J. Biochem. 131, 391–398 (2002)

Genes for a Nuclease and a Protease Are Involved in the Drastic Decrease in Cellular RNA Amount in Fission Yeast Cells during Nitrogen Starvation[1]

Akio Nakashima,* Mayumi Yoshida,* Kazutoshi Nakayama,* Aya Kato-Furuno,* Masaru Ueno,* Takashi Ushimaru,† and Masahiro Uritani*,[2]

**Department of Chemistry and †Department of Biology and Geoscience, Faculty of Science, Shizuoka University, 836 Oya, Shizuoka 422-8529*

Received August 24, 2001; accepted December 14, 2001

Cellular RNA in *Schizosaccharomyces pombe* cells drastically decreases in amount during nitrogen starvation. Previously, we found and purified a soluble RNA-degrading enzyme whose activity drastically increased in the cells of *S. pombe* undergoing nitrogen starvation. The enzyme was a nuclease encoded by *pnu1⁺*. In this study, the increase in the RNA-degrading activity and the decrease in cellular RNA level are examined in a null-mutant of *pnu1⁺* (*pnu1Δ*). During nitrogen starvation, wild-type cells show an apparent increase in RNA-degrading activity, whereas the *pnu1Δ* cells do not. The wild-type cells show a drastic decrease in cellular RNA amount, whereas the *pnu1Δ* cells show only a slight decrease. These results suggest that Pnu1 nuclease is implicated in the decrease in cellular RNA amount during nitrogen starvation, probably *via* the RNA-degrading activity. The increase in the RNA-degrading activity is independent of both the Wis1 stress-activated MAP kinase cascade and Tor1 signaling pathway, but it is strongly dependent on *isp6⁺*, a gene for a possible protease, whose expression is induced during nitrogen starvation. A disruption mutant for *isp6⁺* (*isp6Δ*) is deficient in both the increase in the RNA-degrading activity and the drastic decrease in the cellular RNA amount during nitrogen starvation, which suggests that *isp6⁺* is involved in the RNA degradation *via* regulating the RNA-degrading activity of Pnu1.

Key words: degradation of RNA, mitochondrial nuclease, nitrogen starvation, protease, *Schizosaccharomyces pombe*.

During nitrogen starvation, a drastic decrease occurs in the amount of cellular RNA in the cells of *Schizosaccharomyces pombe* (*1, 2*) and *Saccharomyces cerevisiae* (*3–7*). The RNA degradation is believed to be achieved by certain RNA-degrading activities that specifically appear in the cells during nitrogen starvation. Several studies reported the detection and partial purification of such RNA-degrading enzymes (*5, 8–12*), but the RNA-degrading enzyme in *S. pombe* has yet to be either purified or identified.

Previously, we found that *S. pombe* cells undergoing nitrogen starvation showed a drastic increased in a soluble RNA-degrading activity (Uritani, unpublished data). The purified enzyme showed both RNA-degrading and DNA-degrading activities, suggesting that it was a nuclease. Its gene, referred to as *pnu1⁺* (*S. pombe* nuclease 1), was also cloned; it encoded a possible mitochondrial nuclease, homologous to the mitochondrial nucleases in *S. cerevisiae* and bovine (*13, 14*). The Pnu1-GFP fusion protein is actually transported to the mitochondria.

In this study, we found that a null-mutant of *pnu1⁺* is deficient in both the increase in RNA-degrading activity and the drastic decrease in cellular RNA amount during nitrogen starvation. The *isp6⁺*, a gene for a possible protease, was involved in the decrease in cellular RNA amount *via* regulating the RNA-degrading activity of Pnu1 nuclease.

EXPERIMENTAL PROCEDURES

Reagents—Reagents were obtained from Wako Pure Chemical Industries (Osaka) unless specially noted.

Cell Culture—The *S. pombe* strains used in this study are listed in Table I. Cells were cultured in EMM3 medium as previously described (*15*) except for 2% glucose and 50 mg/liter of each supplement (adenine, uracil, and leucine) when necessary. Culture was carried out at 30°C with shaking (90 rpm), and the cell number was counted in a hematocytometer under a microscope. For the nitrogen starvation treatment, cells at the mid-log phase were collected by centrifugation, washed twice with distilled water, transferred into EMM3-N, which consisted of EMM3 without ammonium chloride, and then cultured. At intervals, aliquots of the culture were withdrawn, and cells were collected and analyzed as described below.

Disruption of pnu1⁺—*pnu1⁺* (C17C9.08) was disrupted using a one-step disruption (*16*). The upstream and down-

[1] This study was supported in part by the Sasakawa Scientific Research Grant from the Japan Science Society.

[2] To whom correspondence should be addressed. Tel: +81-54-238-4761, Fax: +81-54-237-3384, E-mail: scmurit@ipc.shizuoka.ac.jp

Abbreviations: FACS, fluorescence-activated cell sorter; MCS, multi-cloning site; PMSF, phenylmethylsulfonyl fluoride.

© 2002 by The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 29, 2012

stream regions of *pnu1*$^+$ were amplified by PCR using two sets of primers: for the upstream region, 5′-TCTGCAGCG-TTTTGGCTATGCAC-3′ and 5′-TGGAATATTCACTGCGT-GTGAG-3′; for the downstream region, 5′-AAGCTTTGCC-GTCACTTCACAACT-3′ and 5′-GAATTCTAAACTCACCA-AAAGGACC-3′ (underlined sequences were added for *Pst*I, *Hin*dIII, and *Eco*RI sites, respectively). The amplified fragments were digested with restriction enzymes (the upstream fragment with *Hin*dIII and *Pst*I, and the downstream fragment with *Hin*dIII and *Eco*RI), and then ligated to the pUC18 vector that had been double-digested with *Pst*I and *Eco*RI. The resultant plasmid (pYM1) was digested with *Hin*dIII to which *ura4*$^+$ was ligated at the *Hin*dIII site. The resultant plasmid (pYM2) was digested with *Nde*I, and the fragment (upstream-*ura4*$^+$-downstream) was introduced into *S. pombe* cells (JY800) using the lithium acetate method (*17*). The *ura4*$^+$-containing cells were isolated, and the disruption of *pnu1*$^+$ was confirmed by PCR and Southern hybridization (Fig. 1B). The mutant strain obtained from JY800 was referred to as YM31.

