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ARTICLE *in* YEAST · JULY 2006

Impact Factor: 1.63 · DOI: 10.1002/yea.1391 · Source: PubMed

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Published online in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/yea.1391

Research Article

Inorganic polyphosphate and exopolyphosphatase in the nuclei of *Saccharomyces cerevisiae*: dependence on the growth phase and inactivation of the *PPX1* and *PPN1* genes

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Received: 28 February 2006
Accepted: 23 May 2006

Abstract

Nuclei of the yeast *Saccharomyces cerevisiae* possess inorganic polyphosphates (polyP) with chain lengths of ca. 10–200 phosphate residues. Subfractionation of the nuclei reveals that the most part of polyP is not associated with DNA. Transition of the yeast cells from stationary phase to active growth at orthophosphate (P_i) excess in the medium is followed by the synthesis of the shortest polyP (<15 phosphate residues) and hydrolysis of the high-molecular polyP (>45 phosphate residues) in the nuclei. Nuclear exopolyphosphatase (exopolyPase) activity does not depend on the growth phase. The *PPX1* gene encoding the major cytosolic exopolyPase does not encode the nuclear one and its inactivation has no effect on polyP metabolism in this compartment. Under inactivation of the *PPN1* gene encoding another yeast exopolyPase, elimination of the nuclear exopolyPase is observed. The effect of *PPN1* inactivation on the polyP level in the nuclei is insignificant in the stationary phase, while in the exponential phase this level increases 2.3-fold as compared with the parent strain of *S. cerevisiae*. In the active growth phase, no hydrolysis of high-molecular polyP is detected while the synthesis of short-chain polyP is retained. The data obtained indicate substantial changes in polyP metabolism in nuclei under the renewal of active growth, which only partially depends on the genes of polyP metabolism known to date. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: **inorganic polyphosphates; exopolyphosphatase; nuclei; *PPX1* and *PPN1* mutants; *Saccharomyces cerevisiae***

Introduction

Inorganic polyP is a common component of nuclei of the yeast and other eukaryotes. PolyP has been found in the nuclei and DNA preparations in the fungi *Agaricus bisporus* (Kulaev and Vagabov, 1983), *Physarium polycephalum* (Pilatus *et al*., 1989), *Colleotrichum* (Rodrigues, 1993), and in the nuclei of animal cells (Kumble and Kornberg, 1995). PolyP has turned out to be associated with the fraction of non-histone proteins (Offenbacher and Kline, 1984). The most notable changes in the nuclear polyP have been observed under sporulation in which switching and shut-down of large gene groups take place. Degradation of high-polymeric polyP to shorter fragments in the nuclei was observed under sporulation in the fungus *P. polycephalum* (Pilatus *et al*., 1989). These data suggest that nuclear polyP probably plays an important role in gene-expression regulation (Kulaev, 1979).

The most studied enzymes of polyP metabolism in yeast are exopolyphosphatases (exopolyPases) catalysing the hydrolysis of terminal phosphate from polyP and endopolyphosphatases (endopolyPases), also called polyP depolymerases, hydrolysing polyP chains of many hundreds of phosphate residues into shorter chains. We have revealed

and described a nuclear exopolyPase in the yeast (Lichko *et al*., 1995, 1996, 2004a) and demonstrated that it was not encoded by the *PPX1* gene (Lichko *et al*., 2003a). This gene encodes ca. 45 kDa exopolyPase (Wurst *et al*., 1995) localized in the cytosol and soluble mitochondrial fraction (Lichko *et al*., 2002, 2003b). There is another gene encoding a protein exhibiting exopolyPase activity in the yeast: *PPN1* (*PHM5*) (Kumble and Kornberg, 1996; Ogawa *et al*., 2000; Shi and Kornberg, 2005). This gene was originally described as encoding an endopolyphosphatase (Kumble and Kornberg, 1996) and it possessed a domain characteristic of vacuolar proteins (Dove *et al*., 2002; Huh *et al*., 2003).

