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Abstract

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ABSTRACT
To identify genes involved in poly(A) metabolism, we screened the yeast gene deletion collection for growth defects in the presence of cordycepin (3′-deoxyadenosine), a precursor to the RNA chain terminating ATP analog cordycepin triphosphate. Δpho80 and Δpho85 strains, which have a constitutively active phosphate-response pathway, were identified as cordycepin hypersensitive. We show that inorganic polyphosphate (poly P) accumulated in these strains and that poly P is a potent inhibitor of poly(A) polymerase activity in vitro. Binding analyses of poly P and yeast Pap1p revealed an interaction with a $k_D$ in the low nanomolar range. Poly P also bound mammalian poly(A) polymerase, however, with a 10-fold higher $k_D$ compared to yeast Pap1p. Genetic tests with double mutants of Δpho80 and other genes involved in phosphate homeostasis and poly P accumulation suggest that poly P contributed to cordycepin hypersensitivity. Synergistic inhibition of mRNA synthesis through poly P-mediated inhibition of Pap1p and through cordycepin-mediated RNA chain termination may thus account for hypersensitive growth of Δpho80 and Δpho85 strains in the presence of the chain terminator. Consistent with this, a mutation in the 3′-end formation component rna14 was synthetic lethal in combination with Δpho80. Based on these observations, we suggest that binding of poly P to poly(A) polymerase negatively regulates its activity.

INTRODUCTION
Inorganic polyphosphate (poly P) comprises chains of 10s to 100s of phosphate residues, connected by energy-rich phospho-anhydride bonds. Despite the fact that poly P is an ubiquitous molecule detected in every living cell, our knowledge concerning its biochemistry and biological function remains incomplete (1,2). Poly P serves important roles as a substitute for ATP for sugar and adenylate kinases (3,4), as phosphate reservoir with osmotic advantage (5–8), as energy source (9) and reservoir (10), as buffer against alkaline stress (11) and as chelator of divalent ions (1).

In addition to these general functions, poly P has been implicated in a number of regulatory processes both in prokaryotes and eukaryotes. In Escherichia coli, poly P is essential for survival during stationary phase (12). It accumulates into large amounts in response to stress situations brought about by nutritional downshift from rich to minimal media (13), phosphate limitation (14), amino acid depletion (15) or nitrogen starvation. During the latter condition, poly P promotes ribosomal protein degradation by binding and activating the Lon protease in E. coli delivering amino acids needed to respond to starvation (16). Poly P is also ubiquitous in mammalian cells and tissues (17) where it has been implicated in a host of regulatory processes. For example, the mammalian TOR kinase, which is involved in cell growth and proliferation (18), is activated by poly P under conditions of nutritional starvation (19). This observation also led to the proposal of an evolutionary conserved role for poly P in stress response (19). Moreover, poly P was found to enhance proliferation of human fibroblast cells (20), to stimulate calcification of osteoblast-like cells (21),...

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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to inhibit the secretion of immunoglobulin and to stimulate apoptosis in human plasma and myeloma cells (22), and more recently, poly P has been shown to have anti-metastatic and anti-angiogenic activities (23).

Interestingly, there are several reports that link poly P to the regulation of gene expression through effects on RNA polymerase transcription. In E. coli, poly P is associated with RNA polymerase during the stationary phase and inhibits specifically the transcriptional activity of the enzymes associated with the 70 promoter-recognition subunit, which is involved in the transcription of genes during exponential growth phase (24). Moreover, poly P induces transcription of rpoS, the stationary phase σ factor (25). Polyphosphate kinase (Ppk1), the enzyme synthesizing poly P, is a component of the E. coli RNA degradosome (26). Since poly P is a potent inhibitor of the degradosome, it was suggested that Ppk1 might act to maintain a correct microenvironment for proper mRNA degradation (26). In addition, poly P has been shown to associate with ribosomes and to suppress misincorporation of amino acids during translation (27).

In yeast, poly P concentrations can reach ~120 mM (1) and thus poly P contributes up to 20% of the cellular dry weight. Most of the poly P (90–99%) is localized to the vacuole (28,29), but poly P was also detected in the cytoplasm and the nuclei of yeast (30,33). For rat liver nuclei, micromolar poly P concentrations were reported (17) and for yeast nuclei a similar level of poly P is assumed (1). Poly P content of a yeast cell is strongly dependent on the growth phase (31,32) and the average length of nuclear poly P polymers of approximately 45 phosphate residues was found to be changing dynamically with growth conditions (33). Enzymes involved in eukaryotic poly P synthesis remain mostly elusive to this day (1), but activities indicative of an active poly P metabolism (e.g. ATP and phosphate homeostasis) are strongly interdependent. This screen also revealed that poly P metabolism and primary catabolism have been associated with all cellular compartments (31,34), including the nucleus (30,35).

