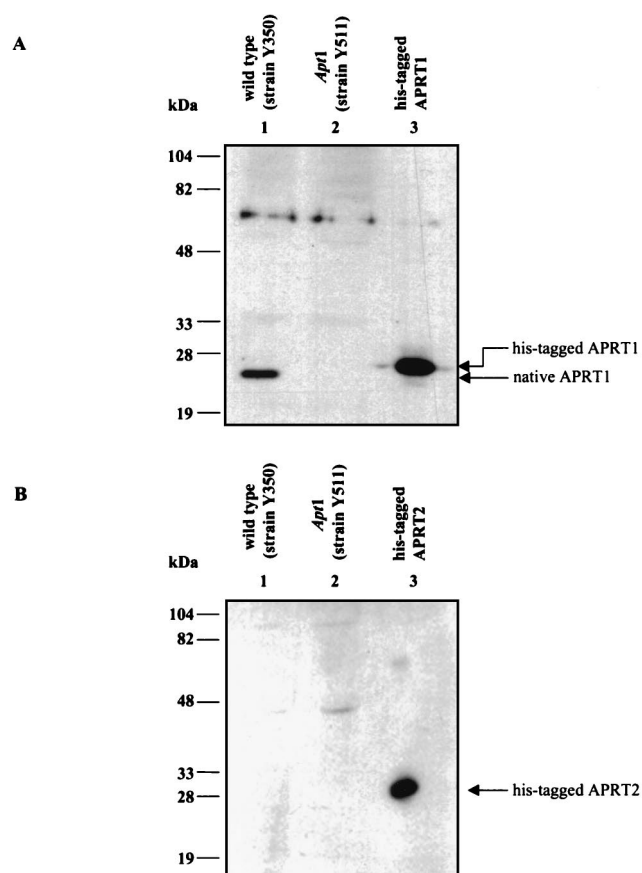


FIG. 3. Western blot analysis of native APRT proteins. (A) Western blot probed with polyclonal anti-APRT1 antibody. Lane 1 contains a cell extract from strain Y350 (wild-type strain), lane 2 contains a cell extract from strain Y511 (*apt1* mutant strain), and lane 3 contains pure recombinant APRT1. (B) Western blot similar to that shown in panel A but probed with polyclonal anti-APRT2 antibody. Lanes 1 and 2 contain cell extracts from strains Y350 and Y511 (respectively), and lane 3 contains pure recombinant APRT2.

Phylogenetic comparison analysis of the APRT1 and APRT2 proteins. Amino acid sequence alignments obtained from the SeqApp program were used in the PAUP program (version 3.11; Smithsonian) to determine the phylogenetic relationship between various APRTs. The analysis was performed by maximum parsimony analysis with the exhaustive-search method. Bacterial sequences were used as outgroups. The *S. cerevisiae* APRT1 (GenBank accession no. S49755) and APRT2 (GenBank accession no. L14434) sequences were used in the alignment, together with the homologous APRTs (GenBank accession numbers in parentheses) from *E. coli* (M14040), *Haemophilus influenzae* (U32748), *Drosophila melanogaster* (S34831), *D. pseudoobscura* AF025800), *Mus musculus* (M86440), *Homo sapiens* (p07741), *Caenorhabditis elegans* (U80438), and *Arabidopsis thaliana* APRT1 (p31166). The phylogenetic analysis was performed on the APRT sequence alignment previously described by us (1a) (Fig. 5). We wished to determine if *APT2* was the result of a duplication of *APT1* (or vice versa) or if *APT2* was more closely related to some other APRT. The phylogenetic analysis showed that *APT1* is most closely related to *APT2*.

Upon completion of the Yeast Genome Project, it was discovered that much of the genome was in fact duplicated. Fifty-five duplicate regions which cover approximately 50% of the *S.*