Thus, the data on polyP metabolism in nuclei remain fragmentary. One of the methods of solving this problem is to study changes in polyP and polyP-dependent enzymes of the nuclei during transition from the stationary phase to new active growth, in both the parent strain and mutants with inactivated genes of polyP-metabolizing enzymes.

The objective of the present work is to study the influence of the growth phase on exopolyPase activities, polyP levels, and polyP chain lengths in the nuclei of *S. cerevisiae* under *PPX1* and *PPN1* inactivation.

Materials and methods

Strains and culture conditions

Strains of the yeast *S. cerevisiae* CRY (parent strain), CRX (a strain with inactivated *PPX1* gene), CRN (a strain with inactivated *PPN1* gene), and CNX (a strain with inactivated *PPX1* and *PPN1* genes) were kindly provided by Professors A. Kornberg and N. Rao (Stanford University, USA). All strains were grown aerobically in a shaker at 30 °C in YPD medium with 1% yeast extract, 2% peptone, and 2% glucose as described earlier (Wurst and Kornberg, 1994).

To obtain partially synchronous cultures with the most part of cells being at the budding stage, the cells from the stationary phase were re-inoculated with a high starting optical density into a fresh YPD medium. In the procedure which involved re-inoculation, 24 h cultures (stationary phase) were collected by centrifugation at 5000 × *g* for 10 min, washed with sterile distilled water, and placed on 250 ml fresh YPD medium (2 g wet biomass for each strain, $OD_{600} = \sim 5$). After cultivation for 5 h (exponential phase), the cells were centrifuged, washed with distilled water as above, and used for further analysis.

Preparation and characterization of nuclei

Spheroplasts were prepared from both 24 h stationary phase cultures and 5 h exponential cells obtained after re-inoculation into fresh YPD medium from all the strains under study (Lichko and Okorokov, 1984). The spheroplasts were used for isolation of nuclei. Preparation of nuclei was described in detail in our previous papers (Lichko *et al*., 1995, 2003a). The purity and intactness of the nuclear fraction was rather satisfactory, as determined by examination with phase-contrast and fluorescence microscopes. The DNA-specific dye Hoechst 33 258 was used in the latter case. The nuclear purity was also characterized by the absence of marker enzymes of other compartments. Isolated nuclei showed no activity of α-mannosidase, a marker enzyme of vacuoles, succinate dehydrogenase, a marker enzyme of mitochondria, or glucose 6-phosphate dehydrogenase, a marker enzyme of cytosol. The protein : DNA ratios of the purified nuclei were 1 : 30 for all the strains under study, close to those obtained previously for the lower eukaryotes (Lichko *et al*., 1995).

Subfractionation of nuclei with KCl and Triton X-100

For nuclei subfractionation, a modified method of Hurt *et al*. was used (Hurt *et al*., 1988). Nuclei obtained from ~20 g wet biomass were suspended in 5 ml ice-cold buffer containing 1% Triton X-100, 150 mM KCl, 1 mM PMSF and 20 mM HEPES-KOH, pH 6.45 (buffer A), and incubated at 0 °C for 10 min. The nuclear suspension was spun at 15 000 × *g* for 25 min. The pellet was suspended in ~2 ml buffer A without Triton X-100. The supernatant (salt-detergent extract) and nuclear pellet (insoluble pellet) was used for further investigation.

Assay of exopolyPase activity

ExopolyPase activities were determined by the rate of P_i formation at 30 °C for 20–30 min in 1 ml

DOI: 10.1002/yea

reaction mixture containing 50 mM Tris–HCl, pH 7.2, 0.1 mM $CoSO_4$, and 9.6 μM $polyP_{208}$ as a polymer (saturated concentration). An activity unit (U) was defined as the quantity of the enzyme catalysing the formation of 1 μmol P_i in 1 min.

Extraction and assay of acid-soluble polyP

Acid-soluble polyP was extracted with 0.5 N $HClO_4$ (Pestov *et al*., 2004). After the removal of nucleotide phosphates by adsorption to Norit A charcoal, the level of polyP was estimated as an increase in the P_i amount after the hydrolysis of samples in the presence of 1 N HCl for 10 min at 100 °C (Pestov *et al*., 2004). P_i was determined with ascorbic acid and SDS (Kulakovskaya *et al*., 1999).

