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The Gene for a Major Exopolyphosphatase of *Saccharomyces cerevisiae*

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The gene encoding a major exopolyphosphatase (scPPX1) in *Saccharomyces cerevisiae* (H. Wurst and A. Kornberg, J. Biol. Chem. 269:10996–11001, 1994) has been isolated from a genomic library. The gene, located at 57 kbp from the end of the right arm of chromosome VIII, encodes a protein of 396 amino acids. Overexpression in *Escherichia coli* allowed the ready purification of a recombinant form of the enzyme. Disruption of the gene did not affect the growth rate of *S. cerevisiae*. Lysates from the mutants displayed considerably lower exopolyphosphatase activity than the wild type. The enzyme is located in the cytosol, whereas the vast accumulation of polyphosphate (polyP) of the yeast is in the vacuole. Disruption of *PPX1* in strains with and without deficiencies in vacuolar proteases allowed the identification of exopolyphosphatase activity in the vacuole. This residual activity was strongly reduced in the absence of vacuolar proteases, indicating a dependence on proteolytic activation. A 50-fold-lower protease-independent activity could be distinguished from this protease-dependent activity by different patterns of expression during growth and activation by arginine. With regard to the levels of polyP in various mutants, those deficient in vacuolar ATPase retain less than 1% of the cellular polyP, a loss that is not offset by additional mutations that eliminate the cytosolic exopolyphosphatase and the vacuolar polyphosphatases dependent on vacuolar protease processing.

Inorganic polyphosphates (polyP) are widespread in nature and are particularly abundant in *Saccharomyces cerevisiae*, accounting for nearly 40% of the total phosphate content (22). The polyP chains of *S. cerevisiae* vary between three and several thousand P_i residues (19). Among the various yeast cell compartments, the bulk is in the vacuoles (38), with lesser amounts and smaller chains found in mitochondria (4); in *Saccharomyces fragilis*, polyP has also been found on the cell surface (37). The distinct locations and sizes of polyP in yeast cells indicate either different pathways of biosynthesis and degradation or a sophisticated sorting system. These different polyP pools might be involved in a variety of functions, such as energy storage, P_i reservoir, metal chelator, buffer against alkali, and regulator. None of these functions for polyP have been established in *S. cerevisiae*, whereas in *Escherichia coli*, a requirement for polyP for survival in stationary phase has been demonstrated (8).

Polyphosphatase (polyPase) activity in *S. cerevisiae* is demonstrably high: a cell extract releases up to 130 nmol of P_i per min per mg of yeast protein (42). In contrast, biosynthetic activity is rather feeble in vitro. Polyphosphate kinase, the enzyme that can make polyP in a reversible reaction by condensing the terminal phosphate of ATP, catalyzes the transfer of only about 4 nmol of phosphate residues from polyP to ADP per min per mg of yeast protein and is even less active in the synthesis of polyP (12, 34). In view of the high level of polyPase activity, effective mechanisms must exist to protect polyP from hydrolytic degradation. Also implied are functions for the polyPase in using polyP as a reservoir for P_i.

Initially, a polyPase activity in *S. cerevisiae* was detected and separated from inorganic pyrophosphatase activity (11). Subsequently, it became evident that *S. cerevisiae* contains several polyPase activities, distinguished by compartmentation and biochemical properties. PolyPases from the cell envelope (3) and from the vacuole (2, 39) were identified. Recently we purified from *S. cerevisiae* an exopolyphosphatase (exopolyPase), named scPPX1 (42), which has a very high turnover number (500 phosphate residues per s at 37°C) and the capacity to degrade a range of chains from three to several hundreds of residues in a highly processive manner. The objective of the present study was to identify the gene for scPPX1 to serve several purposes: (i) inactivation of the gene to help assess the physiological importance of the enzyme and the identity of residual polyPase activities and (ii) overexpression of the gene and overproduction of its product to simplify the purification and to provide adequate quantities of the enzyme to serve as a much-needed reagent for the quantitative analysis of polyP.

MATERIALS AND METHODS

Materials. *S. cerevisiae* strains are derived from W303-1A (R. Rothstein, Columbia University, New York, N.Y.) (Table 1). A genomic library of *S. cerevisiae* in lambda phage (25) was obtained from Sandy Ramer (Stanford University), as was *E. coli* BNN132 (10). A nylon filter containing the DNA of an ordered genomic library of *S. cerevisiae* (27), distributed by the American Type Culture Collection, was provided by Stanley Nelson (Stanford University). Clones 3427 and 9202 of this library were purchased from the American Type Culture Collection. Plasmid pBluescript II KS+ was from Stratagene, La Jolla, Calif., and pTrcHisB was from Invitrogen, San Diego, Calif. A source of *LEU2* was YCp.lac.111 (15), obtained from Kevin Redding (University of Geneva, Geneva, Switzerland). A plasmid containing *vma4*, disrupted with *URA3* (13), was obtained from Françoise Foury (University of Louvain, Louvain, Belgium). *S. cerevisiae* chromosomes in agarose plugs were kindly provided by Christine Alfano (Stanford University); oligodeoxyribonucleotides were prepared in the PAN facility at Stanford University. Restriction and DNA modification enzymes were products from Boehringer Mannheim or New England Biolabs. The digoxigenin (DIG)-based nucleic acid detection system was from Boehringer Mannheim. [^{32}P]polyP, a substrate for polyPase activity, was synthesized as described previously (1) with a chain length near 750 residues and called $polyP_{750}$. Concentrations of polyP are given in terms of phosphate residues.

Isolation of *PPX1*. scPPX1 was purified as described previously (42), and its N-terminal amino acid sequence was determined by the PAN facility with a peptide sequencer from Applied Biosystems (Foster City, Calif.). Approximately 10^5 lambda phages containing a library of the *S. cerevisiae* genome (25) on a lawn of *E. coli* JM101 (43) were screened by hybridization with an oligonucleotide (5′-CCATTGAGAAAGACTGTTCCAGAATTTTTGGCTCATTTGTCTT CTTTGCCAAT-3′) at 49°C in a solution containing 830 mM Na^+; stringent washes were performed two times for 3 min each at 43°C in the presence of 19 mM Na^+. Conversion of isolated lambda phages to pYES-R-derived plasmids by

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TABLE 1. *S. cerevisiae* strains used

Strain[a]	Genotype
CRY	*MAT***a** *his3 ura3 leu2 trp1 ade2*
CRX	*MAT***a** *his3 ura3 ppx1*::*LEU2 trp1 ade2*
CB023	*MAT***a** *pep4*::*HIS3 prb1*::*hisG prc1*Δ::*hisG ura3 leu2 trp1 ade2*
CBX	*MAT***a** *pep4*::*HIS3 prb1*::*hisG prc1*Δ::*hisG ura3 ppx1*::*LEU2 trp1 ade2*
CRY-V4	*MAT***a** *his3 vma4*::*URA3 leu2 trp1 ade2*
CBX-V4	*MAT***a** *pep4*::*HIS3 prb1*::*hisG prc1*Δ::*hisG vma4*::*URA3 ppx1*::*LEU2 trp1 ade2*

[a] Strains CRY and CB023 were furnished by R. S. Fuller and C. Brenner (Stanford University); the other strains were prepared in this study.

using the *cre-lox* system was as described previously (25). To determine the exopolyPase activity in *E. coli*, cultures in exponential growth phase were induced with 4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 60 min at 37°C. Protein extracts were prepared by lysozyme lysis, and exopolyPase activity was determined as described elsewhere (42).

