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Inositol polyphosphate multikinase (ArgRIII) determines nuclear mRNA export in *Saccharomyces cerevisiae*

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Abstract The *ARGRIII* gene of *Saccharomyces cerevisiae* encodes a transcriptional regulator that also has inositol polyphosphate multikinase (ipmk) activity [Saiardi et al. (1999) Curr. Biol. 9, 1323–1326]. To investigate how inositol phosphates regulate gene expression, we disrupted the *ARGRIII* gene. This mutation impaired nuclear mRNA export, slowed cell growth, increased cellular [$InsP_3$] 170-fold and decreased [$InsP_6$] 100-fold, indicating reduced phosphorylation of $InsP_3$ to $InsP_6$. Levels of diphosphoinositol polyphosphates were decreased much less dramatically than was $InsP_6$. Low levels of $InsP_6$, and considerable quantities of Ins(1,3,4,5)P_4, were synthesized by an ipmk-independent route. Transcriptional control by ipmk reflects that it is a pivotal regulator of nuclear mRNA export via inositol phosphate metabolism.

Key words: *ARGRIII*; Inositol polyphosphate multikinase; Inositol phosphate; $InsP_6$; mRNA export; Yeast

1. Introduction

Several years ago, it was shown that a *Saccharomyces cerevisiae* gene, *ARGRIII*, controls the transcription of several enzymes regulating arginine metabolism [1–3]. In addition, this gene was established to be pleiotropic. This idea recently returned to prominence with the discovery that the *ARGRIII* gene product has inositol polyphosphate multikinase (ipmk) activity, which can catalyze the ATP-dependent phosphorylation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 [4]. These developments suggest that inositol phosphates may mediate some of the functions of *ARGRIII* that relate to transcriptional regulation.

Another recent study [5] also strongly indicates that there is a link between the turnover of inositol phosphates and the regulation of gene expression. In the latter investigation, three *S. cerevisiae* genes were identified which, when deleted, resulted in decreased efficiency of mRNA export from the nucleus via the nuclear pore complex [5]. In one of these mutant yeast strains, named gsl3, the deficiency in mRNA export was accompanied by a block in the pathway of $InsP_6$ synthesis, at the stages at which both $InsP_3$ and $InsP_4$ are phosphorylated [5]. Since the nature of the genetic defect in the gsl3 yeast strain was not identified, it was speculated that an essential regulator of separate $InsP_3$ and $InsP_4$ kinase activities was compromised [5]. Alternately, it was also pointed out that the gsl3 strain of yeast might be lacking a single, bi-functional kinase activity that phosphorylates both $InsP_3$ and $InsP_4$ [5].

We have now pursued the possibility that a loss of the Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 kinase activities of ipmk could account for the metabolic defect in the gsl3 strain. We also sought more information concerning the role of the ipmk protein in this newly emerging relationship between inositol phosphate turnover and control over gene expression. By creating and studying a strain of *S. cerevisiae* from which the *ARGRIII* gene is deleted, we reveal new aspects to inositol phosphate turnover in yeast, and we show that ipmk activity regulates nuclear export of mRNA.

2. Materials and methods

2.1. Generation and analysis of ipmkΔ yeast

The YDR173C open reading frame (ORF) in *S. cerevisiae* comprises the *ARGRIII* gene that encodes ipmk activity. The entire *ARGRIII* gene in *S. cerevisiae* (strain PJ69-2A), including start and stop codons, was replaced by the dominant *kan*^{r} marker gene using the KanMX4 expression construct as previously described [6]. The oligonucleotides used for the gene disruption were: 5′-ATGGATATGTGCATACGTGTGCCTAAGTAGAAATTTTTTTCACGCATAGGCCACTAGTGGATCTG-3′ and 5′-GTACCATATCCCATAAACAAGGTAAACTTCACCTCTCAATATACAGCTGAAGCTTCGTACGC-3′. To confirm the correct integration of the marker gene, diagnostic PCR was performed using the following oligonucleotides: 5′-GATAAAGCCATTGGAGATCC-3′ and 5′-AATGCAAACACAATAATACC-3′. The sizes of the diagnostic PCR products derived from the wild-type and mutant (*argRIII::KanMX4* or *ipmk*Δ) strains were 1.4 and 1.8 kb, respectively, close to the sizes of the *ARGRIII* gene (1.3 kb) and the *kan*^{r} gene (1.7 kb). The structures of the PCR products were also verified by direct sequencing.