Electrophoresis of acid-soluble polyP

The acid-soluble polyP fraction was adjusted to pH 4.5 with NaOH and polyP was precipitated with saturated $Ba(NO_3)_2$ by centrifugation at 5000 × *g* for 20 min. The barium salt of polyP was converted to a soluble form by adding cation-exchange resin Dowex 50 WX 8 in the NH_4^+ form and some distilled water. The obtained preparation was subjected to electrophoresis in 20% polyacrylamide gel in the presence of 7 M urea, and the gel was stained with toluidine blue (Kumble and Kornberg, 1995). PolyPs with chain lengths of ~15, 25, 45 (Sigma) and 188 phosphate residues (Monsanto) were used as standards. PolyP was separated from P_i by gel filtration on Sephadex G-10 (Pharmacia) as described earlier (Andreeva and Okorokov, 1993).

Other methods

Protein concentration was assayed by the modified Lowry method (Bensadoun and Weinstein, 1976), using bovine serum albumin as a standard. Molecular masses were determined by gel filtration on Superose 6 column (Pharmacia) according to (Lichko *et al*., 2004a). Quantification of DNA and determination of α-mannosidase, succinate dehydrogenase and glucose 6-phosphate dehydrogenase have been described earlier (Lichko *et al*., 1995). All data in the tables and figures are average values of at least three experiments.

Results

Characterization of nuclear exopolyPase

Only the nuclei of the parent strain CRY and the *PPX1*-mutant CRX of *S. cerevisiae* exhibited detectable exopolyPase activities (Table 1). According to gel filtration of the nuclear extract obtained as described in Materials and methods, the nuclear exopolyPase from CRY had the molecular mass of ~200 kDa. It hydrolysed polyP with average chain lengths of 15–208 phosphate residues to the same extent. This activity was much lower with tripolyphosphate. The nuclear exopolyPase activity was stimulated 1.6- and 2.5-fold by 2.5 mM Mg^{2+} and 0.1 mM Co^{2+}, respectively. All properties of the nuclear exopolyPase from CRY were similar to those of the CRX strain (Lichko *et al*., 2004a). It should be mentioned that the overall physicochemical properties distinguish the nuclear exopolyPase from exopolyPases of other cell compartments (Lichko *et al*., 2003b).

Nuclear PolyP and nuclear subfractionation

Purified nuclei possessed polyP (Table 2). The nuclei were subfractionated using detergent and salt (1% Triton X-100, 0.15 M KCl) into soluble salt-detergent extract and insoluble nuclear pellet. Whereas protein contents and exopolyPase activities were similar in both fractions, 98% of the acid-soluble polyP was found in the soluble fraction

Table 1. ExopolyPase activities (mU/mg protein) in the nuclei of *S. cerevisiae*

Strain	Relevant genotype	Stationary phase	Exponential phase
CRY	Parent strain	100 ± 10	70 ± 5
CRX	*PPX1*-deficient strain	80 ± 5	90 ± 3
CRN	*PPN1*-deficient strain	3 ± 0.2	2 ± 0.5
CNX	*PPX1*- and *PPN1*-deficient strain	0	2 ± 0.1

Table 2. The levels (μmol/mg protein) of acid-soluble polyP in the nuclei of *S. cerevisiae*

Strain	Relevant genotype	Stationary phase	Exponential phase
CRY	Parent strain	0.8 ± 0.04	1.2 ± 0.1
CRX	*PPX1*-deficient strain	0.9 ± 0.06	1.8 ± 0.04
CRN	*PPN1*-deficient strain	0.9 ± 0.01	2.8 ± 0.25
CNX	*PPX1*- and *PPN1*-deficient strain	1.1 ± 0.1	3.0 ± 0.1

DOI: 10.1002/yea

and most of the DNA (70%) was detected in the insoluble nuclear pellet. This observation was true for all strains in both growth phases. Therefore, most of the polyP is not associated with DNA. Purified nuclei were used for further polyP extraction.