Growth of *S. cerevisiae* and *E. coli*; DNA subcloning and sequencing. Growth of lambda phage and general manipulations of DNA were as described previously (32). Nucleic acids were labeled with DIG, hybridized, and detected with protocols from Boehringer Mannheim.

For sequencing of *PPX1*, two fragments were subcloned in both directions into pBluescript II KS+. One fragment, a 900-bp *Hin*dIII fragment adjacent to the 500-bp *Hin*dIII fragment, was subcloned from clone V (Fig. 1A); a 2,000-bp *Xho*I fragment from clone I (Fig. 1A) was subcloned by using an *Xho*I site that flanked the cloned fragment and an interior site. DNA sequencing reactions were performed at the PAN facility. The fragments were sequenced with a primer-walking strategy. Individual sequences were merged and edited with software from Intelligenetics (Mountain View, Calif.). Data bank searches were also performed with software from Intelligenetics.

S. cerevisiae was transformed and grown as described previously (29, 33). The strains were generated by the one-step gene disruption method (30). The constructs were controlled by Southern blotting.

S. cerevisiae chromosomes were fractionated by pulsed-field gel electrophoresis with a Bio-Rad CHEF-Mapper, using an algorithm for resolution between 550 and 850 kbp. The DNA was blotted on a nylon filter and hybridized, first with the 500-bp *Hin*dIII fragment as a DIG-labeled probe and then with total *S. cerevisiae* DNA as a probe.

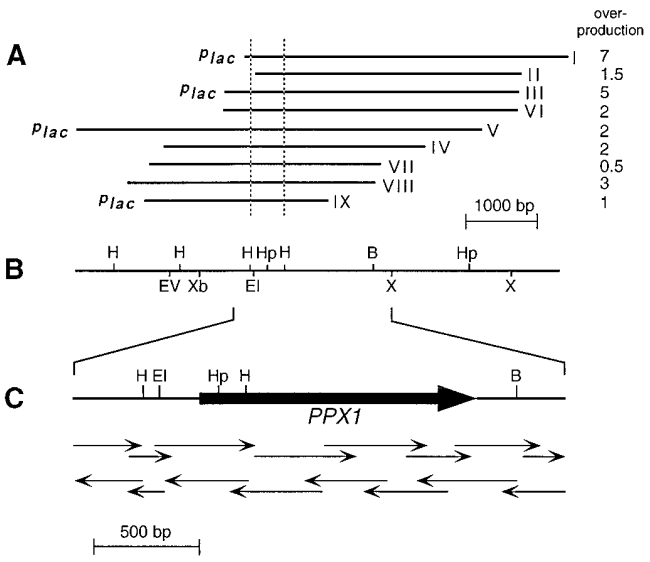

FIG. 1. Restriction map and strategy for sequencing of *PPX1*. (A) Alignment of isolated fragments (roman numbers). Overproduction for each clone is given in multiples of the exopolyPase activity of *E. coli* without the plasmid. p_{lac} denotes the presence of a *lac* promoter to the left of the fragment, directed toward the insert, and provided by the plasmid vector. The dotted vertical lines indicate the 500-bp *Hin*dIII fragment. (B) Restriction map of the isolated region. Restriction sites: B, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; Hp, *Hpa*I; X, *Xho*I; Xb, *Xba*I. (C) Sequenced portion of the isolated region. The open reading frame corresponding to *PPX1* is represented by the thick arrow. Individual sequencing reactions are represented by thin arrows.

PPX1 was amplified by PCR in 0.1 ml of a mixture containing 20 mM Tris-HCl (pH 8.8 at 25°C), 20 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg of bovine serum albumin (BSA) per ml, 0.32 mM (each) dATP, dGTP, dCTP, and dTTP, and 20 U of Vent DNA polymerase per ml (all from New England Biolabs); 250 ng of single-stranded pBSPX (pBluescript II KS+, containing the 2,000-bp *Xho*I fragment) per ml; and 0.22 nmol each of primer 1 (CGCGGATCCGATGTCGCCTTTGAGAAAGA) and primer 2 (AAGGGAA CAAAAGCTGGGTACCGG), which binds to the plasmid vector, per ml during 30 cycles (30 s at 94°C, 30 s at 52°C, and 60 s at 72°C) under a layer of mineral oil. After amplification, the sample was extracted with chloroform-isoamyl alcohol (24:1), with phenol (pH 8), and again with chloroform-isoamylalcohol (24:1) and was precipitated with ethanol. The PCR product was digested with *Bam*HI and subcloned in the *Bam*HI site of pTrcHisB. The orientation of *PPX1* in pTrcHisB was determined by restriction enzyme analysis.

For the deletion and insertion of *LEU2* in *PPX1*, *LEU2* was prepared as a 1,724-bp *Aat*II-*Pvu*I fragment from YCp.lac.111. The pYES-R clone containing fragment IV (Fig. 1A) was digested with *Hin*dIII. Both samples were treated with T4 DNA polymerase to generate blunt ends. Appropriate fragments were purified by agarose gel electrophoresis, ligated, and transformed in *E. coli* DH5α (16). The resulting colonies were scraped from the agar plate, and the plasmid DNA of all colonies was prepared in one batch. This preparation was introduced into *S. cerevisiae* CB023, which was then grown on medium lacking leucine to select for pYES-R-derived plasmids containing the *LEU2* gene. The DNA of leucine auxotrophic yeast clones was isolated and used to transform *E. coli* JM107 (43). Purified plasmid DNA corresponding to the desired construction was treated with *Xho*I. The resulting fragment containing the *LEU2* gene and the truncated *PPX1* gene was purified by agarose gel electrophoresis. This fragment was then introduced into *S. cerevisiae* CRY1 (hereafter referred to as CRY) and CB023.

Preparation of protein and polyP extracts from *S. cerevisiae*. *S. cerevisiae* was aerobically grown at 30°C in SDC medium (29), containing 2% glucose, 0.5% $(NH_4)_2SO_4$, 7.35 mM phosphate, trace elements, salts, and vitamins. At stationary phase, no more than 15% of the phosphate supply was taken up. Growth was monitored at A_{600} in a Beckman DU640 spectrophotometer, using dilutions resulting in an A_{600} of <0.3. After growth, cells were collected by centrifugation, washed with H_2O, and stored in liquid N_2. Protein of *S. cerevisiae* was measured with the bicinchoninic acid reaction (35) with reagents from Sigma, using BSA as a standard.

For protein extracts, cells were suspended in a microcentrifuge tube at an A_{600} of 100 in dilution buffer (50 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM EDTA); then 20 mg of glass beads (0.425- to 0.6-mm diameter; Sigma) per A_{600} unit was added. Lysis was obtained by vortexing the suspension 10 times for 15 s each, with intermittent cooling on ice. The suspension, without glass beads, was transferred to another microcentrifuge tube and centrifuged for 10 min at 16,000 × *g* at 4°C. The supernatant was used as a source for soluble proteins.

