The nucleolus is involved in mRNA export from the nucleus in fission yeast

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Summary

To elucidate the mechanism of mRNA export from the nucleus, we isolated five novel temperature-sensitive mutants (*ptr7* to *ptr11*) that accumulate poly(A)$^+$ RNA in the nuclei at the nonpermissive temperature in *Schizosaccharomyces pombe*. Of those, the *ptr11* mutation was found in the *top2*$^+$ gene encoding DNA topoisomerase II. In addition to the nuclear accumulation of poly(A)$^+$ RNA, *ptr11* exhibited the *cut* (cell untimely torn) phenotype at the nonpermissive temperature, like the previously isolated mutant, *ptr4*. In these two mutants, cytokinesis occurred without prior nuclear division, resulting in cleavage of the undivided nuclei by the septum. To investigate the relationship between mRNA export defects and the *cut* phenotype observed in *ptr4* and *ptr11*, we analyzed 11 other mutants displaying the *cut* phenotype and found that all these tested mutants accumulate poly(A)$^+$ mRNA in the aberrantly cleaved nuclei. Interestingly, nuclear accumulation of poly(A)$^+$ mRNA was observed only in the anucleolate nuclei produced by aberrant cytokinesis. In addition, *nuc1*, the *S. pombe* mutant exhibiting a collapsed nucleolus, trapped poly(A)$^+$ mRNA in the nucleolar region at the nonpermissive temperature. In *ptr11* and *nuc1*, mRNA transcribed from the intron-containing TBP gene showed nuclear accumulation, but not transcripts from the intron-less TBP cDNA, suggesting that the export pathway differs between the spliced and unspliced TBP mRNAs. These findings support the notion that a subset of mRNAs in yeast is exported from the nucleus through transient association with the nucleolus.

Key words: *S. pombe*, *ptr* mutants, *cut*, Nucleolus, mRNA export

Introduction

Transport of mRNA from the nucleus to the cytoplasm is one of the essential steps for gene expression in eukaryotic cells. Several factors required for that process were identified in yeast and mammals (reviewed in Lei and Silver, 2002; Reed and Hurt, 2002; Cullen, 2003). Of those, Mex67p is a major mRNA export factor identified in a synthetic lethal screen with a *NUP85* mutation in *Saccharomyces cerevisiae* (Segref et al., 1997). Mex67p and its metazoan orthologue Tap were shown to shuttle between the cytoplasm and the nucleus. Mex67p/Tap localizes predominantly at the nuclear pore complexes (NPCs), where it associates with phenylalanine-glycine (FG) repeat-containing nucleoporins. The localization of Mex67p to the NPC requires heterodimerization with Mtr2p (Santos-Rosa et al., 1998). In metazoa, p15 is thought to be a functional equivalent of Mtr2p, as the co-expression of mammalian TAP and p15 in the *S. cerevisiae* mutant with double disruption of *MEX67* and *MTR2* complemented its lethality (Katahira et al., 1999).

Mex67p is recruited to spliced mRNA through interaction with Yra1p, a highly conserved REF family protein (Sträßer and Hurt, 2000; Stutz et al., 2000). By contrast, spliceosomal factor Sub2p, a DEAD box RNA helicase involved in pre-mRNA splicing, binds directly to Yra1p and is thought to operate in recruiting Yra1p to mRNA (Sträßer and Hurt, 2001). These factors are believed to couple the machineries that function in pre-mRNA splicing and the export of mRNA.

Recent genome-wide analysis of RNAs co-immunoprecipitated with Mex67p or Yra1p revealed that transcripts representing only one-third of the entire transcriptional events associate with those factors in *S. cerevisiae* (Hieronymus and Silver, 2003). This result suggests that mRNAs are exported through multiple pathways, some of which involve factors other than Mex67p and Yra1p but with similar functions.

To identify novel factors involved in nuclear mRNA export, we isolated five mutants (*ptr7* to *ptr11*) that accumulate poly(A)$^+$ RNA in the nuclei at the nonpermissive temperature in *Schizosaccharomyces pombe*. Through characterization of one of the isolated mutants, *ptr11*, we obtained evidence that the fission yeast nucleolus functions as a subnuclear structure essential for nuclear export of some mRNAs, in addition to functions in ribosome biogenesis.