2.2. Yeast culturing and radiolabeling with [^{3}H]inositol

Yeast cultures were grown in YPD media containing 2% (w/v) glucose. Duplicate 5 ml cultures were seeded at 3×10^4 cells/ml, [^{3}H]inositol (100 μCi/ml, American Radiolabeled Chemicals, MO, USA) was added, and cells were grown at 23°C through at least eight doublings. Cultures were then shifted to 37°C for an additional 2 h. 10^8 cells were harvested from each culture, washed twice with ice-cold water and snap-frozen until lysis. Cell pellets were lysed in 0.2 ml ice-cold lysis buffer (2 M perchloric acid/0.1 mg/ml $InsP_6$/2 mM EDTA) with two cycles of vigorous bead beating (4 mm glass beads pre-soaked in lysis buffer). Lysates were centrifuged for 5 min at 4°C and neutralized [7].

Inositol phosphates were then resolved on two complementary high performance liquid chromatography (HPLC) systems [8,9]: (i) a Partisphere SAX HPLC column (Krackler Scientific, NC, USA) was eluted

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with a gradient generated by mixing buffer A (1 mM Na_2EDTA) and buffer B (buffer A plus 1.3 M $(NH_4)_2HPO_4$, pH 3.85, with H_3PO_4) as follows: 0–5 min, 0% B; 5–10 min, 0–50% B; 10–60 min, 50–100% B; 60–70 min, 100% B. (ii) a SynChropak Q100 SAX HPLC column (Thompson Instrument, VA, USA) was eluted with a gradient generated by mixing buffer A (1 mM Na_2EDTA) with buffer B (buffer A plus 2 M $(NH_4)H_2PO_4$, pH 3.35, with H_3PO_4): 0–5 min, 0% B; 5–120 min, 0–65% B.

Inositol phosphates were identified by their co-elution with the following authentic standards: [^{3}H]Ins(1,4,5)P_3, [^{3}H]Ins(1,3,4,5)P_4 and [^{3}H]InsP_6 (New England Nuclear), [^{3}H]Ins(1,3,4,5,6)P_5 [10], [^{3}H]Ins(1,2,4,5,6)P_5 [11], [^{3}H]PP-InsP_5 and [^{3}H][PP]$_2$-InsP_4 [12] and ^{14}C-labeled Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4, D/L-Ins(1,4,5,6)P_4 and Ins-(1,3,4,5,6)P_5 isolated from [^{14}C]inositol-labeled, parotid acinar glands [13].

2.3. Assay of ipmk activity in vitro

The ORF for *ARGRIII* that encodes ipmk activity was recovered from the pCMV-GST vector [4] after digestion with *Sal*I and *Not*I restriction enzymes, and subcloned into the *Sal*I and *Not*I sites of the pGEX-4T-2 expression vector (Amersham Pharmacia Biotech). The methods used to transform *Escherichia coli* (strain BL21), to induce with IPTG, and to isolate the GST fusion protein using glutathione Sepharose 4B resin were all according to the manufacturer's recommendations (Amersham Pharmacia Biotech). Ipmk activity was assayed by incubating the protein for 5–60 min at 37°C with trace amounts (10 000 dpm) of the appropriate ^{3}H-labeled inositol phosphate in 25 μl assays containing 20 mM HEPES (pH 7.0), 12 mM $MgSO_4$, 10 mM Na_2ATP, 20 mM phosphocreatine, 1 mM DTT, 1 mM EDTA, 360 U phosphocreatine kinase (Calbiochem) and 0.5 mg/ml bovine serum albumin (BSA). Assays were quenched with PCA, neutralized with KCO_3 as described previously [7], and inositol phosphates were resolved by HPLC (see above).