For the preparation of polyP from *S. cerevisiae*, cells were grown in SDC medium including 5 μCi of [^{32}P]phosphate per ml. Cell pellets were extracted on ice with 2% trichloroacetic acid at an A_{600} of 100. The extract was centrifuged for 2 min at 16,000 × *g*, and the supernatant was removed. The pellet was resuspended in a volume corresponding to an A_{600} of 40 in 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.2)–1 M urea–10 mM EDTA–0.1% sodium dodecyl sulfate (SDS). A sample was removed for protein determination; the rest was extracted with phenol-chloroform-isoamyl alcohol (25:24:1). The phases were separated by centrifugation for 2 min at 16,000 × *g*, and the organic phase was reextracted with 0.2 volume of H_2O. After centrifugation, the aqueous phases were pooled and reextracted with equal volumes of phenol, chloroform, and isoamyl alcohol. Finally, the aqueous phase was extracted with phenol-chloroform-isoamyl alcohol (25:24:1). PolyP in the aqueous phase was precipitated by addition of 0.1 volume of a solution containing 1 M barium acetate (equilibrated to pH 4.5 with acetic acid) and incubation for 60 min on ice. The suspension was centrifuged for 30 min at 16,000 × *g* at 4°C, and the pellet was washed with H_2O. A small volume (corresponding to 2.5 μl per A_{600} unit) of TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) and an equal volume of AG 50W-X8 ion-exchanger beads (Bio-Rad) were added to the pellet. After incubation for 14 h at 4°C, the solution was recovered from the beads and the beads were washed with 0.5 volume of TE. The solutions were pooled; less than 10% of the radioactivity remained with the ion-exchanger beads.

Vacuoles were prepared as described previously (28) and frozen in liquid nitrogen. Immediately before use, the vesicles were solubilized by adding 10% Triton X-100 to a final concentration of 0.1%.

ExopolyPase assays and analysis of polyP. Unless otherwise noted, exopolyPase activity was measured as described previously (42). One unit corresponds to the release of 1 pmol of P_i per min at 37°C. The influence of arginine on exopolyPase in vacuolar extracts was measured in 20 mM morpholineethanesulfonic acid (MES)-KOH (pH 5.5)–5 mM $MgCl_2$–0.2 mM $polyP_{750}$–35 or 110 μg of vacuolar protein per ml for 15 min at 37°C.

PolyP levels were determined from the amount of P_i released upon treatment with an excess of recombinant PPX1 (rPPX1). To this end, 2 to 5 μl of polyP extracts (up to 160 nmol) was diluted in 20 μl containing 20 mM Tris-HCl (pH

7.5), 100 mM NH_4 acetate, 4 mM $MgCl_2$, and 15,000 to 40,000 U of rPPX1. Extracts of vacuolar ATPase-deficient mutants were treated in a volume of 10 μl. After 10 and 20 min, aliquots of the reactions were analyzed by thin-layer chromatography (42). By 10 min, 75 to 96% of the radioactivity was degraded. No further degradation could be observed after 20 min. In vacuolar ATPase-deficient mutants, only 0.4 to 13% of the ^{32}P could be released as P_i by rPPX1. To determine P_i levels in untreated samples, the enzyme was omitted from the incubations.

For the determination of average chain length, polyP was degraded with rPPX1 and fractionated together with an untreated sample on a 2 or 8% polyacrylamide gel (7). The radioactivity pattern along the lane of the rPPX1-treated sample was digitized with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) and subtracted from the digitized pattern of the untreated sample. The average chain length was determined as the midpoint of radioactivity in the lane from the mobility of polyP of known chain lengths (7), determined in a separate lane, visualized by toluidine blue staining.

Purification of rPPX1. *E. coli* BL21 (36) containing pTrcPPX1 was grown to an A_{600} of 0.64 in 9 liters of LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl [pH 7.5]) including 100 mg of ampicillin per liter. Production of rPPX1 was induced by adding IPTG (0.5 mM final concentration). After incubation for a further 6 h, the cells were harvested by centrifugation (25 min at 4,500 × *g*). The wet cells (15.5 g) were resuspended in 62 ml of lysis buffer (20 mM Tris-HCl [pH 7.5], 2 mM EDTA), frozen in liquid N_2, and stored at −80°C. Protein concentrations during the purification were determined by the Bradford assay (Bio-Rad), with BSA as a standard.

All purification steps were carried out at 0 to 4°C. Cells (14.8 g) were lysed (21) and centrifuged (60 min, 44,000 × *g*); the clear supernatant was dialyzed against buffer A (10 mM HEPES-KOH [pH 7.6], 5 mM $MgCl_2$, 0.05 mM EDTA, 2 mM β-mercaptoethanol, 10% glycerol) including 100 mM NaCl. The dialyzed solution (fraction I, 75 ml) was applied to a column (2.5 by 6 cm) of Ni-nitrilotriacetic acid agarose (Qiagen, Chatsworth, Calif.) which had been equilibrated with buffer A containing 100 mM NaCl. The column was washed with 225 ml of buffer A containing 500 mM NaCl and reequilibrated in buffer A containing 100 mM NaCl. Proteins were eluted with a linear imidazole gradient (0 to 90 mM, 300 ml) in buffer A containing 100 mM NaCl. Fractions containing exopolyPase activity were pooled (fraction II, 90 ml) and dialyzed against buffer A containing 10 mM KCl. Dialyzed fraction II was applied in five portions to a Mono Q column (5 mg of protein per ml of Mono Q) that was equilibrated in buffer A containing 10 mM KCl. Before elution, the column was washed with buffer A containing 10 mM KCl. Proteins were eluted with a linear KCl gradient (10 to 500 mM KCl, 15 column volumes) in buffer A. Fractions containing exopolyPase activity were pooled (fraction III, 8.4 ml) and applied to a hydroxylapatite column (1.5 by 9 cm) equilibrated with buffer B (20 mM Tris-HCl [pH 7.5], 200 mM KCl, 2 mM β-mercaptoethanol, 10% glycerol). The column was washed with 70 ml of buffer B. Protein was eluted with a linear potassium phosphate gradient (pH 7.4, 120 ml, 0 to 200 mM phosphate) in buffer B. Fractions 26 to 32 were pooled and stored at −80°C (fraction IV, 9.8 ml).

Nucleotide sequence accession number. The DNA sequence of *PPX1* was deposited in the GenBank database under accession number L28711.

RESULTS

Isolation of the gene. The sequence of 20 N-terminal amino acids (SPLRKTVPQFLAHLSSLPIS) of scPPX1 (42) was the basis for the design and synthesis of a 53-mer degenerate deoxyoligonucleotide to screen a genomic library of *S. cerevisiae* (25). Nine independent clones yielded a positive signal upon hybridization with the oligonucleotide (Fig. 1A); the cloned fragments were converted into multicopy plasmids by using the *cre-lox* system in *E. coli* BNN132 (10). All isolated inserts represent the same region. They were flanked by *Xho*I sites originally used to clone the fragments (25) and by *Eco*RI sites provided by the vector. In the restriction map of this region (about 7 kbp) (Fig. 1B), all inserts share at least part of a 500-bp *Hin*dIII fragment. This fragment was also recognized by the 53-mer oligonucleotide in a Southern blot of *Hin*dIII-digested *S. cerevisiae* DNA (data not shown). Therefore, the 5′ end of the open reading frame encoding scPPX1 is located on this 500-bp *Hin*dIII fragment.