Materials and Methods

Yeast in situ hybridization

The general genetic method used for *S. pombe* cells was as described (Gutz et al., 1974). In situ hybridization of poly(A)$^+$ RNA using a biotin-labeled oligo dT probe (50 mer) and triple staining using the oligo dT probe, a monoclonal antibody against fibrillarin (D77) (Aris and Blobel, 1988) and DAPI were carried out as described (Tani et al., 1995). To detect specific cellular mRNA using in situ hybridization, we amplified an entire region of the TATA binding protein (TBP) gene that contains two introns by PCR, and inserted it between the *Sac*I and *Bam*HI sites of the pREP3 vector with the *nmt1* promoter that has potent transcriptional activity (Maundrell, 1993). We also constructed the plasmid containing the TBP cDNA downstream of the *nmt1* promoter. For specific detection of TBP mRNA expressed from the plasmid, we prepared the Cy3-labeled TBP oligonucleotide probe (25 mer) that hybridizes with a region spanning the 3′ end of the TBP gene and the vector. Hybridization using the Cy3-labeled TBP probe was carried out at 37°C in solution containing 4× SSC, 5× Denhardt's, 1 mg/ml tRNA and 10% formaldehyde. Hybridized signals were not observed when 167-fold molar excess of non-labeled TBP oligonucleotide was added to the hybridization buffer, or when the expression of TBP mRNA was repressed by the addition of thiamine (data not shown). These results demonstrate that the oligonucleotide probe hybridized specifically with TBP mRNA expressed from the plasmid. The sequence of the TBP probe is 5′-CCTT*TTACCCGGGGA*TCCCTGTG*TG-3′ (the asterisks indicate the positions of Cy3-fluorochrome labeling).

Analysis of protein import and export

To examine the defects in protein import and export, pR1FPA1 expressing the transcription factor Pap1p tagged with GFP (Kudo et al., 1999) was introduced into the *ptr11-1* strain. The transformant was cultured at 26°C in a MM medium with thiamine overnight. The cells were then transferred to a fresh MM medium without thiamine. After culturing for 16 hours, the cells were shifted to 37°C for 4 hours and treated with leptomycin B at the concentration of 200 ng/ml for 30 minutes. Localization of Pap1p-GFP was examined using an OLYMPUS AX70 fluorescence microscope equipped with a Photometirics Quantix cooled CCD camera.

Electron microscopic analysis

The wild-type 972 or *ptr11-1* cells cultured at 26°C were shifted to 37°C for 4 hours and then fixed with 2.5% glutaraldehyde and 2% osmium tetraoxide. The fixed sample was dehydrated with ethanol and embedded in Quetol-651. Thin sections were cut and counterstained with 2% uranyl acetate and Reynolds lead citrate. The sections were examined at 80 kV in a JEOL GEM 1210 electron microscope.

Localization of the mRNA export factor spMex67p in heat-shocked cells

To examine intracellular distribution of spMex67p, we introduced pREP81-Gmex67 (Yoon et al., 2000) into the wild-type *S. pombe* cells (972). The transformants were grown at 30°C to the mid-log phase in a MM medium and either maintained at 30°C or heat-shocked at 42°C. An aliquot of cells was removed 3 minutes and 30 minutes after heat shock for observation. After staining with 2 μg/ml Hoechst 33342, intracellular distribution of spMex67p-GFP was analyzed using the Olympus AX70 fluorescence microscope equipped with the Photometrics Quantix cooled CCD camera. As a control, cells expressing Cut15p-GFP (Matsusaka et al., 1998) were also cultured to the mid-log phase and then shifted to 42°C for 30 minutes. Cut15p is an *S. pombe* homologue for importin α involved in nuclear protein import, and is known to localize at the nuclear periphery during G2 phase (Matsusaka et al., 1998).

Results

Isolation of novel *ptr* mutants

To isolate novel *ptr* mutants, we screened a *ts* mutant bank consisting of 390 clones (Urushiyama et al., 1996) using in situ hybridization with the oligo dT probe, and identified seven mutants (*ts96*, *ts249*, *ts264*, *ts265*, *ts267*, *ts425* and *ts473*) that accumulate poly(A)$^+$ RNA in the nuclei at the nonpermissive temperature. These mutants were backcrossed three times with a wild-type strain (97*2* or 975) to remove extra mutations. Analysis of tetrads derived from each mutant showed 2:2 segregation of *ts*$^-$ and wild-type phenotypes, suggesting that the *ts*$^-$ phenotype is due to a single mutation. After backcrossing, each mutant was subjected to complementation analysis with the previously isolated *ptr1* [poly(A)$^+$ RNA transport 1] to *ptr6* mutants (Azad et al., 1997; Shibuya et al., 1999). As a result, all the mutants were found to belong to novel *ptr* complementation groups. *ts249*, *ts264* and *ts267* fell into the same complementation groups. Thus, we named *ts425*, *ts473*, *ts265*, *ts96* and *ts249* (*ts264* and *ts267*) as *ptr7-1*, *ptr8-1*, *ptr9-1*, *ptr10-1* and *ptr11-1* (*ptr11-2* and *-3*), respectively. Of these, we chose *ptr11* for further analysis in this study.