2.4. Localization of poly(A)$^+$ RNA by in situ hybridization

Early logarithmic phase cultures of yeast were grown at 23°C before shifting to 37°C for 2 h. Cells were harvested and fixed for 45 min in 4% formaldehyde in 0.1 M phosphate buffer (K^+ salt, pH 6.5) and washed three times in solution A (1.2 M sorbitol, 0.1 M phosphate buffer (K^+ salt, pH 7.5)). Cells were treated for 45 min at 30°C in solution A plus 0.1 mg/ml of lyticase (Sigma); resulting spheroplasts were washed in solution A and plated onto a polylysine-coated slide for 45 min at 4°C. Samples were washed and dehydrated in 70% EtOH overnight at −20°C. Samples were treated for hybridization as previously described [14]. Then, samples were washed three times in 2×SSC at 37°C for 10 min, and then three times in 1×SSC at room temperature for 10 min. Cells were permeabilized for 5 min at room temperature in solution B (150 mM NaCl, 100 mM Tris–HCl pH 7.5) containing 0.1% (v/v) Triton X-100, and then washed in solution B and incubated for 1 h at room temperature with anti-digoxigenin-fluorescein Fab fragment (Boehringer Mannheim) in solution B plus 1% (w/v) BSA. The slides were washed at room temperature in solution B for 5 min, then washed in solution B containing 100 ng/ml 4′,6-diamidino-2-phenylindole for 10 min, followed by a final wash in 300 mM NaCl, 100 mM Tris–HCl pH 7.5 for 15 min. Photographs were taken with a 60× objective on a Zeiss Axioskop microscope with Kodak Elite 400 film.

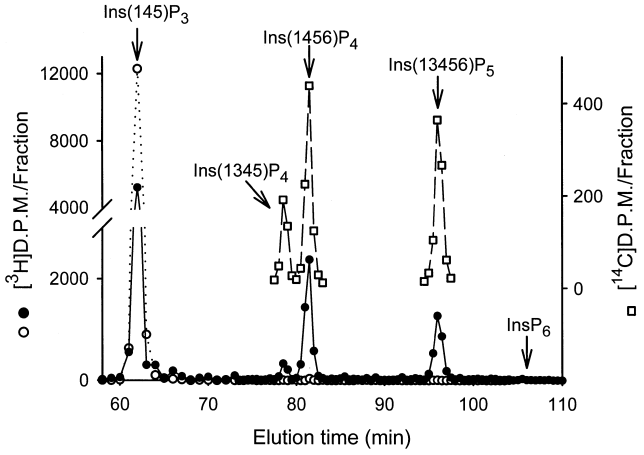

Fig. 1. Products of Ins(1,4,5)P_3 phosphorylation by ipmk in vitro. [^{3}H]Ins(1,4,5)P_3 was incubated for 0 min (open circles) and 10 min (closed circles) with 2 ng ipmk (see Section 2). Samples were quenched and neutralized and chromatographed on a SynChropak Q100 SAX HPLC column (see Section 2); 0.5 ml fractions were collected. The ^{3}H-labeled products, namely, Ins(1,3,4,5)P_4, Ins-(1,4,5,6)P_4 and Ins(1,3,4,5,6)P_5, were all identified by their co-elution with internal ^{14}C-labeled standards (squares) of these three compounds. The elution position of InsP_6 is also indicated by an arrow. Data are representative of three experiments.

3. Results and discussion

3.1. Analysis of ipmk activity in vitro

Ipmk was recently shown to phosphorylate both Ins-(1,4,5)P_3 and Ins(1,3,4,5)P_4, although the products of these reactions were not characterized [4]. We have now used HPLC to determine that ipmk catalyzed the phosphorylation of Ins(1,4,5)P_3 at both the 3- and 6-positions, forming Ins-(1,3,4,5)P_4 and Ins(1,4,5,6)P_4 (Fig. 1). InsP_5 was also formed (Fig. 1) by further phosphorylation of both InsP_4 isomers (data not shown). This InsP_5 was shown to be the 1,3,4,5,6-isomer; no Ins(1,2,4,5,6)P_5 was formed (data not shown). The fact that Ins(1,4,5)P_3 is phosphorylated to Ins(1,4,5,6)P_4 by ipmk (which is 40 kDa [4]) suggests ipmk is equivalent to the Ins(1,4,5)P_3 6-kinase activity of a 40 kDa protein that was partly purified from *S. cerevisiae* several years ago [15]. The authors of the latter study found that their preparations of partly purified Ins(1,4,5)P_3 6-kinase also phosphorylated Ins(1,4,5,6)P_4 to Ins(1,3,4,5,6)P_5, but the latter 3-kinase activity was attributed to a different, contaminating enzyme [15]. In contrast, our data show it is ipmk that phosphorylates