PolyPase activity (up to five- to sevenfold higher than wild-type *E. coli* activity) was induced by plasmids with the 500-bp *Hin*dIII fragment with an extension to the right and a location immediately downstream of a *lac* promoter provided by the plasmid vector (Fig. 1A). In contrast, plasmids with extensions to the other side of this *Hin*dIII fragment induced less additional polyPase activity. Therefore, the direction of *PPX1* in Fig. 1 is to the right starting within the 500-bp *Hin*dIII fragment.

DNA sequence of *PPX1*. When the DNA sequence of 2,304 bp in a region around the 500-bp *Hin*dIII fragment was determined (Fig. 2), a stretch corresponding to the N-terminal amino acid sequence of scPPX1 was found on the 500-bp *Hin*dIII fragment followed by a long open reading frame. Between the first codon and the stop codon, the DNA sequence encodes a protein of 396 amino acids and a molecular mass of 44.942 kDa; this number compares with a value of 40 kDa determined by gel electrophoresis (42). Immediately preceding the N-terminal serine codon is an ATG start codon and a sequence favorable for efficient initiation of translation (17). Three boxes with similarities to the consensus sequence of yeast TATA elements (TATAAA [6]) are at positions 52, 200, and 472. These sequences, however, showed rather little transcriptional activity in vitro (40) and in vivo (6). The one at position 472 seems the most likely to function in initiation of transcription. It is in close proximity to an AT-rich region from bp 487 to 529. This region consists of a T-rich region of 21 bp followed by a A-rich region of 22 bp, a motif that indicates strong promoters with a major transcription start site 8 bp into the A-rich region (24). This potential start site at bp 515 is 37 bp downstream of the TATA element at bp 472. Between the A-rich region and the putative translation start codon are 70 nucleotides, of which 49% are A residues. Following the open reading frame of *PPX1* are TATATA sequences at bp 1804 and 1824 (Fig. 2, underlined), signals known to induce termination of transcription (31).

The predicted protein sequence of scPPX1 did not yield significant homologies with any amino acid sequence in the PIR database (version 40). Comparison of the DNA sequence with the GenBank database (version 78), however, matched with 96% identity a sequence (accession number Z13105) from a cDNA library derived from a human cell line. This library, as stated in the annotations, was contaminated with sequences of fungal and bacterial origin, and thus the sequence is likely to be such a contamination.

Location of *PPX1* in the genome. Hybridization of *PPX1* to chromosomes after separation by pulsed-field gel electrophoresis and blotting onto a nylon membrane identified a band consisting of chromosomes V and VIII (Fig. 3A), which are similar in size (610 and 555 kbp, respectively [14]). With an ordered genomic library of a strain derived from S288C (27) (distributed by the American Type Culture Collection), aligned by using the restriction fragment pattern of double digests with *Eco*RI and *Hin*dIII, the only hybridized clone (clone 3427 [Fig. 3B]) maps on chromosome VIII, 42 to 57 kbp from the distal end of the right arm.

The restriction fragment pattern of this region matched that of *PPX1* only if the fragments of 1,999 and 976 bp were interchanged (Fig. 4). In this corrected map, *PPX1* is located 57 kbp from the right end of chromosome VIII; its expression is directed toward the centromere.

Overexpression of *PPX1* and purification of the recombinant enzyme. For overexpression of *PPX1*, the gene was amplified by PCR using a primer binding at the start codon (at bp 599 to 618 [Fig. 2]) and introducing a *Bam*HI site. The resulting product was digested with *Bam*HI to generate a 1,484-bp fragment extending from bp 599 to 2082 on the DNA sequence, with a *Bam*HI site at the 5′ end of the open reading frame of *PPX1*. This *Bam*HI fragment was subcloned into the *Bam*HI site of expression vector pTrcHisB. In the resulting plasmid, called pTrcPPX1 (Fig. 5), *PPX1* is under control of the *trc* promoter (consisting of parts of the *lac* and *trp* promoters) and the *lac* operator. Translation start signals are also provided by