The observation that a double mutant of PPNI [encoding an endopolyphosphatase; (36)] and PPX1 [encoding an exopolyphosphatase; (37)] rapidly loses viability in stationary phase (36), underscores the biological importance of poly P. We recently screened the entire collection of haploid yeast gene deletion mutants for poly P content (38) and found that poly P metabolism and primary metabolism (e.g. ATP and phosphate homeostasis) were strongly interdependent. This screen also revealed that all cellular compartments are linked to poly P homeostasis (38).

In this work, we establish a connection between cellular accumulation of poly P and inhibition of poly(A) polymerase activity. Yeast strains mutant in central components of the phosphate response pathway (Apso80 and Apho85) contained increased amounts of poly P that contributed to cordycepin-hypersensitive growth. We propose a role for poly P in negatively regulating polyadenylation through inhibition of poly(A) polymerase.

**MATERIALS AND METHODS**

**Yeast strains and plasmids**

Wild-type BY4741 (Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) and isogenic mutant strains were obtained from EUROSCARF. The nra14-1 genotype is Mat a; ura3-1; trp1-1; ade2-1; leu2-3; his3-11; 15; nra14-1 (39). Double mutants were generated by disrupting the PHO80 open reading frame with a NatR cassette by homologous recombination in Apho4, Apho2, Apho84 and nra14-1 strains. PHO80 and PHO85 genes were cloned into pRS313 using BamHI and NotI restriction sites following PCR amplification from genomic DNA with primers PHO80-5′ AAGATCGGATCCCTTTCTATGGAATA TGAATG and PHO80-3′ GATCTTGGCGCCGCAAAAG AACAGTGATGATATGATA AAGAT CGATCTCTTTTATGAAATATGTGC ACT and PHO85-3′ GATCTTGGCGCCGCTTTAAGTCTGTGCT CTCTCACTT. Yeast strains were grown at 30°C either in YPD (1% yeast extract, 2% tryptone, 2% glucose) or in synthetic complete medium (SD, yeast nitrogen base and complete amino acid mixture plus 2% glucose). SD was supplemented with cordycepin (40 μg/ml) purchased from Sigma or 5′-Fluoroorotic acid (1 mg/ml, Zymo Research, Orange, CA, USA).

**Poly(A) length analysis**

Assays were performed essentially as described (40). In the standard reaction, 2 μg of total RNA was incubated with 400 ng recombinantly expressed yeast poly(A) polymerase (a gift from G. Martin, Basel) and 0.2 μl [α-32P]-cordycepin triphosphate (Perkin–Elmer) in reaction buffer (20 mM Tris–HCl pH 7.0, 50 mM KCl, 0.7 mM MnCl2, 10% Glycerol, 100 μg/ml BSA) for 30 min at 30°C in a total volume of 12 μl. After heat inactivation, RNA was digested with RNase A and RNase T1, followed by proteinase K treatment. Precipitated RNAs were resolved on 15%/8.3 M urea polyacrylamide gels, that were exposed and visualized on a FLA-7000 phosphor-imager (Fuji).

**Polyadenylation assays**

Conditions were as described in Refs (41) and (42) with modifications. The yeast poly(A) polymerase reaction mixture of 15 μl contained 100 ng yeast poly(A) polymerase (a generous gift from G. Martin, Basel), 5 pmol of 5′-end labeled A15 RNA primer, 0.5 mM ATP, 5 mM MgCl2, 25 mM Tris–HCl pH 7.9, 20 mM KCl, 10% glycerol, 0.01 mM EDTA, 0.1 mg/ml BSA, 1 mM DTT and 0.02% Nonidet P-40. The reaction mixture with bovine poly(A) polymerase consisted of 200 ng bovine poly(A) polymerase (a gift from G. Martin, Basel), 5 pmol of 5′ end-labeled A15 RNA primer, 0.5 mM ATP, 25 mM Tris–HCl pH 8.3, 40 mM KCl, 6 mM MgCl2, 0.05 mM EDTA, 0.5 mM DTT, 0.01% Nonidet P-40, 10% glycerol and 200 μg/ml BSA in a total volume of 15 μl. Poly P was added to the reactions as indicated in the figure legends.
Reactions were incubated at 30°C for the indicated time and stopped by addition of 25 mM EDTA. Reaction products were precipitated and resolved on 15%/8.3 M urea polyacrylamide gels that were exposed and visualized on a FLA-7000 phosphor-imager (Fuji).