Growth-phase dependence of nuclear polyP and exopolyPase of the parent CRY strain

To study the influence of the growth phase on the nuclear polyP and exopolyPase, two easily distinguishable stages were selected: a stationary phase (~20% of budding cells) and an active budding phase (~90% of budding cells). In the parent strain CRY, transition from stationary to active growth resulted in a decrease in exopolyPase activity and an increase in polyP level in the nuclei (Tables 1 and 2). This was followed by an increase in shorter polyP chains (<15 phosphate residues) and a decrease in high-polymeric ones (~45 phosphate residues) (Figure 1). The most polymeric polyP chains (~100–150 phosphate residues) disappeared (Figure 1). It is thought that polyP depolymerization takes place during the transition from stationary to active growth. However, the 1.5-fold increase of the nuclear polyP level (Table 2) suggests that this process is more complex and involves both polyP depolymerization and synthesis of short-chain polyP *de novo*.

Effect of *PPX1* inactivation

Inactivation of the *PPX1* gene encoding ~45 kDa exopolyPase (CRX) did not appreciably influence nuclear exopolyPase activities both in the stationary and exponential phases (Table 1). In the CRX strain, nuclear exopolyPase activity was similar in both growth phases under study as compared with the CRY strain.

Inactivation of *PPX1* had no effect on the polyP level in nuclei in the stationary phase (Table 2). PolyP level in the nuclei increased 1.5- and 2-fold in the exponential phase in the parent strain and *PPX1* mutant, respectively (Table 2). It should be noted that the increase of polyP level was not clearly connected with the change of exopolyPase activity.

Inactivation of the *PPX1* gene left polyP chain lengths in the nuclei unaffected in both growth phases (Figure 1).

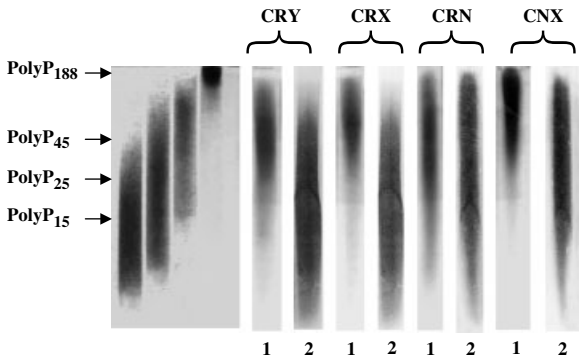

Figure 1. Electrophoresis of nuclear polyP in 20% polyacrylamide gel. 1, stationary phase; 2, exponential phase. Mobility and chain length of polyP size markers are indicated on the left side

Effect of *PPN1* inactivation

Inactivation of the *PPN1* gene encoding another protein, which exhibited exopolyPase activity in the yeast (CRN and CNX strains), resulted in almost total elimination of the nuclear exopolyPase activities in both growth phases as compared with the parent strain CRY and *PPX1* mutant CRX (Table 1).

The effect of *PPN1* inactivation on polyP levels in the nuclei was more complicated. In the stationary phase, nearly the same polyP levels were detected in the nuclei of CRN and CNX strains as compared with the parent CRY strain (Table 2). In the exponential phase, polyP levels in nuclei increased ~ three-fold in *ppn1* mutants as compared with stationary growth, while only 1.5- and 2-fold increases were observed in the CRY and CRX strains, respectively (Table 2). Thus, in this mutation, the increase of polyP level was more expressed during active growth.

In the stationary phase, polyP chain lengths in CRN were just the same as in CRY (Figure 1). In the double mutant CNX, the chain-length spectrum was also similar to that in CRY, except the presence of extremely long-chain polyP that was not observed in the nuclei of other strains. Under exponential phase, shorter polyP chains (<15 phosphate residues) were observed, along with the high-polymeric polyP chains (15–100 phosphate residues) characteristic of stationary phase in both CRN and CNX strains (Figure 1).