Table 1
Levels of inositol phosphates in wild-type and *ipmk*Δ yeast

	DPM in wild-type	DPM in *ipmk*Δ	*ipmk*Δ/wild-type
InsP_2	3 447 ± 571	248 502 ± 43 908	72
Ins(1,4,5)P_3	921 ± 178	159 709 ± 11 642	173
Ins(1,4,5,6)P_4	349 ± 38	0	–
Ins(1,3,4,5)P_4	0	6 046 ± 316	–
X	490 ± 161	1 659 ± 582	3.4
Ins(1,3,4,5,6)P_5	1 104 ± 300	159 ± 48	0.14
D/L-Ins(1,2,3,4,5)P_5	148 ± 23	2 724 ± 631	18.4
D/L-Ins(1,2,4,5,6)P_5	2 750 ± 852	0	–
InsP_6	56 167 ± 7 502	519 ± 106	0.009
PP-InsP_5	420 ± 78	133 ± 22	0.32
[PP]$_2$-InsP_4	460 ± 53	65 ± 48	0.14

Data (mean ± S.E.M.) are taken from three experiments similar to those described in Fig. 2.

Ins(1,4,5,6)P_4 to Ins(1,3,4,5,6)P_5 (Fig. 1). Ipmk is also probably very similar to a 41 kDa kinase purified from *Schizosaccharomyces pombe* that phosphorylates Ins(1,4,5)P_3 to Ins(1,3,4,5,6)P_5, via Ins(1,4,5,6)P_4 and Ins(1,3,4,5)P_4 [16].

3.2. The inositol phosphate profile in wild-type and ipmkΔ strains of S. cerevisiae

The inositol phosphate profiles of the wild-type and *ipmk*Δ strains were compared. A major difference was the accumulation of large quantities of Ins(1,4,5)P_3 in the mutant strain, to levels that were 173-fold above those of the wild-type (Fig. 2; Table 1). Ins(1,4,5)P_3 accumulated because it continued to be produced from the yeast's ongoing PLC activity [5], but it was no longer phosphorylated by ipmk. The *ipmk*Δ yeast also had 72-fold higher levels of $InsP_2$ than did the wild-type (Fig. 2, Table 1), presumably reflecting some dephosphorylation of the excess Ins(1,4,5)P_3. In yeasts, Ins(1,4,5)P_3 has been proposed to be a precursor for the synthesis of $InsP_5$ and $InsP_6$ [5,16]. This hypothesis is supported by the observations that, relative to the wild-type yeast, the *ipmk*Δ strain contained 100-fold lower levels of $InsP_6$, and at least 10-fold lower levels of D/L-Ins(1,2,4,5,6)P_5 and Ins(1,3,4,5,6)P_5 (Fig. 2).