Expression Plasmid of pnu1$^+$—The cDNA of *pnu1*$^+$ was amplified from the *S. pombe* cDNA library (Clontech). The fragment amplified was inserted into pT7 Blue vector to generate pNA1, which was then double-digested with *Nde*I and *Sma*I (*Sma*I site located on the MCS of the vector), and the insert was ligated to the pREP1 vector (*18*) that had been double-digested with *Nde*I and *Sma*I. The resultant plasmid (pREP1-*pnu1*$^+$) was used for the over-expression of *pnu1*$^+$. The pREP1 contains a thiamine-repressible *nmt1*$^+$ promoter.

Western Blotting—Cells (2×10^7 cells) were collected by centrifugation and suspended in 20 μl of 20% (w/v) trichloroacetic acid, then glass beads (0.35–0.50 mm) were added to the meniscus, and the mixture was vigorously shaken using a microtube shaker (TOMY MT-300, Tokyo) for 30 min at 4°C. After centrifugation, the supernatant was removed, and the precipitate was washed with ethanol several times thoroughly to remove the trichloroacetic acid. The washed precipitate was dried under vacuum, resolved in 50 μl of a sample buffer [50 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol, and 0.002% (w/v) bromophenol blue], and boiled. Samples were subjected to SDS-PAGE (*19*), and protein was transferred onto a nitrocellulose membrane. Protein was detected with an antiserum against Pnu1 that was raised using an ImmunoStar kit for rabbit according to the manufacturer's protocol.

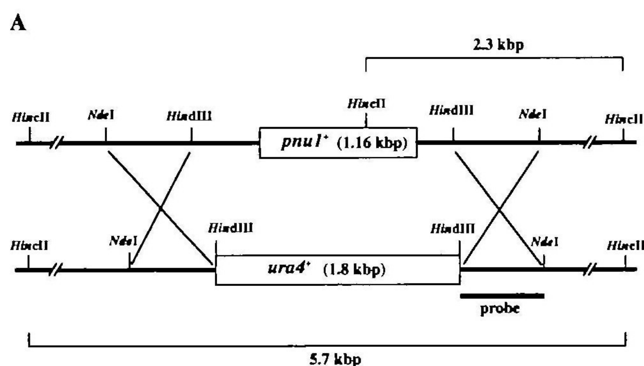
Assay for RNA-Degrading Activity and DNA-Degrading Activity—Cells of a 20-ml culture were collected by centrifugation, washed twice with distilled water, and then suspended in 100 μl of buffer A [50 mM Tris-HCl (pH 7.6), 30 mM potassium chloride, 10 mM magnesium chloride, 10 mM 2-mercaptoethanol, and 10% (v/v) glycerol] containing 1 mM PMSF (Sigma) and 50 mg/liter pepstatin A (The Peptide Institute). Glass beads were added to the meniscus of the suspension, and the mixture was vigorously shaken in the microtube shaker for 80 min at 4°C. To the homogenate, 150 μl of the same buffer was added. After centrifugation for 10 min at 14,000 ×*g* at 4°C, the supernatant was pooled, which was further centrifuged for 1 h at 100,000 ×*g* at 4°C. The supernatant was used as the enzyme fraction. Protein concentrations were measured using a dotMETRIC 1 μl Protein Assay Kit (Geno Technology). For the RNA-de-grading activity, the total RNA from *S. pombe* was used as the substrate; for the DNA-degrading activity, pUC18 or pSP1 (*20*) was used as the substrate. A standard reaction mixture (30 μl) contained 30 mM Tris-HCl (pH 7.6), 30 mM potassium chloride, 5 mM magnesium chloride, 4 μl (2.5 μg of protein) of enzyme fraction and 1 μl of substrate (5 μg of RNA or 1 μg of DNA). The mixture was incubated at 30°C for the time indicated in the figures, and the reaction was terminated with 1 μl of 0.5 M EDTA. A half volume of the mixture (15 μl) was subjected to agarose gel electrophoresis to analyze the nucleic acids.

RNA Analysis—Cells were collected from the culture, washed twice with distilled water, and stored at −80°C until use. RNA was extracted from the cells as previously described (*21*) with modifications. In brief, the frozen cells were suspended in the ice-cooled LETS buffer [10 mM Tris-HCl (pH 7.6), 0.1 M lithium chloride, 10 mM EDTA, and 0.2% (w/v) SDS]. To the mixture, the Ice-cooled LETS-saturated phenol and glass beads were added immediately,

TABLE I. **The fission yeast strains used in this study.**

Strain	Genotype	Refs.
JY800	*h*^{90} *ade6-M210 leu1 ura4-D18*	23
JY476	*h*^{90} *ade6-M210 leu1*	29
JZ858	*h*^{90} *ade6-M216 leu1 ura4-D18 cgs1::ura4*$^+$	29
SS006	*h*^{90} *ade6-M210 leu1 ura4-D18 isp6::ura4*$^+$	23
KM30	*h*^{90} *ade6-M210 leu1 ura4-D18 tor1::ura4*$^+$	30
NS4	*h*^{90} *ura4-D18 rcd1::ura4*$^+$	27
NS13	*h*^{90} *his1-102 wis1::ura4*$^+$	27
NS11	*h*^{90} *ura4-D18 pka1::ura4*$^+$	27
CN803	*h*$^-$ *ade6-M216 leu1 ura4-D18 atf1::ura4*$^+$	32
YM31	*h*^{90} *ade6-M210 leu1 ura4-D18 pnu1::ura4*$^+$	This study
AN1181	*h*$^-$ *ade6-M216 leu1 ura4-D18 pnu1::ura4*$^+$ *isp6::ura4*	This study

A



B

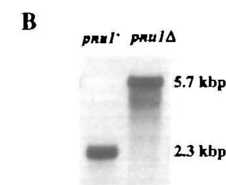


Fig. 1. **Gene disruption of *pnu1*$^+$.** A: Schematic representation of the disruption of *pnu1*$^+$. The entire ORF of *pnu1*$^+$ was replaced with the *ura4*$^+$ gene. B: Southern hybridization of *pnu1*$^+$ in *pnu1Δ*. Total DNA from JY800 (*pnu1*$^+$) or YM31 (*pnu1Δ*) was subjected to Southern hybridization after digestion with *Hin*cII. The *Hin*dIII–*Nde*I fragment of the downstream region of *pnu1*$^+$ was used as a probe.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 29, 2012

then the mixture was shaken vigorously using the microtube shaker for 30 min at 4°C. RNA was purified from the extract by two times of acid phenol-extraction and ethanol-precipitation. RNA was dissolved in the TE buffer [10 mM Tris-HCl (pH 7.6) and 1 mM EDTA] and analyzed as follows. The buffers contained EDTA, a strong inhibitor of the Pnu1 nuclease (Fig. 2B). The amount of RNA was determined by UV absorption at 260 nm. For analysis of the rRNA, an aliquot of each RNA sample was subjected to agarose gel electrophoresis; rRNA was visualized by ethidium bromide staining. For northern blotting, the RNA (25 μg) was subjected to the electrophoresis in agarose gel with formaldehyde; the RNA was transferred to a nylon membrane (Hybond N+) and hybridized with a labeled probe using the AlkPhos Direct system (Amersham-Pharmacia Biotech). For probes, the entire ORFs of *rps6*+ (*22*), *isp6*+ (*23*), and *cox2*+ (*24*) were used.