Phenotypes of the *ptr11* mutant

In addition to the nuclear accumulation of poly(A)$^+$ RNA, the *ptr11-1* mutant showed abnormal cell division at the nonpermissive temperature. At the nonpermissive temperature, cytokinesis occurred without prior nuclear division, resulting in cleavage of the undivided nuclei in this mutant (Fig. 1A). The percentage of cells exhibiting such a phenotype reached 45% two hours after shifting to the nonpermissive temperature. Two other *ptr11* alleles, *ptr11-2* and *ptr11-3*, also displayed almost identical cytological phenotypes at the nonpermissive temperature (data not shown). Such a cytological phenotype of *ptr11* is very similar to that observed in a series of '*cut*' (cell untimely torn) mutants (Su and Yanagida, 1997; Yanagida, 1998).

Interestingly, nuclear accumulation of poly(A)$^+$ RNA was observed only in cells with the *cut* phenotype in *ptr11-1*. Poly(A)$^+$ RNA was distributed throughout the wild-type 972 cells at both 26 and 37°C, whereas poly(A)$^+$ RNA accumulated in the nuclei of the *ptr11-1* cells exhibiting the *cut* phenotype at the non-permissive temperature of 37°C (Fig. 1B; most of the cells exhibiting the *cut* phenotype were separated into a single cell during the process of in situ hybridization). No nuclear accumulation was observed in cells without the *cut* phenotype at 37°C, or cells cultured at the permissive temperature of 26°C in *ptr11-1*, indicating a possible relationship between the *cut* phenotype and the nuclear accumulation of poly(A)$^+$ RNA in this mutant.

Cloning of the *ptr11*$^+$ gene

To clone the *ptr11*$^+$ gene, we transformed *ptr11-1* using the *S. pombe* genomic library and isolated cosmid clones that complemented the temperature-sensitive phenotype of the *ptr11-1* mutation. The identified cosmid clone contained a genomic fragment of 36 kb in length. We subcloned several DNA fragments into the pSP1 vector and found complementation activity in the 8 kb *Hin*dIII/*Pst*I fragment. A

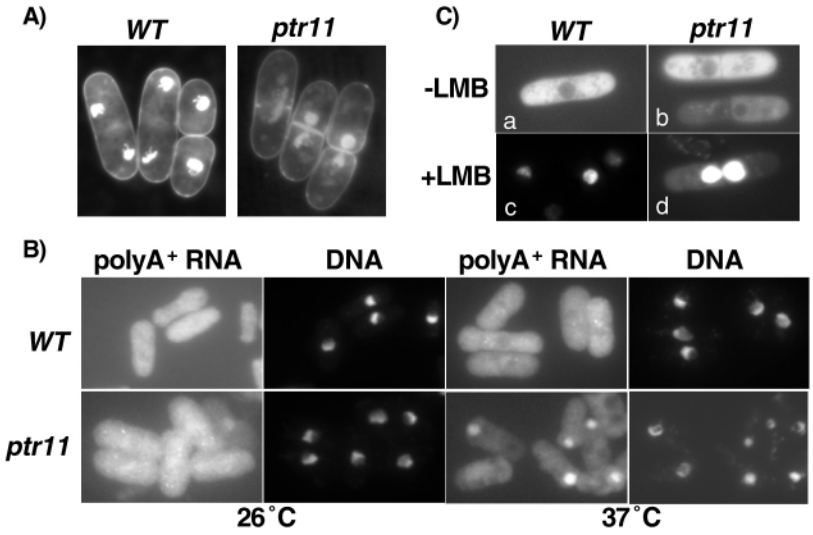

Fig. 1. Phenotypes of the *ptr11* mutant. (A) The *cut* phenotype observed in *ptr11-1*. *ptr11-1* and wild-type 972 cells cultured at 37°C for 4 hours were stained by DAPI. The undivided nucleus was cleaved by a septum in *ptr11-1*. (B) *ptr11-1* cells cultured at 26°C were shifted to 37°C for 4 hours and then subjected to in situ hybridization with the oligo dT probe. Poly(A)$^+$ RNA accumulated in the *cut* phenotype nuclei at 37°C in *ptr11-1*. (C) *ptr11-1* has no apparent defects in protein transport. The plasmid expressing Pap1p fused with GFP was introduced into cells and then localization of the expressed fusion protein was examined in the absence (a,b) or presence (c,d) of LMB at 37°C.