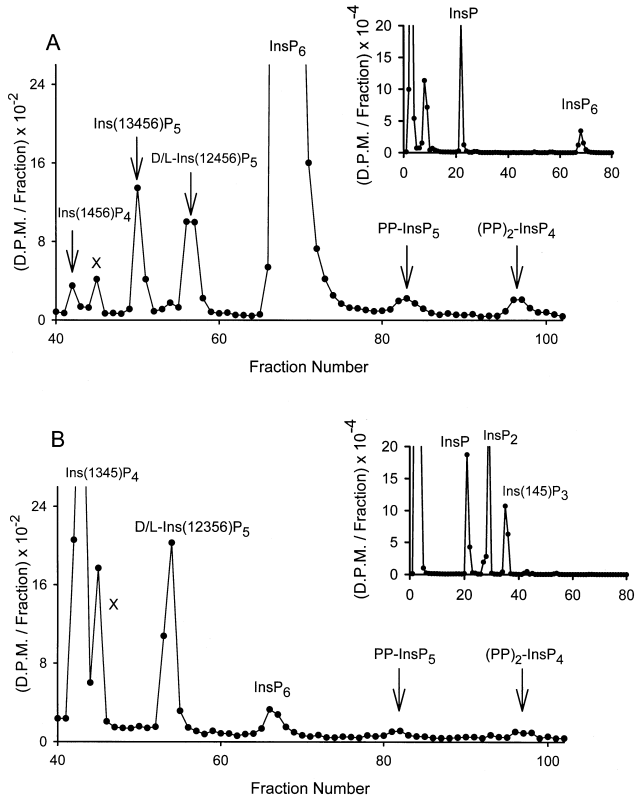

Fig. 2. Partisphere SAX HPLC analysis of inositol phosphates in wild-type and *ipmk*Δ yeast. [^{3}H]Inositol phosphates were resolved from [^{3}H]inositol-labeled wild-type (A) and *ipmk*Δ cells (B), using a Partisphere SAX column (see Section 2); 1 ml fractions were collected. The inset to each panel shows the same data using a different *y*-axis scale. The structures of several inositol phosphates (Ins(1,4,5)P_3, Ins(1,3,4,5,6)P_5, D/L-Ins(1,2,3,5,6)P_5, $InsP_6$, PP-$InsP_5$ and $[PP]_2$-$InsP_4$) were assigned from their elution times matching those of corresponding, authentic ^{3}H-labeled standards, which were separately determined in parallel HPLC runs. The D/L-Ins(1,2,3,5,6)P_5 was tentatively identified from its known [8] elution position between Ins(1,3,4,5,6)P_5 and D/L-Ins(1,2,4,5,6)P_5. The identification of Ins(1,3,4,5)P_4 and Ins(1,4,5,6)P_4 is described by Fig. 3. Peak 'X' was not identified. Data are representative of three experiments.

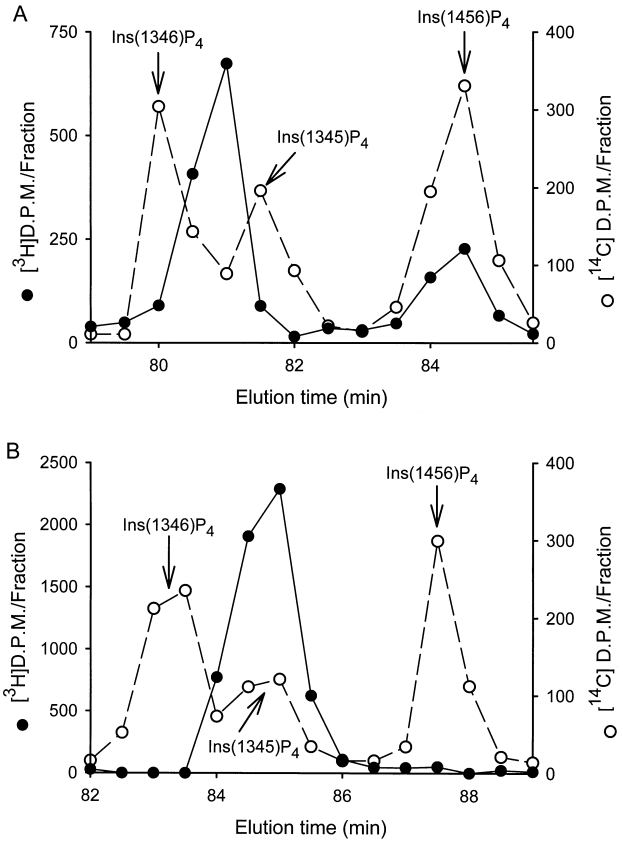

Fig. 3. SynChropak Q100 SAX HPLC analysis of inositol phosphates in wild-type and *ipmk*Δ yeast. [^{3}H]InsP_4 isomers (filled circles) from wild-type (A) and *ipmk*Δ cells (B) were resolved using a SynChropak Q100 SAX column (see Section 2); 0.5 ml fractions were collected. Elution positions of authentic ^{14}C-labeled internal standards (open circles) of Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4 and Ins(1,4,5,6)P_4 are marked. In extracts prepared from wild-type cells (A), two ^{3}H-labeled InsP_4 isomers were observed. One co-eluted with [^{14}C]Ins(1,4,5,6)P_4 and the other (corresponding to peak 'X' in Fig. 2) eluted between standards of Ins(1,3,4,6)P_4 and Ins(1,3,4,5)P_4. In extracts prepared from *ipmk*Δ cells, one ^{3}H-labeled peak co-eluted with a standard of [^{14}C]Ins(1,3,4,5)P_4. Note that this SynChropak HPLC column does not resolve Ins(1,3,4,5)P_4 from the minor, contaminating peak 'X'. These two peaks were, however, separated on a Partisphere SAX column (Fig. 2). Data are representative of three independent experiments. Absolute elution times differ in A and B because of batch variation in the two Synchropak columns that were used for these experiments.