RESULTS

Disruption of pnu1+—We obtained a null-mutant for *pnu1*+ by one-step disruption (Fig. 1, A and B). Haploid cells of the null-mutant for *pnu1*+ (*pnu1Δ*) grew as well as the cells of *pnu1*+ in the synthetic medium containing glucose (data not shown), suggesting that *pnu1*+ is not essential for cell growth. Also, the *pnu1Δ* cells grew well on the medium where the carbon source is glycerol, a non-fermentable sugar, suggesting that *pnu1*+ is dispensable for the mitochondrial function. Similar results were obtained for a null-mutant for *NUC1* in *S. cerevisiae* (*25*), which encodes the mitochondrial nuclease homologous to Pnu1 nuclease.

RNA-Degrading Activity in pnu1Δ during Nitrogen Starvation—We examined whether *pnu1*+ is responsible for the increase in the RNA-degrading activity level during nitrogen starvation. The soluble fraction (S-100 fraction) from either *pnu1*+ or *pnu1Δ* was incubated with the *S. pombe* total RNA fraction, which was then analyzed by agarose gel electrophoresis. The RNA fraction mainly contained the 26S and 17S rRNAs, which were relatively stable during the incubation with the S-100 fraction from the *pnu1*+ cells that were not nitrogen-starved, while they were drastically degraded with the fraction from the cells that were starved for nitrogen (Fig. 2A). The RNA-degrading activity was

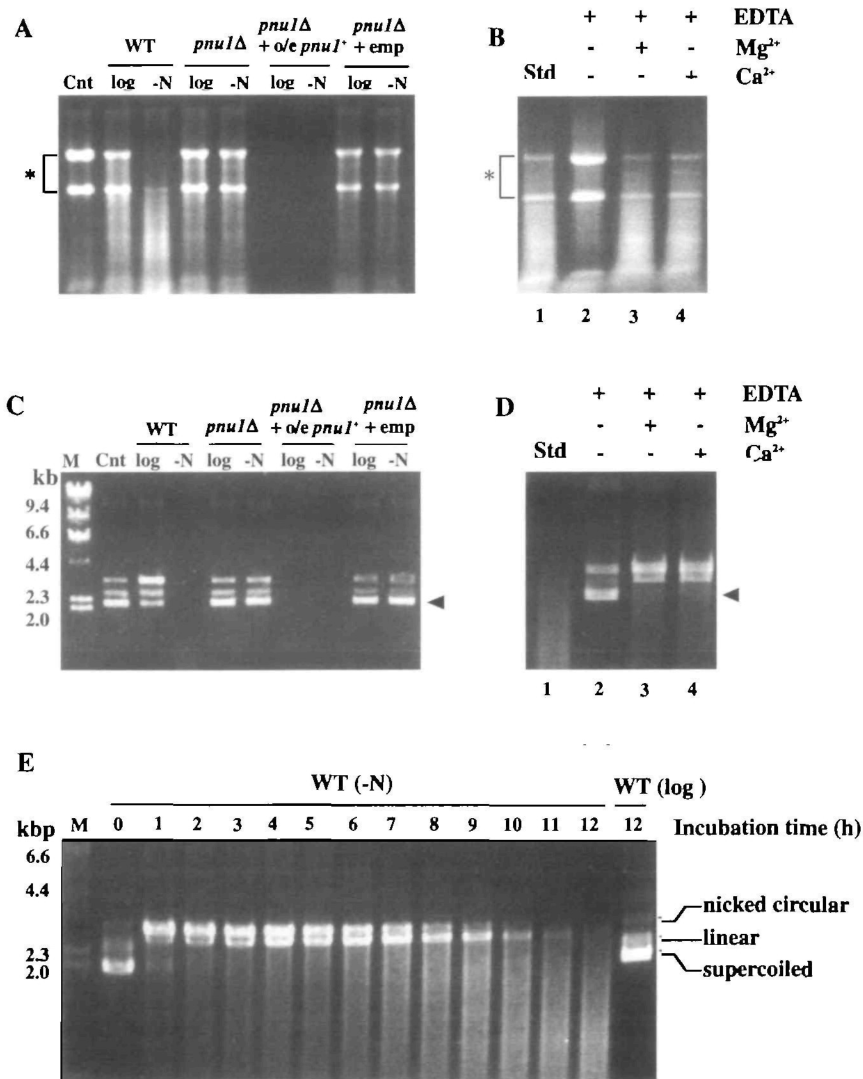


Fig. 2. **The RNA-degrading and DNA-degrading activities in *pnu1Δ* cells during nitrogen starvation.** The RNA-degrading activity (A and B) and the DNA-degrading activity (C, D, and E) in the S-100 fractions were measured from the cells of mid-log phase (log) or with nitrogen starvation for 4 h (–N). A: The RNA-degrading activity in JY800 (WT), YM31 (*pnu1Δ*), YM31 with over-expression of *pnu1*+ in pREP1 plasmid (*pnu1Δ*+o/e*pnu1*+), and YM31 with the vacant pREP1 plasmid (*pnu1Δ*+emp). The activity was measured after incubation for 80 min. The substrate RNA incubated without the soluble fraction is shown as the control (Cnt). An asterisk shows the 26 S rRNA and 17 S rRNA. B: The RNA-degrading activity in the S-100 fraction from the nitrogen-starved JY800 cells. The RNA-degrading activity was measured under the standard conditions (lane 1, Std), with 15 mM EDTA (lane 2), lane 2 plus 20 mM magnesium chloride (lane 3), or lane 2 plus 20 mM calcium chloride (lane 4). The incubation time was 60 min. C: The same as A, except that the DNA substrate (pUC18) was used instead of the RNA substrate. The activity was measured after incubation for 18 h. The substrate DNA incubated without the soluble fraction is shown as the control (Cnt). The arrowhead shows the supercoiled DNA. M, Size markers. D: The same as B, except that the DNA substrate (pUC18) was used. Incubation time was 12 h. E: Time course for the DNA-degrading activity. The S-100 fractions were obtained from the cells of JY800 cultured without (WT, [log]) or with nitrogen starvation for 4 h (WT, [–N]).