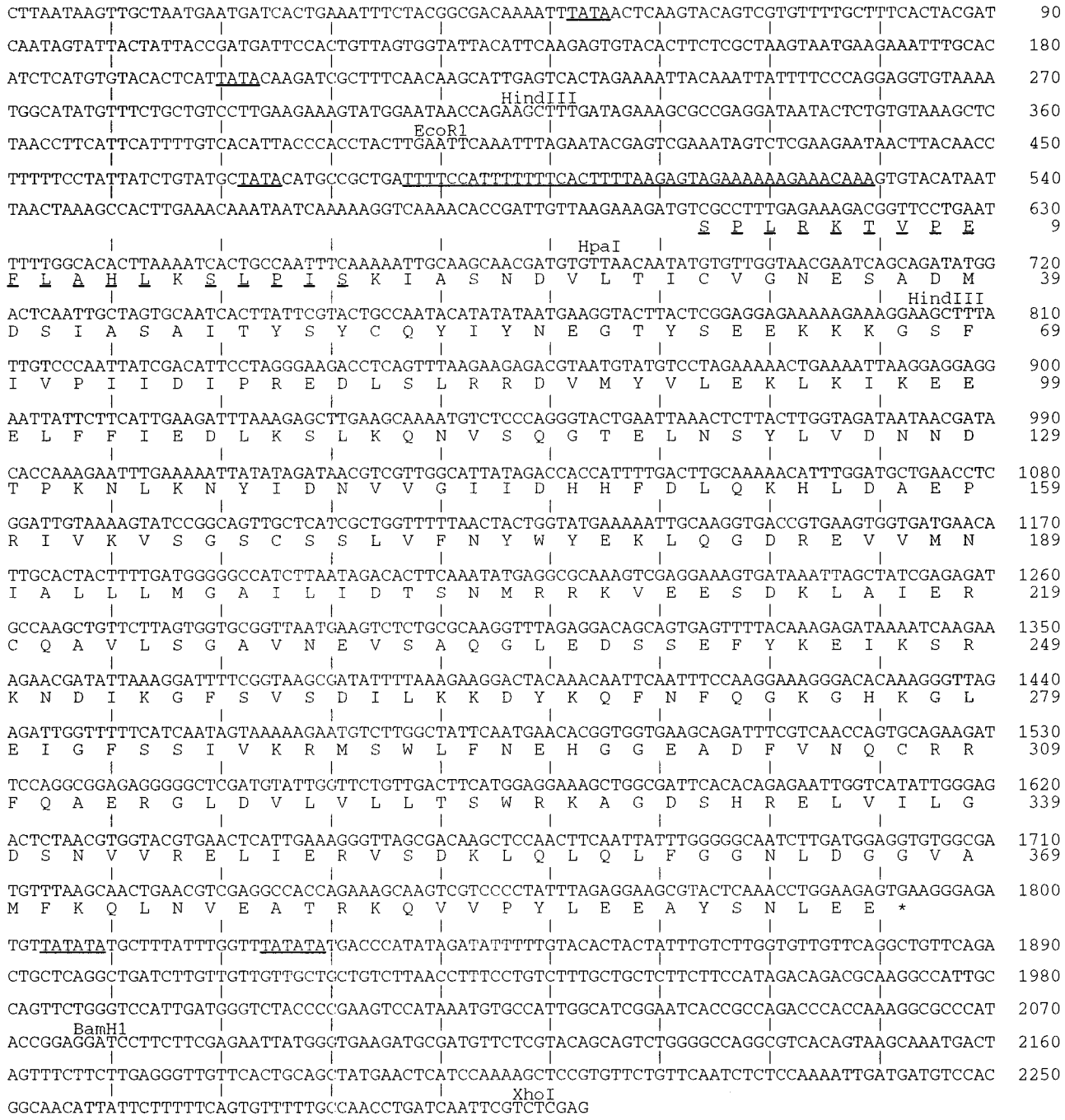

FIG. 2. DNA sequence of *PPX1*. Underlined are the TATA elements, AT-rich regions, transcription termination signals (TATATA), and N-terminal amino acids determined by protein sequencing.

the vector. The 5′ end of the open reading frame of *PPX1* is linked to a fusion sequence including the codes for six consecutive histidine residues. The high affinity of an array of histidines for Ni^{2+} has been exploited for affinity chromatography of histidine-tagged proteins (20). The fusion sequence also contains a signal for restriction protease cleavage by enterokinase (23) close to the fusion site. The resulting plasmid allowed an IPTG-inducible production of recombinant polyPase activity in *E. coli* about 40-fold higher than production in *S. cerevisiae* cells. The recombinant fusion protein was called rPPX1 to distinguish it from the wild-type scPPX1. *PPX1* was also amplified directly from genomic DNA of CB023, the source of scPPX1, and cloned into the *Bam*HI site of pTrcHisB. This construction induced overproduction of polyPase activity similar to the level produced by pTrcPPX1 (data not shown).

Purification of rPPX1 from *E. coli* (Table 2) involved affinity chromatography with nickel-agarose which made use of the stretch of histidines at the N terminus of rPPX1. This step was followed by ion-exchange chromatography, mainly to concentrate the sample. As a final step, chromatography on hydroxylapatite yielded a homogeneous protein, as judged by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 6), with a

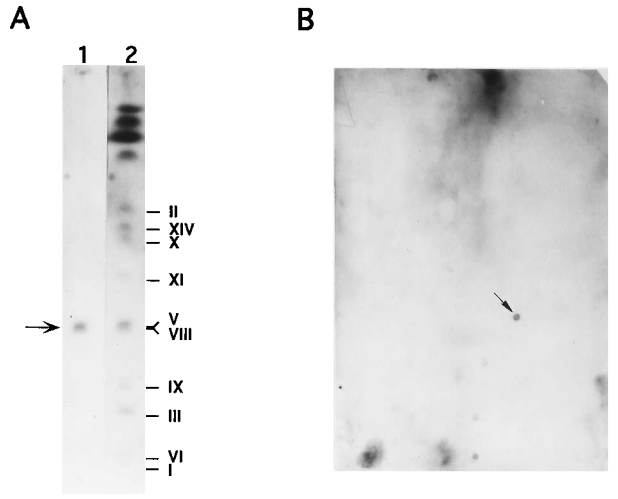

FIG. 3. Chromosomal location of *PPX1*. (A) Hybridization of *PPX1* to *S. cerevisiae* chromosomes immobilized on a nylon membrane after pulsed-field gel electrophoresis. Lane 1, hybridization with a 2,000-bp *Xho*I fragment from clone I; lane 2, hybridization with total *S. cerevisiae* DNA as a probe. Identified chromosomes are indicated on the right (roman numbers). Chromosomes larger than chromosome II were not resolved. The band hybridized by the *PPX1* probe is indicated by the arrow. (B) Hybridization of the 500-bp *Hin*dIII fragment to a set of ordered lambda clones covering the *S. cerevisiae* genome. The clone hybridized by this probe is indicated by the arrow. The weak hybridization signals displayed by several clones with a large amount of DNA can be attributed to incomplete removal of the probe as observed when total yeast DNA is used.

25-fold increase in specific activity compared with the lysate and a yield of 1.4 mg of rPPX1 per liter of growth medium. The mobility of 46 kDa for rPPX1 compared with 43.5 kDa for wild-type scPPX1 is due to the 3,787 Da contributed by the N-terminal fusion peptide. The specific activity of purified rPPX1 was about 10-fold lower than that of scPPX1, possibly indicating a perturbation of the native enzyme structure by the fusion. Despite the presence of a cleavage site for enterokinase, the fusion sequence was not removed by the enzyme; even with a 1,000-fold-larger amount of enterokinase as recommended by the supplier (Biozyme, San Diego, Calif.), only slight cleavage of the fusion peptide was observed without any increase in polyPase activity (data not shown). Presumably, the cleavage site for enterokinase is inaccessible in this particular peptide.

Disruption of the gene. To inactivate *PPX1* in CB023 (lacking genes for three vacuolar proteases) and the parental CRY strain, a *LEU2* marker gene was inserted in *PPX1* replacing the 500-bp *Hin*dIII fragment (Fig. 7A). The promoter of *PPX1* and the codons for 69 amino-terminal residues of scPPX1 (17% of the protein) were replaced by this construct, which was introduced to replace wild-type *PPX1*. The resulting strains are called CRX (derived from CRY) and CBX (derived from CB023). Inactivation of *PPX1* did not significantly alter the growth pattern of the strains in rich medium (Fig. 7B), nor was *PPX1* required for growth in a medium in which polyP was the source of P_i (data not shown). Also, no difference in growth was noted when the strains were shifted from a medium rich in phosphate to a medium without phosphate (data not shown).

The *PPX1*-deficient strains were clearly distinguishable in exopolyPase activity (Fig. 7C), an activity most prominent in early logarithmic growth. The maximal level of CB023 was about 60% of that of the parental CRY (data not shown). The CBX strain, lacking both the vacuolar proteases and *PPX1*, has a very low residual activity, less than 1% of that of the wild type; on the other hand, CRX, with active proteases but lacking *PPX1*, had an activity near 50% of the wild-type level. Inasmuch as large portions of *PPX1* were deleted in CRX and CBX, no residual activity of scPPX1 is expected in these strains. Thus, the measured activities were produced by other exopolyPases: the one in CRX, dependent on proteolytic activation, is designated scPPX2; the small amount of residual activity is designated scPPX3 (Table 3).