**Purification of recombinant E. coli polyphosphate kinase**

The gene encoding the E. coli polyphosphate kinase [EcPPK; (43)] was amplified with the primers CATG(16). Purified poly(A) polymerase from Poly P binding 5 mM MgCl₂. Equal volumes of radioactive poly P 750 and cerevisiae poly P 88,1 ATP synthesis at 37°C was determined with the reverse reaction by measuring purified on amylose resin following standard protocols (New England BioLabs, Beverly, MA, USA). The activity was determined with the reverse reaction by measuring ATP synthesis at 37°C in 50 μl reactions containing 10 μM poly P₈₈, 1 μM ADP, 50 mM Tris and 50 mM malate, pH 6.5 and purified EcPPK. The reactions were stopped by heat inactivation at 90°C for 2 min. ATP was quantified after the addition of 50 μl luciferase reaction mixture (ATP bioluminescence assay kit CLS II, Roche Molecular Biochemicals) in a luminometer [Lumat LB 9507 (Berthold Technologies GmbH & Co. KG)].

**Poly P measurements and poly P synthesis in vitro**

Determination of poly P concentration was performed as previously described (32). Poly P with an average chain length of 750 residues was synthesized as described (13) except for some modifications. Poly P was synthesized in 200 μl reactions containing 138 nM [γ-³²P]-ATP, 1 mM ATP, 2 mM creatine-phosphate, 6 U creatine phosphokinase (from rabbit muscle, Sigma-Aldrich), 150 U polyphosphate kinase (1 unit corresponds to the transfer of 1 pmol P₁ to ADP/min), 5 mM MgCl₂, 50 mM Tris and 50 mM malate (pH 6.5). The reactions were incubated for 16 h at 30°C. ³²P-labeled poly P was purified as described (33) and was eluted in 100 μl water. Radioactivity was measured in a scintillation counter (LS1801, Beckman Instruments, Fullerton, California). The poly P concentration was calculated based on the fraction of ³²P that was incorporated in poly P (resulting typically in ~3 mM considering phosphate residues).

**Poly P binding**

Poly P binding assays were performed as reported earlier (16). Purified poly(A) polymerase from Saccharomyces cerevisiae and Bos taurus was diluted to a concentration of 200 nM in 100 μl reaction buffer (50 mM Tris–HCl pH 7.5, 5 mM MgCl₂). Equal volumes of radioactive poly Pₐₕ₀ (0.3–30 nM, 890 c.p.m./pmol for yeast and 132 000 c.p.m./pmol for bovine poly(A) polymerase) were added. After 5 min at 37°C, the mixtures were applied to nitrocellulose filter discs (0.45 μm) and washed twice with 1 ml ice cold TBS (50 mM Tris–HCl pH 7.5, 100 mM NaCl). The remaining radioactivity on the filters, corresponding to the amount of poly P–protein complex, was measured in a scintillation counter.

**RESULTS**

**Δpho85 and Δpho80 strains are hypersensitive towards cordycepin**

Poly(A) addition by poly(A) polymerase is terminated in vitro in the presence of cordycepin triphosphate (CoTP) (44,45). Since temperature-sensitive mutations in the essential 3' end formation factors Rna14p and Rna15p render yeast cells sensitive to cordycepin (3'−deoxyadenosine) (46), we reasoned that a growth phenotype in the presence of this drug may identify mutant strains involved in poly(A) metabolism. Cordycepin is taken up by the yeast (47) and converted into the RNA-chain terminating CoTP, which is a substrate for RNA synthesis. The presence of 40 μg/ml cordycepin in the medium has only mild toxic effects on wild-type cells and increased the doubling time in liquid culture by ~20% (data not shown). In contrast, a strain lacking the 3' end formation factor Ref2p (48) showed severe growth inhibition (Figure 1A). Thus, strains lacking non-essential genes involved in poly(A) metabolism can be identified through cordycepin-hypersensitive growth.