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 29, 2012

EDTA-sensitive and was recovered by either magnesium chloride or calcium chloride (Fig. 2B). In case of *pnu1Δ*, the RNA-degrading activity was not seen in either nitrogen starved or not starved.

When expression of the Pnu1 protein was examined by Western blotting, a 38-kDa band was missing in *pnu1Δ* (Fig. 3). We note that the molecular mass of the purified nuclease was also 38 kDa (Uritani, unpublished data). We then examined whether the lack of the RNA-degrading activity could be restored by the introduction of a functional $pnu1^+$ gene in *pnu1Δ*. The *pnu1Δ* cells harboring a thiamine-repressible plasmid, pREP1-$pnu1^+$, were cultured without thiamine to over-produce Pnu1. The S-100 fraction from the cells exhibited a very strong RNA-degrading activity (Fig. 2A). Since the pREP1 contains a potent $nmt1^+$ promoter (*18*), Pnu1 was produced in a large amount (Fig. 3), which could explain such a high level RNA-degrading activity in the cells with pREP1-$pnu1^+$.

The $pnu1^+$ encodes a nuclease, so that we examined the DNA-degrading activity in *pnu1Δ*. The pUC18 plasmid DNA was incubated with the S-100 fraction, which was then analyzed by gel electrophoresis. Since the plasmid was circular, it separated into three discrete bands according to its topology; the supercoiled form, nicked circular form and linear form. In the $pnu1^+$ cells, the plasmid DNA was stable during the incubation with the S-100 fraction from the cells before starvation, and it was degraded with that from nitrogen-starved cells (Fig. 2C). The DNA-degrading activity was inhibited by EDTA, and recovered by the addition of either magnesium chloride or calcium chloride (Fig. 2D). In contrast, *pnu1Δ* did not show the DNA-degrading activity even when the cells were nitrogen-starved. Introduction of the pREP1-$pnu1^+$ into the cells of *pnu1Δ* resulted in the appearance of a very strong DNA-degrading activity. The S-100 fraction from $pnu1^+$ undergoing nitrogen starvation also showed the DNA-nicking activity (Fig. 2E).

Decrease in RNA Amount in pnu1Δ during Nitrogen Starvation—We next examined whether $pnu1^+$ is involved in the decrease of cellular RNA level during nitrogen starvation. Cells at the mid-log phase (5×10^6 cells/ml) were transferred into a nitrogen-free medium, and aliquots of the culture were withdrawn at intervals. RNA was carefully isolated from the cells and the amount of RNA was measured by UV absorption. It should be noted that we took special care to keep the RNA from degradation during extraction and isolation (see "EXPERIMENTAL PROCEDURES"). Figure 4A presents the results of the changes in the RNA amounts. In $pnu1^+$, the RNA level began to de-

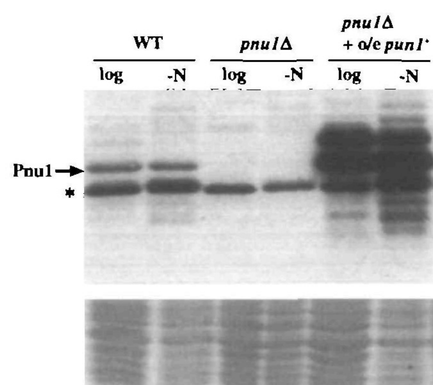


Fig. 3. **Detection of Pnu1 by Western blotting.** The cells were harvested either at the mid-log phase (log) or after being starved of nitrogen for 4 h (–N), and whole protein was analyzed by the Western blotting using the antibody against Pnu1. The cells of wild-type JY800 (WT), YM31 (*pnu1Δ*), and YM31 with over-expression of $pnu1^+$ in pREP1 plasmid (*pnu1Δ*+o/e$pnu1^+$) were used. The upper panel shows the results of the Western blotting, and the lower panel shows the Coomassie Brilliant Blue staining of the gel for control of the protein amounts loaded. The arrow indicates the position of (38 kDa). A non-specific protein band was marked with an asterisk.

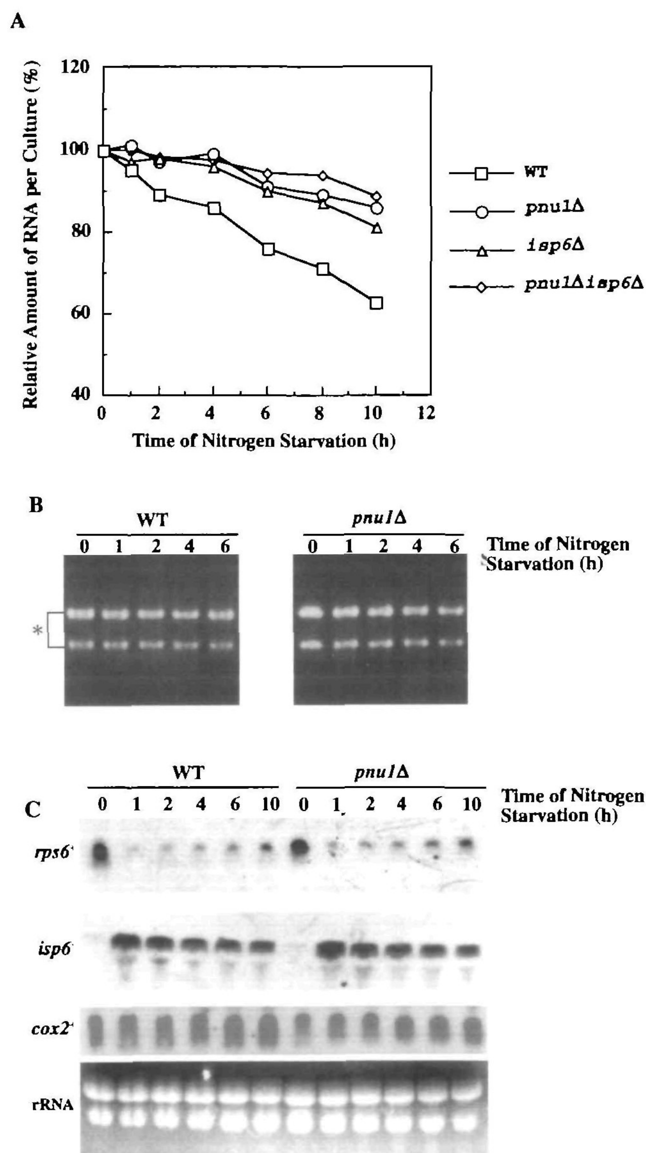


Fig. 4. **Decrease in the RNA amount in *pnu1Δ* cells during nitrogen starvation.** A: Total RNA from the cells with various nitrogen starvation times was measured. RNA amounts were represented as a ratio to the initial level (0 h of nitrogen starvation) of RNA. Square, WT (JY476); circle, *pnu1Δ* (YM31); triangle, *isp6Δ* (SS006); diamond, *pnu1Δisp6Δ* (AN1181). The data represent the average values of three independent experiments. In each spot, the standard error was within 10%. B: Total RNA was analyzed by agarose gel electrophoresis and stained with ethidium chloride. C: Northern blotting of the mRNA for $rsp6^+$, $isp6^+$, and $cox2^+$.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 29, 2012