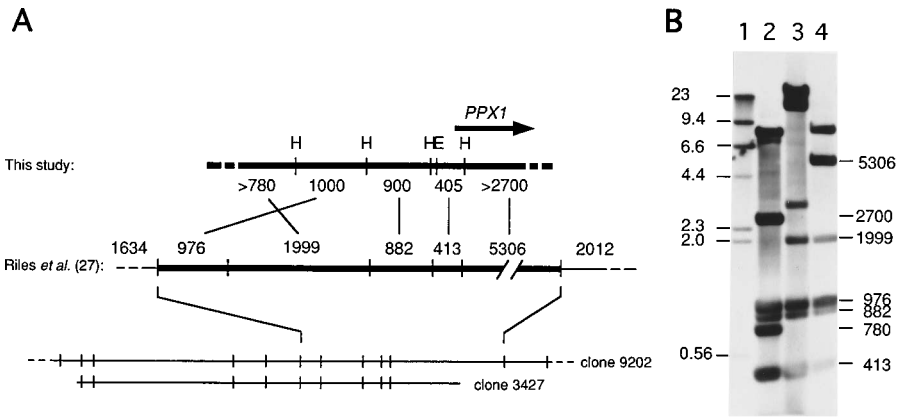

FIG. 4. Chromosomal location of *PPX1*. Two overlapping clones from the ordered library (27) that map in the questionable region were compared with a plasmid containing PPX1. (A) *Eco*RI-*Hin*dIII restriction fragments observed in this study (top) are aligned with published values (27) for a corresponding region on chromosome VIII (bottom). This region was covered by clones 9202 and 3427 (27). Numbers indicate lengths in base pairs. Fragments detected by the *PPX1* probe in panel B are drawn in bold lines. The arrow indicates the open reading frame of *PPX1*. Vertical and diagonal lines connect identical fragments. (B) Southern blot with clone V as a probe. Lane 1, molecular weight marker (DIG-labeled, *Hin*dIII-digested lambda DNA); lane 2, clone V, *Eco*RI-*Hin*dIII digested; lane 3, clone 3427, *Eco*RI-*Hin*dIII digested; lane 4, clone 9202, *Eco*RI-*Hin*dIII digested. The numbers on the left are fragment lengths (in kilobase pairs) of the marker in lane 1; numbers on the right indicate lengths (in base pairs) of fragments detected by the probe. The pattern (B) shows fragments with identical mobilities for the three clones that correspond to sizes of 413, 882, and 976 bp in the published map. This result confirms that *PPX1* is located in this region. In the clones from the ordered library (lanes 3 and 4), a fragment corresponding to 1,999 bp can also be identified, in contrast to the *PPX1* clone (lane 2). Thus, the 1,999-bp fragment is not located between the 882- and 976-bp fragments. On the other hand, the 1,999-bp fragment shares sequences with clone V, since it was detected by the probe. Apparently, the 1,999-bp fragment is on clone V in a truncated form, 780 bp long, adjacent to the 882- and 976-bp fragments. The 5,306-bp fragment, detected in clone 9202 (lane 4), is seen in clone 3427 (lane 3) in a truncated, 3,000-bp form and in clone V as an even shorter 2,700-bp form (lane 2). Thus, the order of the 1,999- and 976-bp fragments in the published restriction map should be reversed.

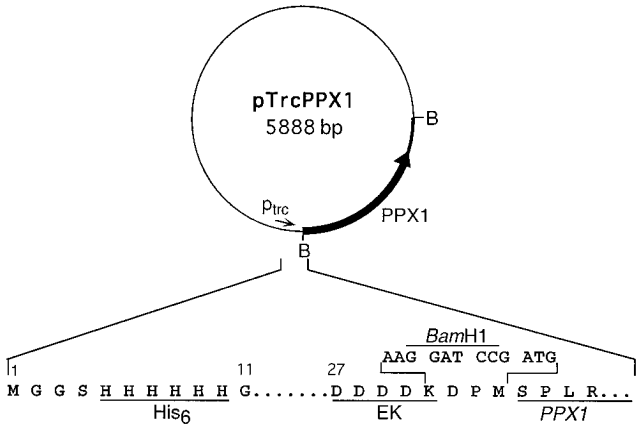

FIG. 5. Construction of a plasmid to overproduce rPPX1. The first 32 amino acids, provided by the vector, include six consecutive histidines (His_6) and an enterokinase cleavage site (EK). B, *Bam*HI restriction site.

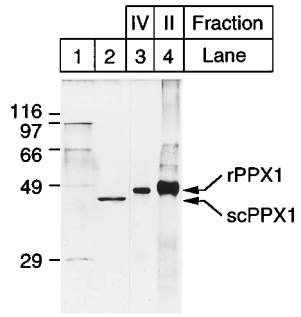

FIG. 6. PAGE analysis of purified rPPX1. Lane 1, molecular mass markers (numbers on the left in kilodaltons); lane 2, purified scPPX1 (0.5 μg [42]); lane 3, rPPX1 fraction IV (0.65 μg); lane 4; rPPX1 fraction II (2.5 μg). Arrows on the right indicate positions of scPPX1 and rPPX1.

ExopolyPase activity in vacuoles. Since scPPX2 depends on vacuolar proteases, vacuoles were isolated to confirm the location of this polyPase activity. Activity enrichments near 20-fold were observed compared with the lysate activities, suggesting that not only scPPX2 but also scPPX3 is a vacuolar enzyme (Table 4); vacuoles from CB023 were only 1.7-fold enriched compared with the lysate. When the respective values of scPPX3 were subtracted from those of the lysate and vacuole fractions of CB023, no enrichment for scPPX1 in the vacuoles could be observed. Thus, scPPX1 is not a vacuolar enzyme. Furthermore, mitochondrial and nuclear preparations from CB023 also showed no enrichment for scPPX1 (data not shown).

Distinction between scPPX2 and scPPX3. Inasmuch as scPPX2 and scPPX3 are vacuolar activities, but only scPPX2 depends on proteolytic activation, it was uncertain whether scPPX3 is a distinctive enzyme or the residual activity of an unprocessed scPPX2. The two activities were therefore compared in vacuolar preparations with respect to the influence of arginine which is accumulated in the vacuole, where it acts as a counterion for polyP (9). With increasing arginine, the activity of scPPX3 increased, whereas the scPPX2 activity displayed a different pattern (Fig. 8). The difference in the response to arginine, in addition to the differences in activity patterns of scPPX2 and scPPX3 during growth (Fig. 7), suggests that the two activities are due to distinctive enzymes.

PolyP levels and chain lengths. To determine whether the accumulation of polyP and its chain length are affected by reduced polyPase activity, polyP levels and size were determined by using ^{32}P in SDC medium with 7.35 mM phosphate. The extracts were obtained from acid-insoluble material after treatment with organic solvents and precipitation with Ba^{2+}, a procedure which yields up to a 96% recovery of [^{32}P]$polyP_{750}$ added to unlabeled cells; acid-soluble polyP ($polyP_{3-10}$) is not recovered in this procedure. PolyP, measured by the release of P_i after treatment with an excess of rPPX1, showed a slow accumulation during growth (Fig. 9). Strains with deficiencies in the vacuolar proteases contained somewhat less polyP; strains lacking scPPX1 showed no significantly greater accumulation of polyP. Thus, the lack of polyPases did not appreciably influence polyP levels.