We screened a haploid S. cerevisiae gene deletion collection (49) for growth defects in the presence of cordycepin using a simple drop test on agar plates. The entire results of this screening will be presented elsewhere (S.H. and B.D., unpublished data). Here we focus on the further characterization of cordycepin-hypersensitive growth observed with Δpho85 and Δpho80 strains (Figure 1A). Complementation of the mutant strains with plasmids carrying wild-type PHO85 and PHO80 genes, respectively, demonstrated a direct requirement for these genes for cordycepin resistance. Empty plasmids, in contrast, did not complement. Pho85p kinase and its cyclin partner Pho80p form a complex and are central regulators of the PHO pathway in yeast (Figure 1B) (50). However, Pho85p can associate with nine other cyclins to perform functions in various cellular pathways including the cell cycle, carbon-source utilization and glycogen metabolism (51). We also tested mutant strains of seven other Pho85p cyclins (Figure 1C; Δpcl1, Δpcl2, Δpcl6, Δpcl7, Δpcl8, Δpcl9 and Δclgl). Since we could not observe sensitivity with these strains, the cordycepin-hypersensitive growth of Δpho85 and Δpho80 appeared to be mainly linked to their function in phosphate homeostasis. The absence of Pho85p or Pho80p results in constitutively active transcription of phosphate-dependent genes (50). To evaluate the relation of other PHO pathway components and the observed cordycepin sensitivity, we analyzed strains lacking the transcription factors Pho4p and Pho2p, the cyclin-dependent kinase inhibitor Pho81p, the high-affinity phosphate transporter Pho84p and the target gene PHO5 (encoding a secreted acid
phosphatase). None of these strains reacted to the drug (Figure 1C and data not shown). We conclude that a constitutively active PHO pathway underlies the observed cordycepin hypersensitivity of Δpho85 and Δpho80 mutants.

**Δpho85 and Δpho80 strains accumulate an inhibitor of poly(A) polymerase**

Since CoTP can act as a terminator of the poly(A) addition reaction in vitro (44,45), we considered the possibility that cordycepin hypersensitivity of Δpho85 and Δpho80 strains may relate to this process. Therefore, we isolated total RNA from wild-type and mutant strains and analyzed the length distribution of cellular poly(A).

The protocol for poly(A) labeling that we employed included the transfer of radioactively labeled [α-32P]-CoTP to 3’-OH groups present in the RNA preparation with recombinant yeast poly(A) polymerase (40). With wild-type RNA, a uniformly distributed length of approximately 10–70 adenosines was observed (Figure 2A). Quite unexpectedly, we were unable to label any poly(A) isolated from Δpho85 and Δpho80 strains under standard assay conditions (i.e. 1–2 μg of total RNA; Figure 2A). We considered the possibility that some inhibitory activity may be present in the RNA preparations of the mutant strains interfering with the activity of poly(A) polymerase and performed the labeling assay with decreasing amounts of total RNA. We observed that a 4- to 8-fold dilution of the total RNA from Δpho85 and Δpho80 strains indeed allowed poly(A) labeling (Figure 2A). Furthermore, reduced amounts of wild-type RNA gave increased labeling efficiency. We conclude that an inhibitory activity was present in the total RNA preparations from Δpho85 and Δpho80 strains interfering with the poly(A) labeling test and that the same inhibitor was also present in wild-type RNA, albeit at lower concentration.

**Poly P accumulates in Δpho85 and Δpho80 strains and interferes with RNA 3’ end labeling by poly(A) polymerase**

We hypothesized that the co-purification of a metabolite with Δpho85 and Δpho80 RNA may cause the inhibition of the poly(A) labeling reaction. It was suggested that the PHO pathway is involved in regulating the synthesis of poly P (6). However, inconsistent results were obtained when Δpho85 and Δpho80 strains were previously analyzed for poly P contents (6,52). Therefore, we measured poly P levels in wild-type and mutant strains with a recently developed assay (32) and found that Δpho85 and Δpho80 strains had approximately 3-fold more poly P than wild type (Figure 2B). In contrast,
mutants in other components of the \textit{PHO} pathway did not show this accumulation; indeed poly P concentrations were reduced in \textit{D\textsubscript{pho4}}, \textit{D\textsubscript{pho2}} and \textit{D\textsubscript{pho84}} mutant strains (Figure 5D) \cite{6,53}. These results support the idea that poly P metabolism is regulated by the \textit{PHO} pathway and that constitutive activity of this pathway (in the absence of Pho85p and Pho80p) resulted in increased cellular poly P concentrations.