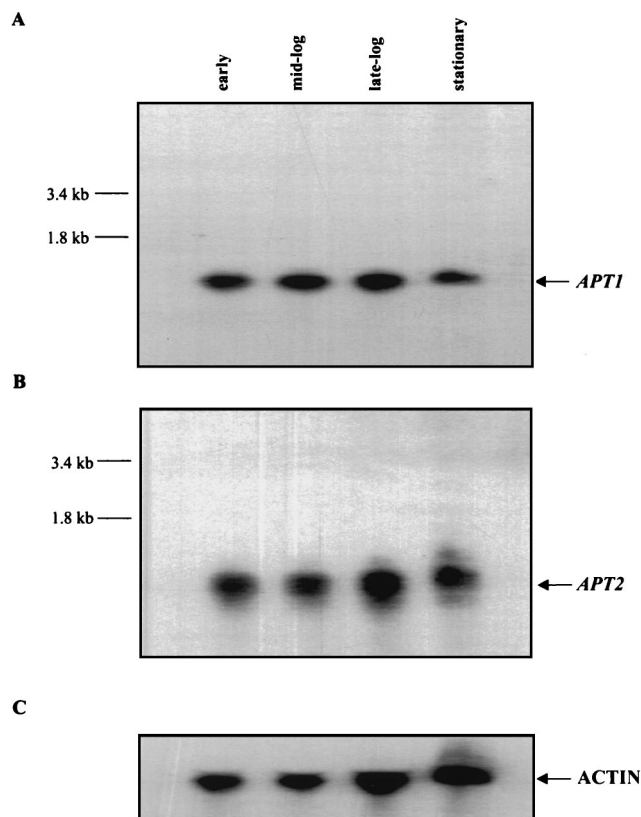


FIG. 4. Northern blot analysis of the *APT1* and *APT2* gene products from strain X79 (wild type). (A) Total RNA was extracted at various times during growth and probed with a radiolabeled *APT1* probe. (B) Same membrane as that shown in panel A, probed with a radiolabeled *APT2* probe. (C) Same membrane as that shown in panel A, probed with a radiolabeled yeast actin used as a loading control.

cerevisiae genome have been found (10). It has been proposed that the entire yeast genome was duplicated and that subsequently many of the duplicated regions were lost (31). *APT1* and *APT2* are both found in duplicated regions of each other: *APT1* on chromosome XIII and *APT2* on chromosome IV (31). Southern blot analysis was used to determine if this *APT* duplication event was found only in *S. cerevisiae*. The closely related yeast *Picchia pastoris* was examined for the presence of two *APTs*. With *APT1* and *APT2* probes, it appears that *P. pastoris* also contains two *APTs* (data not shown).

Taken together, the complementation and the expression data lead us to conclude that *APT1* is by itself sufficient to code for APRT in *S. cerevisiae*. Furthermore, from the phylogenetic comparative analysis, we propose that the *APT2* gene is a pseudogene, perhaps derived from an aberrant gene duplication of the *APT1* gene. The occurrence of pseudogenes in yeast, although rare, is not unprecedented. It is possible that *APT2* codes for an APRT homolog that has diverged to the point of possessing a different function. However, our failure to detect APRT2 protein in various yeast strains makes this possibility unlikely and makes our original proposal of two forms of APRT in yeast untenable.

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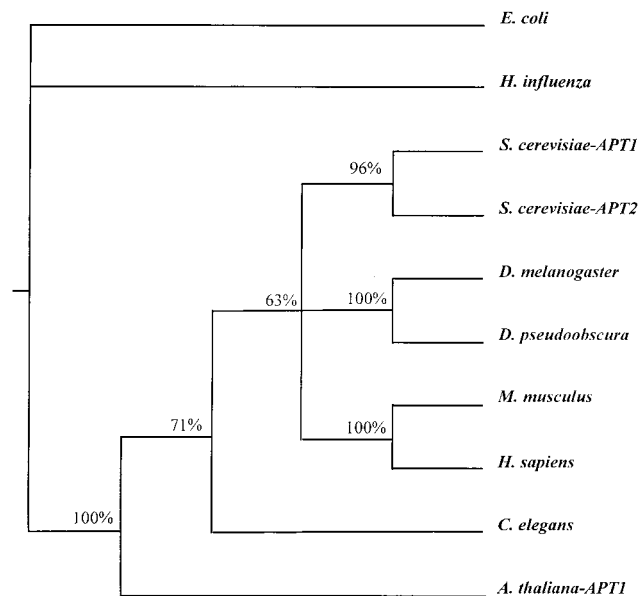


FIG. 5. Comparative phylogenetic reconstruction of APRT1 and APRT2. A phylogenetic tree of various deduced APRT sequences constructed with PAUP version 3.11 is shown. Numbers indicate the probability of two sequences branching together.

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