PolyP chains analyzed by PAGE with an 8% polyacrylamide gel indicated longer chains when CRY entered stationary phase (Fig. 10); this tendency was much stronger in CB023. A quantitative analysis of the patterns in 2 and 8% polyacrylamide gels revealed that polyP chains in the stationary phase

TABLE 2. Purification of rPPX1

Fraction	Total protein (mg)	Total activity (10^6 U)	Sp act (10^6 U/mg)
I (lysate supernatant)	1,030	1,220	1.2
II (Ni-agarose)	55	870	15.8
III (Mono Q)	29	520	17.9
IV (hydroxylapatite)	12.7	370	29.1

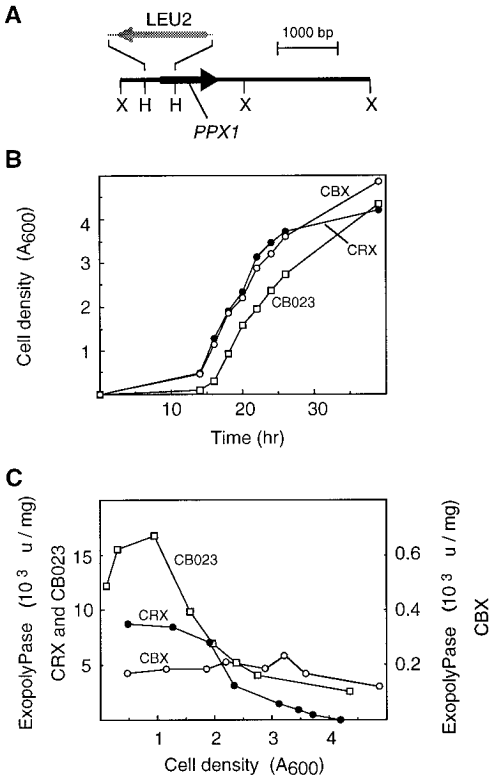

FIG. 7. Gene disruption of *PPX1*. (A) Construction of a DNA fragment with an inactivated *PPX1* gene. The 500-bp *Hin*dIII fragment was replaced with a 1,724-bp fragment containing the *LEU2* marker gene. X, *Xho*I; H, *Hin*dIII. (B) Growth curves of strains in SDC medium (29) at 30°C. (C) ExopolyPase activities of the samples in panel B.

TABLE 3. PolyPase activities in *S. cerevisiae* strains

Strain	Activity			
	Protease	scPPX1	scPPX2	scPPX3
CRY	+	+	+	+
CRX	+	−	+	+
CB023	−	+	−	+
CBX	−	−	−	+

were three- to fourfold longer in strains lacking the vacuolar proteases (Fig. 9C).

PolyP levels in vacuolar ATPase mutants. Complete loss of polyP was reported (5) in strains deficient in *VMA4* (13), the gene involved in assembly of the vacuolar ATPase (18) required for acidification of the vacuolar lumen. With more sensitive methods than the nuclear magnetic resonance analyses previously used (5), a very small residual amount of polyP was detected in the *vma4* mutant, below 1% of that of wild-type *S. cerevisiae* (data not shown). Since a rapid loss of polyP was also reported (5), accompanied by an increase in cytosolic P_i after addition of protonophores, the vacuolar polyPases might be implicated in this degradation. To determine whether this rapid loss of polyP was catalyzed by scPPX1 or scPPX2, the *vma4* mutation was introduced into CRY and CBX. These strains also contained polyP at between 0.03 and 0.4% of the respective wild-type levels (Fig. 11). Inasmuch as the absence of scPPX1 and scPPX2 in CBX-V4 did not result in increased levels of polyP, neither of these polyPases can be implicated in the breakdown of polyP in the absence of a pH gradient.

DISCUSSION

The gene for scPPX1, a major exopolyPase in *S. cerevisiae*, was isolated by using the amino-terminal sequence of the purified protein. *PPX1* represents the first gene related to polyP metabolism isolated from a eukaryotic cell. The gene is located 57 kbp from the right end of chromosome VIII and is directed toward the centromere. The open reading frame corresponding to scPPX1 encodes a protein of 396 amino acids and a molecular mass of 44,942 Da. AT-rich elements preceding the open reading frame indicate a strongly expressed gene. Since the enzyme needs to be purified more than 7,000-fold to attain homogeneity (42), it represents only 0.014% of the total cellular protein. These data suggest additional, yet unidentified regulatory elements for expression of *PPX1*. The gene may be expressed constitutively at a low level during regular growth and highly expressed under certain conditions which require high polyPase activity. This supposition is supported by identical growth rates under standard conditions of *ppx1* mutants compared with the wild type.

PPX1 was overexpressed in a bacterial host to a level about 40-fold that of wild-type *S. cerevisiae*. Furthermore, the addition of an N-terminal histidine tag to the recombinant protein allowed efficient purification by affinity chromatography, but with a 10-fold-reduced specific activity compared with the wild-type enzyme. Presumably, the N-terminal appendix interacts with the protein since it could not be removed by a enterokinase, despite possessing the cleavage site for the protease. The recombinant enzyme rPPX1 has provided an excellent reagent for enzymatic analysis of polyP. The enzyme acts on polyP of all chain lengths with approximately equal rates; unlike polyphosphate kinase, it catalyzes an irreversible hydrolysis resulting in an almost complete breakdown of polyP.

Inactivation of *PPX1* in two different strains led to the biochemical identification of two additional soluble polyPase ac-

TABLE 4. ExopolyPase activities in vacuoles

Strain	ExopolyPase (10^3 U/mg)		Fold enrichment
	Lysate	Vacuoles	
CRX	1.2	24.8	21
CB023	6.2	10.6	1.7
CBX	0.3	5.1	17

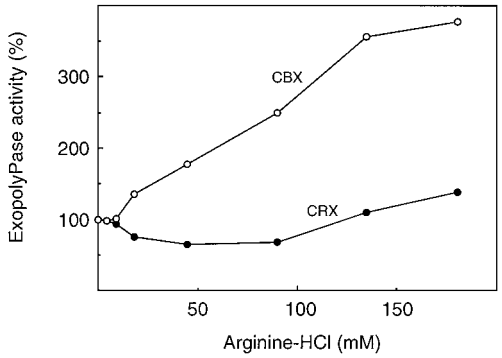

FIG. 8. Arginine dependence of the exopolyPase activity in vacuoles.

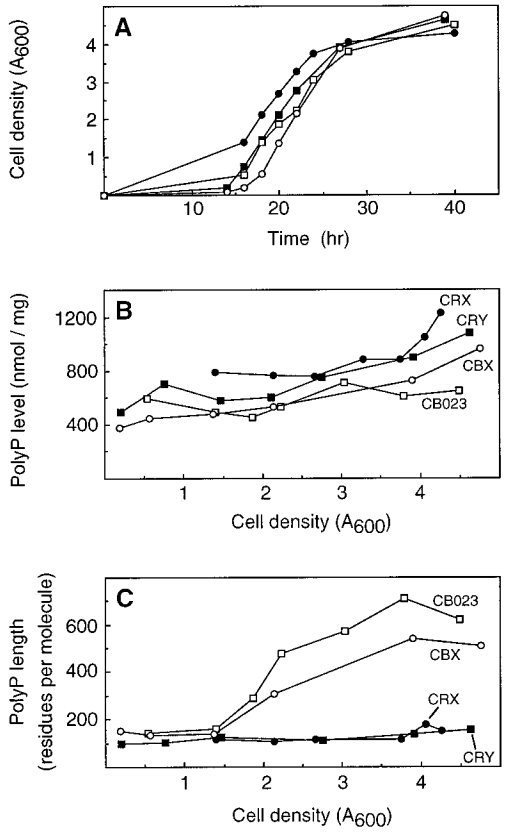

FIG. 9. PolyP levels and chain lengths during growth of *S. cerevisiae*. (A) Growth curves; see panel B for definition of symbols. (B) PolyP levels of the samples in panel A. (C) Average chain lengths of polyP in the samples in panel A. PolyP levels and chain lengths were determined as described in Materials and Methods.