The accumulation of poly P in \textit{D\textsubscript{pho85}} and \textit{D\textsubscript{pho80}} strains prompted us to test whether poly P is an inhibitor of poly(A) polymerase. Therefore, we repeated the poly(A) labeling test with standard amounts of RNA (2 \textmu g, 1 \textmu g, 0.5 \textmu g, 250 ng, 125 ng and 75 ng) were included with recombinant yeast Pap1p in the reactions. Following RNase A/T1 digestion, the labeled poly(A) tails were loaded on a 15\% denaturing polyacrylamide gel. Poly(A) labeling was observed in \textit{D\textsubscript{pho80}} and \textit{D\textsubscript{pho85}} only following dilution of the RNA included in the reactions. HpaI-digested pBR322 fragments were 5\'-end labeled and served as marker bands (M) with the indicated size. (B) Poly P contents in wild-type, \textit{D\textsubscript{pho80}} and \textit{D\textsubscript{pho85}} strains following growth in YPD medium for 6 h. (C) Poly(A) labeling assay as described in (A) with the addition of \textit{D\textsubscript{pho80}} RNA (lane 3) or of increasing amounts of poly P (60 nM to 6 \textmu M poly P\textsubscript{28}; 27 nM to 2.7 \textmu M poly P\textsubscript{61}; and 2 nM to 200 nM poly P\textsubscript{750}) with the indicated average chain length (lanes 4–12) to poly(A)-labeling assays with wild-type RNA. Lane 2 shows the reaction of wild-type RNA without addition of inhibitors. (D) Poly(A)-labeling assay as described in (A) with total RNA obtained from wild-type, \textit{D\textsubscript{pho80}} and \textit{D\textsubscript{pho85}} strains. RNAs were treated as indicated with yeast exopolyphosphatase (scPpx1p), with heat-denatured exopolypolyphosphatase (denat. Ppx1p) or buffer (mock) before the labeling reaction was performed.

![Figure 2. \textit{D\textsubscript{pho85}} and \textit{D\textsubscript{pho80}} strains accumulate poly P, an inhibitor of poly(A) polymerase. (A) Poly(A)-labeling assays with total RNA extracted from wild-type, \textit{D\textsubscript{pho80}} and \textit{D\textsubscript{pho85}} strains. Decreasing amounts of total RNA (2 \textmu g, 1 \textmu g, 0.5 \textmu g, 250 ng, 125 ng and 75 ng) were included with recombinant yeast Pap1p in the reactions. Following RNase A/T1 digestion, the labeled poly(A) tails were loaded on a 15\% denaturing polyacrylamide gel. Poly(A) labeling was observed in \textit{D\textsubscript{pho80}} and \textit{D\textsubscript{pho85}} only following dilution of the RNA included in the reactions. HpaI-digested pBR322 fragments were 5\'-end labeled and served as marker bands (M) with the indicated size. (B) Poly P contents in wild-type, \textit{D\textsubscript{pho80}} and \textit{D\textsubscript{pho85}} strains following growth in YPD medium for 6 h. (C) Poly(A) labeling assay as described in (A) with the addition of \textit{D\textsubscript{pho80}} RNA (lane 3) or of increasing amounts of poly P (60 nM to 6 \textmu M poly P\textsubscript{28}; 27 nM to 2.7 \textmu M poly P\textsubscript{61}; and 2 nM to 200 nM poly P\textsubscript{750}) with the indicated average chain length (lanes 4–12) to poly(A)-labeling assays with wild-type RNA. Lane 2 shows the reaction of wild-type RNA without addition of inhibitors. (D) Poly(A)-labeling assay as described in (A) with total RNA obtained from wild-type, \textit{D\textsubscript{pho80}} and \textit{D\textsubscript{pho85}} strains. RNAs were treated as indicated with yeast exopolyphosphatase (scPpx1p), with heat-denatured exopolypolyphosphatase (denat. Ppx1p) or buffer (mock) before the labeling reaction was performed.]

![Image 1](image1.png)

![Image 2](image2.png)

![Image 3](image3.png)

![Image 4](image4.png)
Ppx1p pre-treatment was also enhanced, demonstrating the presence of poly P also in wild-type RNA, consistent with our poly P measurements in wild-type strains (Figure 2B). Taken together the results from this figure show that poly P accumulated in \( \text{Dpho85} \) and \( \text{Dpho80} \) strains, that this metabolite co-purified with total RNA extracted from mutant and wild-type strains and that poly P was responsible for the observed inhibition of poly(A) polymerase.