The profile of [^{3}H]InsP_4 isomers was altered in the *ipmk*Δ yeast. Wild-type cells contained two InsP_4 peaks. One of these, which was not identified (peak 'X'), eluted between standards of Ins(1,3,4,6)P_4 and Ins(1,3,4,5)P_4 on a SynChropak SAX HPLC column (Fig. 3A). The other InsP_4 co-eluted with Ins(1,4,5,6)P_4 (Fig. 3A). In contrast, Ins(1,4,5,6)P_4 was absent in the *ipmk*Δ strain (Fig. 3B), due to the elimination of the Ins(1,4,5)P_3 6-kinase activity of ipmk. In addition, the *ipmk*Δ cells were found to contain considerable quantities of another InsP_4 isomer, namely Ins(1,3,4,5)P_4 (on the Synchropak HPLC column, Ins(1,3,4,5)P_4 was contaminated with peak 'X' (Fig. 3B), but these two compounds were resolved when cell extracts were analyzed with a Partisphere SAX column (Fig. 2B)). Since Ins(1,3,4,5)P_4 was not detected in wild-type yeast (Fig. 3A), there is a dramatic up-regulation of Ins(1,3,4,5)P_4 levels in the absence of $InsP_3$ phosphorylation

by ipmk. Thus, *S. cerevisiae* synthesizes Ins(1,3,4,5)P_4 using a different inositol phosphate kinase that by-passes ipmk. Furthermore, the persistence of low levels of $InsP_6$ in the *ipmk*Δ yeast suggests that, to a limited extent, this auxiliary metabolic pathway extends to the synthesis of $InsP_6$. D/L-Ins(1,2,3,4,5)P_5 might participate in this alternate metabolic route, since its levels increased 18-fold in the *ipmk*Δ cells (Table 1). It has previously not been considered that there might be more than one pathway of $InsP_6$ synthesis in yeast.

We also identified both PP-$InsP_5$ and $[PP]_2$-$InsP_4$ in *S. cerevisiae* (Fig. 1). In mammalian cells, these 'high-energy' diphosphoinositol polyphosphates are candidate molecular switches [17,18] that are rapidly metabolized through substrate cycles that are regulated by cAMP, cGMP and Ca^{2+} [8,19]. PP-$InsP_5$ and $[PP]_2$-$InsP_4$ are present at 100-fold lower levels than $InsP_6$, as is the case in higher eukaryotes [17]. This is the first time that PP-$InsP_5$ and $[PP]_2$-$InsP_4$ have been identified in any yeast, although their presence was anticipated, following earlier identification of a kinase from *S. cerevisiae* that synthesizes PP-$InsP_5$ [4] and the discovery of a diphosphoinositol polyphosphate phosphatase from the same organism [12].

It should be noted that the *ipmk*Δ strain of yeast grew more slowly than the wild-type at both 23°C and 30°C, and growth was completely arrested at 37°C ([2] and data not shown). Despite this temperature-sensitive growth phenotype, all of the differences in the inositol phosphate profile between wild-type and *ipmk*Δ yeast strains that were observed at 37°C (Figs. 2 and 3) were also observed at 23°C (data not shown).

3.3. Inositol phosphates and the regulation of nuclear mRNA export

Recently, York et al. [5] described a mutant strain of yeast, named gsl3, that displayed changes in inositol phosphate turnover that, in some cases, are similar to those of our *ipmk*Δ strain. These include decreases in levels of $InsP_6$, accompanied by increases in levels of $InsP_2$, Ins(1,4,5)P_3 and $InsP_4$ [5]. The protein product that was disrupted in the gsl3 strain was not identified, although it was suggested that it might be either (i) a regulator of kinase activities against $InsP_3$ and $InsP_4$, or (ii) a multifunctional $InsP_3$/$InsP_4$ kinase [5]. Our data indicate for the first time that deletion of a genuine $InsP_3$/$InsP_4$ kinase does promote the changes in inositol phosphate turnover that typified the gsl3 strain [5]. Thus, our data are consistent with the gsl3 strain being equivalent, and probably identical, to the *ipmk*Δ strain. York et al. [5] reported that the gsl3 variant was one of three yeast strains displaying impaired mRNA export from the nucleus. Thus, it was important to determine if mRNA export was also inhibited in our own *ipmk*Δ mutant.

Poly(A)$^+$ RNA was detected with a fluorescein isothiocyanate-coupled antibody against digoxigenin. In wild-type cells, a diffuse signal was distributed throughout the cell (Fig. 4). However, the *ipmk*Δ cells contained more punctate signals that were restricted to the nuclei, as revealed by coincident staining with 4′,6′-diamidino-2-phenylindole (Fig. 4). These results are consistent with mRNA export from the nucleus being strongly impaired in the *ipmk*Δ yeast, although we cannot exclude the possibility that there may also be some decreased stability of cytoplasmic mRNA. mRNA export itself comprises a series of molecular events including RNA synthesis, polyadenylation and capping, ribonucleoprotein targeting to the pore complex as well as transport through it [20]. A defect in any of these individual steps could account for the loss of cytoplasmic poly(A)$^+$ in the *ipmk*Δ cells (Fig. 4).