crease upon nitrogen starvation and the decrease progressed with starvation. Finally, the RNA level was reduced to 60% of the initial amount by 10 h. The results are in good agreement with the previous results (*1–7*). Notably, in *pnu1Δ*, the RNA-decreasing rate was less than half that in *pnu1*+ (Fig. 4A), suggesting that *pnu1*+ may function to promote the degradation of RNA during nitrogen starvation. It is also noted that the RNA-degrading activity in *pnu1*+ began to increase after 2 h and it increased with nitrogen starvation (Fig. 5A). Thus, the time course of the RNA degradation was parallel to that of the RNA-degrading activity. Interestingly, such an aberrant decrease in the cellular RNA amount was also observed in *isp6Δ* mutant (Fig. 4A). As will be described below, *isp6*+ was responsible for the expression of the RNA-degrading activity during nitrogen starvation (Fig. 6A). A double mutant with *pnu1* and *isp6* (*pnu1Δisp6Δ*) also gave the same RNA-decreasing pattern as single mutants (*pnu1Δ* and *isp6Δ*), suggesting that *pnu1*+ and *isp6*+ may function in the same pathway.

The total RNA fraction was analyzed by agarose gel electrophoresis to obtain information about the RNA species that are degraded during the starvation. Both 26S and 17S rRNAs decreased with starvation in *pnu1*+ and *pnu1Δ*, suggesting that *pnu1*+ is not necessary for the degradation of these rRNAs. The cytoplasmic mRNAs for *rps6*+ and *isp6*+ were analyzed by Northern blotting (Fig. 4C). The *rps6*+ encodes a ribosomal protein, which is expressed during growth but is repressed during nitrogen starvation (*22*). The mRNA for *rps6*+ drastically decreased in amount within 1 h in either *pnu1*+ or *pnu1Δ*. The *isp6*+ encodes a protease, whose expression is repressed in exponentially growing cells but is induced by nitrogen starvation (*23*). In *pnu1*+, the mRNA for *isp6*+ was present only in a small amount before starvation and it drastically increased in amount within 1 h; after that its amount was relatively constant during the starvation. The same results were obtained in *pnu1Δ*. A mitochondrial gene *cox2*+ encodes a cytochrome oxidase and its mRNA is present in the mitochondria (*24*). The Northern blotting of *cox2*+ revealed that the amount of the mRNA was constant during nitrogen starvation in either *pnu1*+ or *pnu1Δ*.

The implication of Pnu1 in the metabolism of DNA during nitrogen starvation was also investigated. The FACS analysis demonstrated that the patterns were essentially the same in *pnu1*+ and in *pnu1Δ* (data not shown). By Southern blotting using *cox2*+ ORF as a probe, the amount of mitochondrial DNA was monitored. These results indicated that the amount of the mitochondrial DNA was constant during nitrogen starvation in the cells of both *pnu1*+ and *pnu1Δ* (data not shown).

Increase in RNA-Degrading Activity and Decrease in Cellular RNA Level in Various Mutants—As described above, *pnu1Δ* cells showed no apparent increase in both RNA-degrading and DNA-degrading activities, suggesting that Pnu1 specifically exhibits the activities during nitrogen starvation. We then examined whether the activities of Pnu1 are enhanced in mutants that are defective in adaptation to nitrogen starvation. Pnu1 was normally activated in the cells of *wis1Δ* (*26, 27*), *cgs1Δ* (*28, 29*), *tor1Δ* (*30*), *rcd1Δ* (*27*), and *atf1Δ* (*31, 32*). In *isp6Δ*, however, either RNA-degrading activity or DNA-degrading activity was absent (Fig. 6, A and B). The *isp6*+ was repressed in the

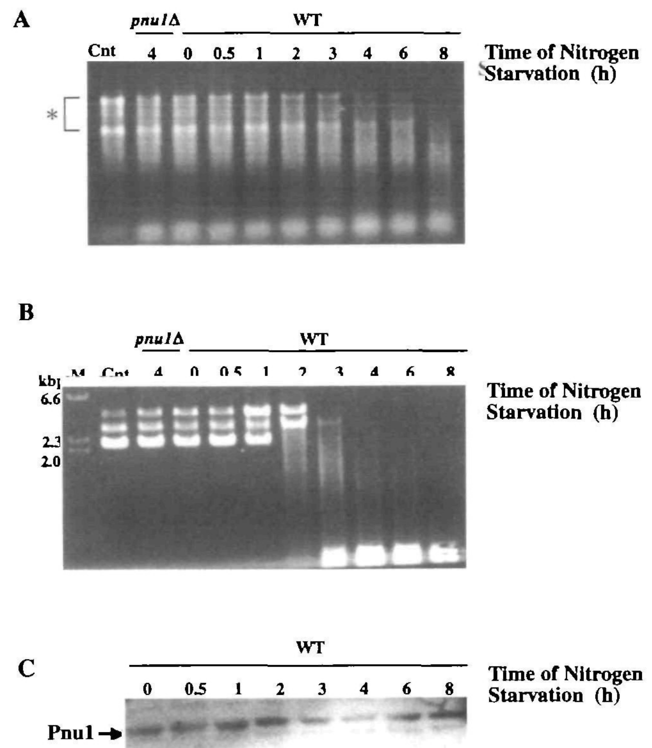


Fig. 5. **Increase in the RNA-degrading and DNA-degrading activity levels during nitrogen starvation.** The RNA-degrading activity (A) and the DNA-degrading activity (B) in the S-100 fraction from the cells of JY476 (WT) were monitored during nitrogen starvation. For controls, the substrate RNA or DNA was incubated without the S-100 fraction (Con) or incubated with the fraction from YM31 (*pnu1Δ*) after 4 h of nitrogen starvation. The asterisk indicates the 26 S rRNA and 17 S rRNA. M, size markers. Incubation time was 80 min for the RNA-degrading activity and 16 h for the DNA-degrading activity. The Pnu1 protein in the S-100 fraction was detected by Western blotting using anti-Pnu1 antibody (C).