Poly P binds poly(A) polymerase

Since the poly(A)-labeling assay uses CoTP, poly(A) polymerase activity is restricted to a single round of nucleoside addition. To examine the effects of poly P under multiple turn-over conditions, we performed poly(A) polymerase assays with an end-labeled A\(_{15}\) RNA primer, cold ATP and recombinant yeast Pap1p (Figure 3A). Under the conditions employed, yeast Pap1p extended the primer to \( \sim 300 \) to 400 adenosines in 60 min. In the presence of 2 nM poly P\(_{750}\), we observed a slight reduction of the length of the polyadenylation products during the time course. This effect was stronger with 20 nM poly P\(_{750}\) and 200 nM resulted in almost complete inhibition. Similar effects were observed when poly P of varying length (average of 28 and 61 phosphate residues) was tested (data not shown). The poly P concentrations used for these assays are likely to be physiologically relevant, as yeast nuclei are thought to harbor poly P concentrations of up to 89 \( \mu \text{M} \) (1).

Next, we tested whether poly P also inhibited the activity of bovine poly(A) polymerase. Under the employed conditions bovine Pap extended the A\(_{15}\) RNA primer by approximately 100 adenosines within 1 hour (Figure 3B). When 20 nM to 2 \( \mu \text{M} \) of poly P\(_{750}\) were included in the reaction, the length of the products was reduced but inhibition was not complete. Therefore, poly P was a less potent inhibitor of bovine poly(A) polymerase compared to the yeast enzyme.
Poly P is an efficient chelator of divalent metal ions (1). A possible explanation for the inhibitory effect on poly(A) polymerase activity could be the chelation of magnesium ions which are essential for Pap1p function. However, the magnesium in our assays is present in large molar excess over inhibitory poly P concentrations (calculated as the amount of free inorganic phosphate). Therefore, we considered the possibility that poly P may inhibit the enzymes by direct binding. To test this, we performed filter-binding assays with radioactively labeled poly P 750 and yeast or bovine poly(A) polymerase. We determined an apparent dissociation constant of 0.38 nM for the yeast enzyme and of 2.7 nM for the bovine enzyme (Figure 4). It has to be pointed out, however, that one poly P molecule is likely to provide multiple binding sites for Pap1p. Therefore, it seems possible that binding was influenced by avidity effects. Irrespective of that, the tight binding of poly P to poly(A) polymerase was most likely responsible for the observed inhibitory effects of poly P in polyadenylation assays.

To address the mechanisms of inhibition, we considered the possibility that poly P binding may result in unspecific aggregation or precipitation of the enzyme. To test this idea we incubated Pap1p with increasing amounts of poly P, collected protein aggregates by centrifugation and resolved remaining soluble material by SDS–PAGE. As shown in Figure 3C, poly P binding did not result in precipitation of the enzyme. Thus, unspecific aggregation can be excluded as a possible reason for the observed inhibition. To test whether the inhibition of poly P on Pap1p activity was reversible, we pre-incubated Pap1p with 200 nM poly P to allow binding of poly P and Pap1p. As expected, this resulted in strong inhibition of polyadenylation activity (Figure 3D, lanes 7–11). However, Pap1p activity could be almost completely restored by treatment of the Pap1p–poly P complexes with Ppx1p (Figure 3D, lanes 12–16). This demonstrated that the inhibition of Pap1p by poly P was reversible.

The Pap1p that we used in the poly(A) length analysis and in the polyadenylation assay was purified from E. coli, which also contains poly P (1). Thus, purified Pap1p may already be partially bound to poly P causing a partial inhibition of the enzymatic activity. To test this, we performed a polyadenylation assay with Pap1p that was pretreated with Ppx1p. Since the treatment did not noticeably increase the activity of Pap1p preparation (data not shown), we conclude that most of Pap1p that we used in our experiments was not pre-bound by poly P. This indicated that the conditions used for over-expression of Pap1p did not induce significant poly P accumulation in E. coli.