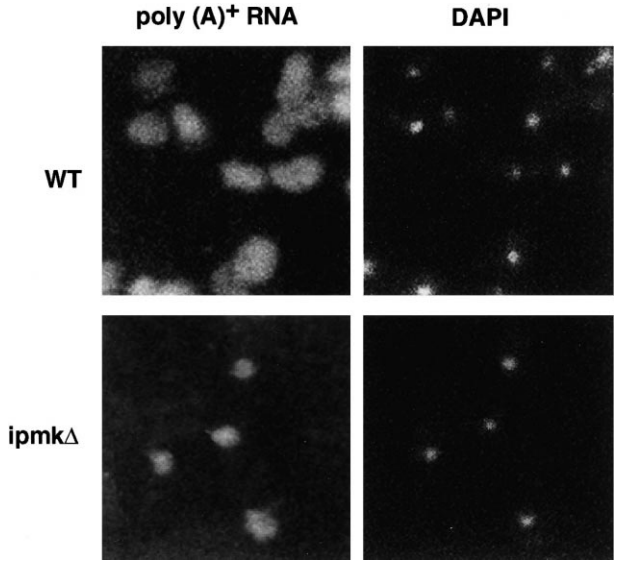

Fig. 4. Nuclear mRNA export in wild-type and *ipmk*Δ yeast. The upper panels show poly(A)$^+$ RNA labeling. The lower panels show the same fields processed for DAPI staining. Data are representative of three experiments.

Ipmk was first discovered as a product of the *ARGRIII* gene [4], which controls the transcription of enzymes involved in arginine metabolism [1–3]. The latter workers have also indicated that *ARGRIII* is pleiotropic, although it was unclear what molecular mechanisms underlie the additional gene functions [2]. Therefore, our demonstration that disruption of this same gene both affects mRNA dynamics and causes global changes in inositol phosphate turnover represents major findings in our study.

How might these changes in inositol phosphate turnover regulate nuclear mRNA export in yeast? [$InsP_6$] may be one factor that is important; other genetic mutations in *S. cerevisiae* that also impaired mRNA export, namely, deletion of either the Ins(1,3,4,5,6)P_5 2-kinase or PLC, both have in common with the *ipmk*Δ cells a dramatic decrease in $InsP_6$ levels [5]. However, our results indicate that it may be an over-simplification to attribute a reduced rate of mRNA export to a decrease in [$InsP_6$], in part because we have shown that $InsP_6$ does not represent a metabolic end-point in yeast. The concentrations of two $InsP_6$ metabolites, namely, PP-$InsP_5$ and $[PP]_2$-$InsP_4$, also decrease in the *ipmk*Δ yeast (Table 1). Moreover, the *ipmk*Δ cells also have substantial increases in levels of D/L-Ins(1,2,3,4,5)P_5, Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3, as well as decreased concentrations of Ins(1,4,5,6)P_4 and D/L-Ins(1,2,4,5,6)P_5 (Table 1). It could be that one or more of these other polyphosphates, rather than $InsP_6$ itself, are the compounds that play an important role in mRNA export. In particular, it is notable that the turnover of the diphosphoinositol phosphates is generally a rapid, ongoing activity that demands a considerable investment of cellular energy [17]; nuclear mRNA export is also an energy-intensive process [20]. In any case, the particular importance to the yeast cell of PP-$InsP_5$ and $[PP]_2$-$InsP_4$ is indicated by the fact that, in

the face of 100-fold decreases in the levels of $InsP_6$, the yeast cells apparently change the poise of the kinase/phosphatase substrate cycles that turn over these diphosphoinositol polyphosphates; thus, the ratio of PP-$InsP_5$:$InsP_6$ increased nearly 40-fold, from a value of 0.007 in the wild-type cells to 0.26 in the *ipmk*Δ yeast (Table 1).

Our data establish that complex changes in inositol phosphate metabolism are associated with decreased efficiency of nuclear export of mRNA. Analysis of signal transduction by inositol phosphates in the nuclei or at the level of the nuclear pore complex may elucidate the mechanism of nuclear mRNA transport at the molecular level.

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