growing cells and was induced within 1 h upon nitrogen starvation (Fig. 4C in this study, and Ref. *23*). Thus, the time course of the expression of *isp6*+ was parallel with that of the activation of both RNA-degrading and DNA-degrading activities (Fig. 5, A and B). Notably, the decreasing curve for the RNA amount was the same in *pnu1Δ*, *isp6Δ*, and *pnu1Δisp6Δ* (Fig. 4A). The Western blotting of Pnu1 protein in *isp6Δ* revealed that the amount of Pnu1 protein was not affected by *isp6*+ although Pnu1 migrated faster in *isp6Δ* (Fig. 6C).

The *pka1*+ encodes the catalytic subunit of the cAMP-dependent protein kinase (*27, 33*). In *pka1Δ*, the DNA-degrading activity was activated prominently and the RNA-degrading was also activated, although less prominently, even without nitrogen starvation (Fig. 6, A and B). The degradation of RNA became more marked with prolonged incubation (3 h, data not shown). We obtained the results that *isp6*+ was expressed in the cells of *pka1Δ* even in a nitrogen source-rich medium (Nakashima and Uritani unpublished data), suggesting that the cAMP-dependent protein kinase cascade activates the RNA-degrading and DNA-degrading activities of Pnu1 *via* induction of *isp6*+.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 29, 2012

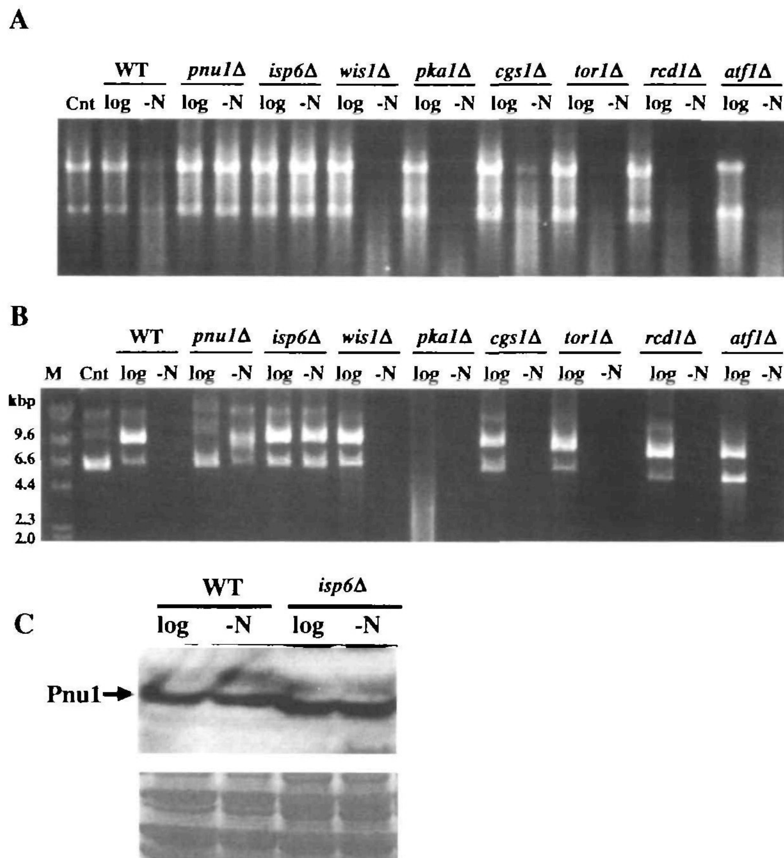


Fig. 6. **RNA-degrading and DNA-degrading activities in the mutants defective in the response to nitrogen starvation.** The RNA-degrading activity (A) and DNA-degrading activity (B) were measured in the S-100 fraction from the cells at a mid-log phase (log) or under nitrogen starvation for 4 h (–N). For controls, the RNA or DNA substrate was incubated without the S-100 fraction (Cnt). For DNA-degrading assay, pSP1 plasmid was used as the substrate. Incubation time was 80 min for the RNA-degrading activity and 13 h for the DNA-degrading activity. The following strains were used in this experiment: JY800 (WT), YM31 (*pnu1Δ*), SS006 (*isp6Δ*), NS13 (*wis1Δ*), NS11 (*pka1Δ*), JZ858 (*cgs1Δ*), KM30 (*tor1Δ*), NS4 (*rcd1Δ*), and CN803 (*atf1Δ*). The Pnu1 protein in the S-100 fractions was detected from JY476 (WT) and *isp6Δ* (SS006) (C). The upper panel shows the results of Western blotting using the anti-Pnu1 antibody, and the lower panel shows the Coomassie Brilliant Blue staining of proteins.

DISCUSSION

During nitrogen starvation, a drastic decrease occurs in the amount of cellular RNA in the cells of *S. pombe* by an unknown mechanism (*1, 2*). Previously, we purified the RNA-degrading enzyme whose activity specifically appears during nitrogen starvation and cloned its gene (*pnu1⁺*). In this study, we obtained results indicating that *pnu1⁺* is involved in the apparent increase in RNA-degrading activity (Figs. 2A and 5A) and the drastic decrease in cellular RNA amount during nitrogen starvation (Fig. 4A). These findings suggest that Pnu1 may be implicated in degradation of RNA due to its RNA-degrading activity.

The RNA-degrading activity of Pnu1 was highly specific to nitrogen starvation. However, the increase in RNA-degrading activity was independent of the Wis1 stress-activated MAP kinase cascade (*26*), the Tor1-dependent pathway (*30, 34*), and the Rcd1-dependent pathway (*27*) although they play pivotal roles in the signaling of nitrogen starvation. Interestingly, activation of the RNA-degrading activity was dependent on *isp6⁺*, a gene for a possible protease (*23*). The *isp6Δ* cells showed neither RNA-degrading activity nor DNA-degrading activity and were also defective in decrease in the cellular RNA amount during nitrogen starvation (Fig. 5, A and B). Thus, *isp6⁺* is responsible for the increase in the RNA-degrading activity of Pnu1, thereby regulating the cellular RNA level. The *isp6Δ* cells are deficient in the response to nitrogen starvation (*23*). The *isp6⁺* is homologous to the *PRB1* in *S. cerevisiae* (*35, 36*). Interestingly, a mutant for *PRB1* is also deficient in the response to nitrogen starvation and is defective in the RNA-degrading activity during nitrogen starvation although the mechanism is unknown (*12, 35, 36*).