### Poly P accumulation contributes to cordycepin sensitivity

We showed that Apho85 and Apho80 strains are cordycepin hypersensitive, that these strains accumulated poly P and that poly P acted as an efficient inhibitor of poly(A) polymerase. These observations suggested a
correlation between cellular poly P accumulation and the drug-dependent growth defect. Ogawa and co-workers (6) reported accumulation of poly P in strains mutant for PHM5, PHM6, PHM2 and CTF19 during the so-called ‘overplus’ conditions. However, we could not observe cordycepin-hypersensitive growth associated with these mutant strains (Figure 5A). Moreover, our poly P measurements in these strains did not indicate a significant increase of the polymer under conditions, which revealed a strong increase in Δpho85 and Δpho80 strains (Figure 5B). The differences between the measurements presented here and in Ogawa et al. are possibly due to differences in growth conditions at the time of measurement. Furthermore, our recent genome-wide assessment of poly P metabolism failed to identify other mutants that would result in poly P accumulation comparable to levels observed in Δpho85 and Δpho80 strains (38). It seems possible, therefore, that cordycepin-hypersensitive growth is a consequence of elevated poly P levels exceeding a certain threshold in Δpho85 and Δpho80 strains and that such levels are not achieved or stably maintained in other mutants defective in poly P metabolism.
To further test whether cordycepin sensitivity was due to poly P accumulation in \( \Delta \text{pho85} \) and \( \Delta \text{pho80} \) mutants, we interfered with the ability of the strains to synthesize poly P and asked whether this alleviated cordycepin sensitivity. When low concentrations of phosphate (0.1 mM) are present in the medium growth of the yeast is only marginally reduced, but no significant poly P accumulation can be measured (32). We tested \( \Delta \text{pho80} \) strains on plates containing cordycepin but low phosphate and found that \( \Delta \text{pho80} \) strains showed significant resistance to cordycepin under these conditions (Figure 5C). This observation suggested that abundant phosphate was required for the observed cordycepin hypersensitivity. Furthermore, the \( \text{PHO} \) pathway regulates poly P accumulation in yeast (6). Figure 5D shows that cellular levels of poly P were significantly reduced in \( \Delta \text{pho}2 \), \( \Delta \text{pho}4 \) and \( \Delta \text{pho84} \) mutants compared to wild type. We produced double mutants of \( \Delta \text{pho80} \) and other components of the \( \text{PHO} \) pathway and found that \( \Delta \text{pho80} \), \( \Delta \text{pho}4 \), \( \Delta \text{pho80} \Delta \text{pho2} \) and \( \Delta \text{pho80} \Delta \text{pho84} \) double mutants did not accumulate poly P (Figure 5D). The cellular poly P content of the analyzed double mutants correlated well with a loss of cordycepin hypersensitivity associated with the single \( \Delta \text{pho80} \) mutation (Figure 5C). These observations are consistent with the proposal that poly P accumulation in \( \Delta \text{pho80} \) strains contributed to cordycepin hypersensitivity. Increased cellular poly P levels may interfere with poly(A) polymerase activity, and this may cause hypersensitive growth in the presence of cordycepin, another inhibitor of RNA synthesis.

To further test this idea we asked whether a temperature-sensitive mutation in the 3' end formation factor Rna14p (\( rna14-1 \)) can duplicate the effects observed with cordycepin in \( \Delta \text{pho80} \) mutants. It was previously shown that combination of mutations in \( rna14 \) and \( pap1 \) resulted in a synthetic growth defect (39) and \( rna14 \) mutant strains are cordycepin hypersensitive ([46]; our unpublished data). Figure 5E shows that combination of the \( \Delta \text{pho80} \) mutation with the \( rna14-1 \) allele indeed resulted in synthetic lethality. This genetic interaction strongly supported the proposal that accumulation of poly P in \( \Delta \text{pho80} \) strains affected 3' end formation via inhibition of poly(A) polymerase activity and that this inhibition in combination with another defect in 3' end formation led to the observed synthetic growth defect.

**DISCUSSION**

We provide evidence for an unexpected link between poly P metabolism and polyadenylation in yeast. We propose that a synergistic effect of poly P-mediated inhibition of poly(A) polymerase together with inhibition of RNA synthesis by CoTP can cause lethality. Synthetic lethality observed between \( \text{PHO80} \) and an essential factor involved in 3' end formation underscores this assumption. Poly P may therefore play a role in the regulation of poly(A) polymerase activity.

The starting point of this work was an unbiased genome-wide screen for mutants that showed hypersensitive and/or resistant growth in the presence of cordycepin. The rational for this screen was that cordycepin will be converted to CoTP, which will then provoke RNA-chain termination in the nucleus. Although CoTP will terminate RNA synthesis when incorporated during transcription, it seems possible that polyadenylation would be a more sensitive target since up to 70 consecutive adenosines are incorporated during this process. This may be particularly pronounced under the low cordycepin conditions (40 μg/ml) that were employed during our screening; as expected, mutants in 3' end formation factors displayed hypersensitive growth in the presence of such cordycepin concentrations (Figure 1A and data not shown). Although it remains unclear which other cellular ATP-consuming activities are sensitive to cordycepin or CoTP, cordycepin-hypersensitive growth can be indicative of a functional involvement of a mutation in 3' end formation and poly(A) metabolism. A complete account of the results obtained from our cordycepin-dependent growth screening will be presented elsewhere (S.H. and B.D., unpublished data). However, no additional links emerged between cordycepin hypersensitivity and poly P metabolism other than the one presented here for \( \Delta \text{pho85} \) and \( \Delta \text{pho80} \) strains.