The double mutation of both *PPX1* and *PPN1* (CNX strain) left exopolyPase activity, polyP level

DOI: 10.1002/yea

and polyP chain lengths of nuclei essentially unchanged in exponential phase as compared with the CRN strain, in contrast to the stationary phase, in which some elongation of polyP chains of the nuclei was observed.

The emergence of short polyP chains was not associated with the functioning of exopolyPase, since its activity was negligible in these strains (Table 1). It seems that the short-chain polyP was not a product of defragmentation but synthesized *de novo.*

Discussion

The study of polyP is of interest because these polymers have a regulatory role in metabolism correction and control of both genetic and enzymatic levels (Reusch, 1989; Kornberg *et al*., 1999; Kulaev *et al*., 2004; Kuroda, 2006). Earlier, there has been experimental evidence that the yeast nuclei possess polyP (Kulaev, 1979). However, the methods of isolation of nuclei and polyP analysis have improved over the years. In the present work, the modern technique of nuclei isolation and polyP analysis using electrophoresis has made it possible to reveal polyP in nuclei with reliability. It should be noted that metachromatic red-violet staining characteristic of polyP is observed at electrophoregram. The content and chain length of polyP in a cell as a whole undergo essential changes, depending on the growth phase, even at phosphate excess in the medium (Vagabov *et al*., 1998; Werner *et al*., 2005). In exponential phase, the polyP levels of the nuclei increase in all the strains under study. In this growth phase, shorter polyP chains (<15 phosphate residues) appear in the nuclei along with the high-polymeric ones characteristic of nuclear polyP chains in stationary phase. The most polymeric polyP disappears during active growth. These changes are probably independent of P_i level changes in the medium. In our experimental conditions, P_i concentration in the medium was 7 mM and the cells took up no more than 1 mM. It seems that there are two simultaneous processes: depolymerization of high-polymeric polyPs and synthesis of short polyP chains *de novo.*

The present work demonstrates that the product of the *PPX1* gene is not involved in polyP metabolism in the nuclei. This supports our earlier findings that the nuclear exopolyPase differing from all the known exopolyPases by molecular mass and other properties is not encoded by the *PPX1* gene (Lichko *et al*., 2004a). On the contrary, the *PPN1* gene has a pronounced effect on this metabolism. Inactivation of *PPN1* encoding another yeast exopolyPase results in elimination of nuclear exopolyPase activity (Table 1). Negligible activities in the nuclei of the *ppn1* mutant strains might be due to the presence of the cytosol Ppx1p in minor amounts. It is possible that just *PPN1* encodes the nuclear exopolyPase and its distinction in some properties from the enzyme found earlier (Shi and Kornberg, 2005) is due to its post-translational modification. At least, expression of nuclear exopolyPase is closely associated with the *PPN1* gene, just as it was observed for the high-molecular-mass exopolyPase of the cytosol (Lichko *et al*., 2004b).

Inactivation of *PPN1* has no effect on the levels and polyP chain lengths in the nuclei in the stationary phase as compared with the parent strain CRY (Table 2, Figure 1). Under active growth of the CRN strain, the polyP level increases. At the same time, high-molecular polyP (>45 phosphate residues) is kept constant, while it disappears in the parent strain. This suggests that the nuclear exopolyPase hydrolyses just this polyP fraction. However, the amount of short-chain polyP in the CRN strain still increases. Therefore, short-chain polyPs are not products of defragmentation of the high-polymeric polyP but synthesized *de novo.* The problem of polyP synthesis in the yeast remains unsolved. However, there are indications on possibility of their biosynthesis using ATP (McGrath *et al*., 2005) or the proton motive force (Ogawa *et al*., 2000) as a source of energy.

Thus, transition to active growth is followed by the synthesis of low-polymeric polyP in the yeast nuclei and this process does not depend on the genes of polyP metabolism in the yeast known to date. The functional significance of this synthesis requires further investigation.

Acknowledgements

This work was supported by the Russian Foundation for Basic Research (Grant No. 05-04-48175) and a grant of Presidium of RAS (Programme No. 18). We thank E. Kulakovskaya for her help with polyP electrophoresis, and L. Mihailina and N. Kosenkova for excellent technical assistance.

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