The RNA-degrading activity and DNA-degrading activity of Pnu1 was activated during nitrogen starvation in an *isp6⁺*-dependent manner (Fig. 6, A and B). Ikeda and Kawasaki recently reported that a full-length of Pnu1 (SpNuc1) showed a low nuclease activity, while the truncated form of Pnu1 without the N-terminal 40 amino acids showed a high activity (*37*), suggesting that Pnu1 may be activated through processing. Since *isp6⁺* is suggested to play a role in the processing of a protein (*38*), it is possible that a precursor form of Pnu1 is directly processed by Isp6 to produce a mature form of Pnu1. This assumption predicts that a fast migrating mature form of Pnu1 is observed in SDS-PAGE in a manner that is dependent on both nitrogen starvation and *isp6⁺*. However, the mobility of the Pnu1 protein in SDS-PAGE was not obviously changed during nitrogen starvation (Figs. 3 and 6C). It is possible that Isp6 cleaves only a small peptide from the precursor form of Pnu1, which results in a mature form of Pnu1 with a similar molecular mass to the precursor form of Pnu1. Alternatively, Pnu1 might be activated in a manner other than processing, such as the degradation of a protein that inhibits Pnu1, although such an inhibitory protein has not been found yet.

It is also noted that *PRB1* is necessary for autophagy, which occurs during nitrogen starvation (*39, 40*). Since PRB1p is the homolog of Isp6, Isp6 could also play such a

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 29, 2012

pivotal role in the autophagy in *S. pombe*, which may be required in the activation of the nuclease activity of Pnu1. In this model, Pnu1 should be transported to the vacuole during nitrogen starvation. We examined the localization of a fusion protein of Pnu1 and a green fluorescence protein during nitrogen starvation, but the fusion protein was not accumulated in vacuolar space (data not shown). Further study is necessary to resolve the mechanism for the activation of Pnu1 by Isp6.

In this study, rRNA and a few mRNAs decreased in a Pnu1-independent manner during nitrogen starvation (Fig. 4, B and C). It is possible that Pnu1 non-specifically degrades a number of RNA molecules only slightly, which results in a significant amount of RNA being degraded in total. It is also possible that other RNA molecules specifically targeted by Pnu1 are yet to be found. The *NUC1* in *S. cerevisiae* is homologous to Pnu1. Interestingly, *NUC1* is reported to be involved in the catabolism of the cytoplasmic double-stranded RNA, which is the RNA genome of a virus-like particle (*41*); recessive mutations in the *NUC1* resulted in a 10-fold accumulation of the double-stranded RNA (4.5 kbp) that encodes a coat protein of the particle (86 kDa). Other mutations in the mitochondrial outer membrane also showed the same results. In *S. pombe*, it is not known, whether such a viral genome RNA is present in the cells. Further study to specify the RNA molecules targeted by Pnu1 would clarify the function and the biological meaning of Pnu1 in nitrogen starvation.

The authors thank Prof. M. Yamamoto (the University of Tokyo), Dr. N. Mutoh (Institute for Developmental Research), and Dr. K. Okazaki and Dr. S. Sato (Kazusa DNA Institute) for the fission yeast strains.