The kinase activity of the Pho85p/Pho80p CDK–cyclin complex controls phosphate-dependent gene expression by regulating both the activity and localization of the Pho4p transcription factor (50,55). Interestingly, poly P was proposed to act as a phosphate buffer that can be mobilized to maintain constant internal phosphate levels under conditions where external phosphate levels fluctuate (7,8) and the \( \text{PHO} \) pathway was linked to expression of genes that are involved in poly P homeostasis (6). As a consequence, constitutive activation of the \( \text{PHO} \) pathway (e.g. in \( \Delta \text{pho80} \) strains) was expected to result in increased poly P levels. However, Ogawa and co-workers (6) could not observe increased levels of poly P in \( \Delta \text{pho80} \) strains. In contrast, it was reported that \( \Delta \text{pho85} \) strains have increased poly P (52). We re-evaluated the poly P content of \( \Delta \text{pho85} \) and \( \Delta \text{pho80} \) strains and detected an approximately 3-fold increase in both strains compared to wild-type (Figure 2B). We attribute these conflicting poly P measurements to fluctuations of poly P levels in the yeast depending on the growth state and recently proposed a standardized procedure for poly P determination (32).

We found that the presence of increased amounts of co-purified poly P in RNA preparations from \( \Delta \text{pho85} \) and \( \Delta \text{pho80} \) strains was accompanied by a strong inhibition of poly(A) polymerase activity. Consistent with our observations, previous biochemical work on the effects of poly P on yeast Pap1p revealed an inhibitory activity on tRNA primed poly(A) synthesis (56). We show in this work by filter-binding assays that poly P directly binds to yeast Pap1p with a \( k_D \) in the low nanomolar range (0.38 nM). This value is comparable to the \( k_D \) of 0.48 nM that was determined for the poly P–Lon interaction, which stimulates Lon protease activity (16). Pap1p and Lon are thus the interactors with the highest reported affinities for poly P. Consistent with a physiological relevant role for the interaction of poly P and Pap1p, nuclear poly P levels were reported to reach micromolar concentrations (1); furthermore, nuclei predominantly contain...
poly P of an average chain length of approximately 45 phosphate residues (33), and poly P with similar length (poly P$_{28}$ and poly P$_{69}$) acted as efficient inhibitor of Pap1p in our in vitro experiments (Figure 2C). We have not delineated the binding site of poly P on Pap1p, but potential interaction surfaces include regions of RNA primer binding or of ATP binding (57,58). Notably, binding of poly P to bovine poly(A) polymerase and inhibition of its activity is approximately 10-fold weaker compared to the yeast enzyme. This may have important consequences on a potential in vivo interaction of poly P and poly(A) polymerases in higher eukaryotes.

What is the biological role of poly P-mediated inhibition of poly(A) polymerase activity? We predict that such a function must be connected to growth conditions that are accompanied by high poly P concentrations and that the control of poly P levels is at least partially under control of the PHO pathway. We recently showed that poly P content reaches a peak when cells experience the diauxic shift following depletion of glucose from the medium (32). This growth phase is characterized by major changes in the gene expression program (59,60). It seems possible that the down-regulation of many hundreds of genes during diauxic shift is accompanied by an inhibition of polyadenylation. In this context, it is worth pointing out that cellular poly(A) levels inversely correlate with poly P levels when cells enter diauxic shift (32,60). Our observation that the inhibitory effects of poly P on Pap1p were completely reversible (Figure 3D) may indicate that poly P action can be transient and controlled through a dynamic nuclear poly P metabolism (33). Furthermore, fluctuations of nuclear poly P levels may interfere with RNA processing in a microenvironment as was suggested for the regulation of the bacterial degradosome (26). Since no global polyadenylation defect could be observed at steady state in Apho80 and Apho85 strains (Figure 2D) poly P may act on specific gene loci and not as a general suppressor of Pap1p activity. A deeper understanding of poly P biochemistry and metabolism will be required to address these possibilities in future experiments.

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REFERENCES