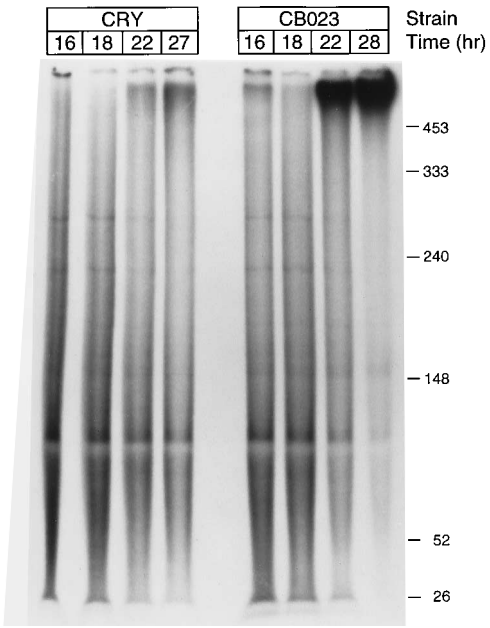

FIG. 10. PAGE analysis of polyP. Samples from CRY and CB023 were extracted after the indicated times of growth (see Fig. 9) and fractionated on 8% polyacrylamide gels. Numbers on the right indicate the mobility and chain length of polyP size markers (7).

tivities (scPPX2 and scPPX3), both located in the vacuole. scPPX2, which requires proteolytic processing, may be the enzyme described by Andreeva and colleagues (2), and like scPPX1, it is not active at early stages of growth. At later growth stages, scPPX2 is no longer detectable, while scPPX1 remains active but declines with dilution by the increase in cell mass. The scPPX3 activity, only about 2% of that of scPPX2, is distinguished from it by its constant activity during growth and stationary phase and by its activation pattern by arginine, a counterion of polyP in the yeast vacuole (9). These data suggest that the scPPX2 and scPPX3 activities are carried out by two different enzymes. But it is also possible that the two activities are performed by one enzyme which has different properties in the presence and absence of proteolytic activation. scPPX1 is not enriched in vacuoles. The lack of a signal sequence indicates that it does not enter the secretory pathway; loss of the N-terminal methionine is further evidence for, at least, a transient location of scPPX1 in the cytosol.

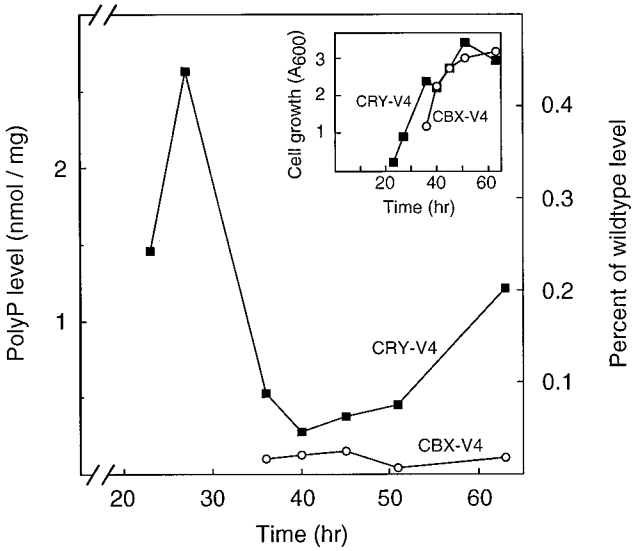

FIG. 11. PolyP levels in vacuolar ATPase mutants. CRY-V4 and CBX-V4 were grown in SDC (see inset). At indicated times, polyP was extracted and quantified. Percentages were calculated by using 600 nmol/mg of protein as a wild-type level.

The vast bulk of polyP in *S. cerevisiae* is located in the vacuole. Thus, it was expected that inactivation of *PPX1* did not significantly affect polyP levels. The absence of the vacuolar proteases resulted in 10 to 20% reduced levels of polyP, which could be the result of a partial loss of vacuolar functions in these strains. More strikingly, absence of vacuolar proteases results in a dramatic increase in the average chain length of polyP in stationary phase from about 140 residues per molecule to about 600. In strains with fully functional vacuoles, the chain length increases only slightly, from about 100 to 160 residues per molecule. It seems unlikely that the lack of scPPX2 is responsible for the increased chain length, because this enzyme is completely turned off in the later stages of growth. Furthermore, all known exopolyPases are processive enzymes and would therefore be expected to have more impact on the levels of polyP than on chain length. More likely, the increased chain length is the result of a lack of an endopolyPase activity. Such an enzyme, which reduces the size of polyP without affecting its levels, has been identified in vacuoles (39) and requires proteolytic activation (26).

Crucial for the accumulation of polyP is the presence of an active vacuolar ATPase which acidifies the vacuolar lumen and provides a pH gradient across the vacuolar membrane. The pH gradient, in turn, serves as a energy reservoir for the accumulation of a number of cellular metabolites that include Ca^{2+}, basic amino acids, and, apparently, also polyP. The lack of vacuolar polyP in the absence of the pH gradient and the rapid decrease of polyP accompanied by an increase in cytosolic P_i after addition of protonophoric compounds (5) suggest an involvement of the gradient either in biosynthesis of polyP or in preventing it from being degraded by polyPases. When vacuolar ATPase was genetically inactivated in strains lacking the two major polyPases (scPPX1 and scPPX2), the polyP level remained low, indicating that these polyPases do not contribute to the loss of polyP in vacuolar ATPase mutants.

Thus far, five hydrolytic activities specific for polyP have been identified in *S. cerevisiae*: a vacuolar endopolyPase (39) and four exopolyPases located in different compartments, the cell envelope (3), the cytosol (scPPX1), and the vacuole (scPPX2 and scPPX3). PolyP kinase, the enzyme that catalyzes the reversible formation of polyP from ATP, is much more active in the breakdown of polyP in vitro than in its biosynthesis (12, 34). The presence of so many enzymes involved in the utilization of polyP suggests important, yet unexplored functions for this polymer. PolyPases in the cell envelope and in the cytosol also suggest that polyP is present, at least transiently, in these compartments. While polyP has been found on the surface of *Saccharomyces* strains (37), the cytosol deserves more attention with respect to the occurrence of polyP. *vma4* mutants provide a low background of polyP which might be helpful in the search of small amounts of the cytosolic material.

It remains unclear how *S. cerevisiae* maintains enormous deposits and low turnover of polyP (41) in the face of potent degradative activities in the vacuole. A clear definition of the features and regulation of the biosynthetic pathways is needed before the physiology and function of polyP can be evaluated.

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