REFERENCES

1. Egel, R. and Egel-Mitani, M. (1974) Premeiotic DNA synthesis in fission yeast. *Exp. Cell Res.* **88**, 127–134
2. Calleja, G.B., Johnson, B.F., and Yoo, B.Y. (1980) Macromolecular changes and commitment to sporulation in the fission yeast *Schizosaccharomyces pombe. Plant Cell Physiol.* **21**, 613–625
3. Hopper, A.K., Magee, P.T., Welch, S.K., Friedman, M., and Hall, B.D. (1974) Macromolecule synthesis and breakdown in relation to sporulation and meiosis in yeast. *J. Bacteriol.* **119**, 619–628
4. Tsuboi, M. and Yanagishima, N. (1975) Promotion of sporulation by caffeine pretreatment in *Saccharomyces cerevisiae*. I. Metabolism on nucleic acids and protein during sporulation. *Arch. Microbiol.* **106**, 159–164
5. Tsuboi, M. and Yanagishima, N. (1976) Promotion of sporulation by caffeine pretreatment in *Saccharomyces cerevisiae*. II. Changes in ribonuclease activity during sporulation. *Arch. Microbiol.* **108**, 149–152
6. Tsuboi, M. (1976) Correlation among turnover of nucleic acids, ribonuclease activity and sporulation ability of *Saccharomyces cerevisiae. Arch. Microbiol.* **111**, 13–19
7. Johnston, G.C., Singer, R.A., and McFarlane, E.S. (1977) Growth and cell division during nitrogen starvation of the yeast *Saccharomyces cerevisiae. J. Bacteriol.* **132**, 723–730
8. Tsuboi, M. (1978) Species of ribonucleases and changes of their activities during sporulation of *Saccharomyces cerevisiae. Spores* **7**, 344–349
9. McFarlane, E.S. (1980) Ribonuclease activity during G1 arrest of the yeast *Saccharomyces cerevisiae. Arch. Microbiol.* **124**, 243–247
10. Swida, U., Schulz-Harder, B., Kücherer, C., and Käufer, N. (1981) The occurrence of two ribosomal ribonucleases depending on growth phase in yeast: induction of ribonuclease in glucose-starved cells. *Biochim. Biophys. Acta* **652**, 129–138
11. Ryk, D.V. and von Tigerstrom, R.G. (1985) Activity changes of three nucleolytic enzymes during the life cycle of *Saccharomyces cerevisiae. Can. J. Microbiol.* **31**, 1095–1102
12. Jones, E.W. (1991) Three proteolytic systems in the yeast *Saccharomyces cerevisiae. J. Biol. Chem.* **266**, 7963–7966
13. Vincent, R.D., Hofmann, T.J., and Zassenhaus, H.P. (1988) Sequence and expression of *NUC1*, the gene encoding the mitochondrial nuclease in *Saccharomyces cerevisiae. Nucleic Acids Res.* **16**, 3297–3312
14. Côté, J. and Ruiz-Carrillo, A. (1993) Primers for mitochondrial DNA replication generated by endonuclease G. *Science* **261**, 765–769
15. Creanor, J. and Mitchison, J.M. (1982) Patterns of protein synthesis during the cell cycle of the fission yeast *Schizosaccharomyces pombe. J. Cell Sci.* **58**, 263–285
16. Grimm, C., Kohli, J., Murray, J., and Maundrell, K. (1988) Genetic engineering of *Schizosaccharomyces pombe*: a system for gene disruption and replacement using the *ura4*$^+$ gene as a selectable marker. *Mol. Gen. Genet.* **215**, 81–86
17. Okazaki, K., Okazaki, N., Kume, K., Jinno, S., Tanaka, K., and Okayama, H. (1990) High-frequency transformation method and library transducing vectors for cloning mammalian cDNAs by trans-complementation of *Schizosaccharomyces pombe. Nucleic Acids Res.* **18**, 6485–6489
18. Maundrell, K. (1993) Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene* **123**, 127–130
19. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
20. Cottarel, G., Beach, D., and Deuschle, U. (1993) Two new multipurpose multicopy *Schizosaccharomyces pombe* shuttle vectors, pSP1 and pSP2. *Curr. Genet.* **23**, 547–548
21. Nielsen, O. and Egel, R. (1990) The *pat1* protein kinase controls transcription of the mating-type gene in fission yeast. *EMBO J.* **9**, 1401–1406
22. Bonnet, C., Perret, E., Dumont, X., Picard, A., Caput, D., and Lenaers, G. (2000) Identification and transcription control of fission yeast genes repressed by an ammonium starvation growth arrest. *Yeast* **16**, 23–33
23. Sato, S., Suzuki, H., Widyastuti, U., Hotta, Y., and Tabata, S. (1994) Identification and characterization of genes induced during sexual differentiation in *Schizosaccharomyces pombe. Curr. Genet.* **26**, 31–37
24. Lang, B.F. and Wolf, K. (1984) The mitochondrial genome of the fission yeast *Schizosaccharomyces pombe*. 2. Localization of genes by interspecific hybridization in strain *ade7-50h*$^-$ and cloning of the genome in small fragments. *Mol. Gen. Genet.* **196**, 465–472
25. Zassenhaus, H.P. and Hofmann, T.J., Uthayashanker, R., Vincent, R.D., and Zona, M. (1988) Construction of a yeast mutant lacking the mitochondrial nuclease. *Nucleic Acids Res.* **16**, 3283–3296
26. Warbrick, E. and Fantes, P.A. (1991) The Wis1 protein kinase is a dosage-dependent regulator of mitosis in *Schizosaccharomyces pombe. EMBO J.* **10**, 4291–4299
27. Okazaki, N., Okazaki, K., Watanabe, Y., Kato-Hayashi, M., Yamamoto, M., and Okayama, H. (1998) Novel factor highly conserved among eukaryotes controls sexual development in fission yeast. *Mol. Cell. Biol.* **18**, 887–895
28. DeVoti, J., Seydoux, G., Beach, D., and McLeod, M. (1991) Interaction between *ran1*$^+$ protein kinase and camp dependent protein kinase as negative regulators of fission yeast meiosis. *EMBO J.* **10**, 3759–3768
29. Kanoh, J., Watanabe, Y., Ohsugi, M., Iino, Y., and Yamamoto, M. (1996) *Schizosaccharomyces pombe gad7*$^+$ encodes a phosphoprotein with a bZIP1 domain, which is required for proper G1 arrest and gene expression under nitrogen starvation. *Genes Cells* **1**, 391–408
30. Kawai, M., Nakashima, N., Ueno, M., Ushimaru, T., Aiba, K., Doi, H., and Uritani, M. (2001) Fission yeast Tor1 functions in

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 29, 2012

response to various stresses including nitrogen starvation, high osmolarity, and high temperature. *Curr. Genet.* **39**, 166–174
31. Takeda, T., Toda, T., Kominami, K., Kohnosu, A., Yanagida, M., and Jones, N. (1995) *Schizosaccharomyces pombe atf1*⁺ encodes a transcription factor required for sexual development and entry into a stationary phase. *EMBO J.* **14**, 6193–6208
32. Nakagawa, C., Yamada, K., and Mutoh, N. (1998) Two distinct upstream regions are involved in expression of the catalase gene in *Schizosaccharomyces pombe* in response to oxidative stress. *J. Biochem.* **123**, 1048–1054
33. Maeda, T., Watanabe, Y., Kunitomo, H., and Yamamoto, M. (1994) Cloning of the pka1 gene encoding the catalytic subunit of the cAMP-dependent protein kinase in *Schizosaccharomyces pombe*. *J. Biol. Chem.* **269**, 9632–9637
34. Weisman, R. and Choder, M. (2001) The fission yeast TOR homolog, *tor1*⁺, is required for the response to starvation and other stresses via a conserved serine. *J. Biol. Chem.* **276**, 7027–7032
35. Moehle, C.M., Tizard, R., Lemmon, S.K., Smart, J., and Jones, E.W. (1987) Protease B of the lysosomallike vacuole of the yeast *Saccharomyces cerevisiae* is homologous to the subtilisin family of serine proteases. *Mol. Cell. Biol.* **7**, 4390–4399
36. Teichert, U., Mechler, B., Müller, H., and Wolf, D.H. (1989) Lysosomal (vacuolar) proteinases of yeast are essential catalysts for protein degradation, differentiation, and cell survival. *J. Biol. Chem.* **264**, 16037–16045
37. Ikeda, S. and Kawasaki, N. (2001) Isolation and characterization of the *Schizosaccharomyces pombe* cDNA encoding the mitochondrial endonuclease (1). *Biochim. Biophys. Acta* **1519**, 111–116
38. Ladds, G. and Davey, J. (2000) Identification of proteases with shared functions to the proprotein processing protease Krp1 in the fission yeast *Schizosaccharomyces pombe*. *Mol. Microbiol.* **38**, 839–853
39. Takeshige, K., Baba, M., Tsuboi, S., Noda, T., and Ohsumi, Y. (1992) Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *J. Cell Biol.* **119**, 301–311
40. Baba, M., Takeshige, T., Baba, N., and Ohsumi, Y. (1994) Ultrastructural analysis of the autophagic process in yeast: Detection of autophagosomes and their characterization. *J. Cell Biol.* **124**, 903–913
41. Liu, Y. and Dieckmann, C.L. (1989) Overproduction of yeast viruslike particles by strains deficient in a mitochondrial nuclease. *Mol. Cell. Biol.* **9**, 3323–3331

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 29, 2012