single ORF encoding 1431 amino acids protein was found in that fragment. Database searching revealed that the ORF is *top2*$^+$ encoding DNA topoisomerase II. In the *top2* mutant, condensed chromosomes are not formed at the time of mitosis at the nonpermissive temperature, yielding cells exhibiting the typical '*cut*' phenotype (Uemura and Yanagida, 1984; Uemura et al., 1986; Uemura and Yanagida, 1986; Uemura et al., 1987). The DNA fragment containing the *top2*$^+$ gene could rescue temperature-sensitive growth of *ptr11-1* as well as *top2-191*. It also complemented the *cut* phenotype and the defect of mRNA export at the nonpermissive temperature (data not shown). In addition, the diploid containing the *ptr11-1* and *top2-191* could not grow at the nonpermissive temperature, demonstrating that *ptr11-1* is allelic with *top2-191* (data not shown). We identified mutations that result in replacements of glycine to serine at amino acid position 438, leucine to isoleucine at 431 and arginine to histidine at 1008 in the corresponding genes in *ptr11-1*, *ptr11-2* and *ptr11-3*, respectively.

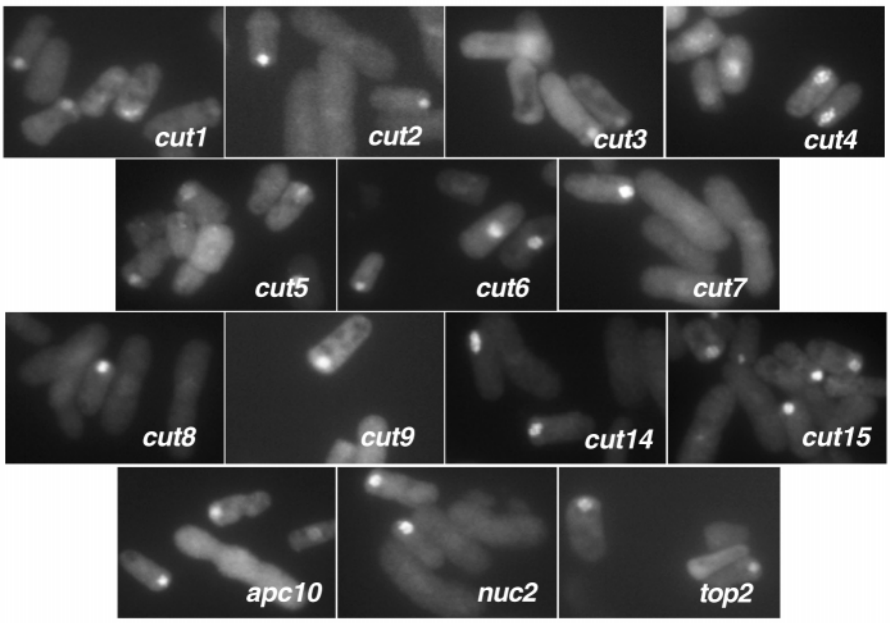

Fig. 2. In situ hybridization of poly(A)$^+$ RNA in mutants with the *cut* phenotype. After shifting to 37°C for 2 hours, each indicated mutant was subjected to in situ hybridization with the biotin-labeled oligo dT probe. Photographs were taken with the same exposure time.

ptr11 has no defects in nucleocytoplasmic transport of a protein with a NLS and a NES

To determine whether the *ptr11* mutation causes a defect in nuclear protein transport in addition to the defect in mRNA export, we introduced a plasmid expressing a Pap1p-GFP protein (Kudo et al., 1999) into the *ptr11-1* mutant and analyzed the intracellular distribution of the expressed fusion protein. Pap1p is a shuttling transcription factor containing a basic-type nuclear localization signal (NLS) and a leucine-rich nuclear export signal (NES), the export of which is mediated by Crm1p (Toda et al., 1992; Toone et al., 1998). The Pap1p-GFP protein was predominantly distributed in the cytoplasm in the wild-type cells and *ptr11-1* cells exhibiting the *cut* phenotype at 37°C (Fig. 1Ca,b). After addition of leptomycin B (LMB), which inhibits the CRM1-dependent protein export into the cytoplasm, the reporter protein accumulates into the nuclei of the *ptr11-1* cells with the *cut* phenotype as observed in the wild-type cells, demonstrating that there are no defects in protein import (Fig. 1Cd). Also, the cytoplasmic distribution of Pap1p-GFP in the absence of LMB implies that protein export continues at the nonpermissive temperature in *ptr11-1* (Fig. 1Cb). Based on these observations, we concluded that blockage of export in *ptr11-1* is specific for poly(A)$^+$ RNA.

Other mutants with the *cut* phenotype also accumulate poly(A)$^+$ RNA at the non-permissive temperature

To date, more than a dozen of mutants that show the *cut* phenotype at the nonpermissive temperature have been isolated (reviewed in Su and Yanagida, 1997; Yanagida, 1998). Thus, we wanted to know whether such mutants with the *cut* phenotype also show defects in mRNA export. After shifting to the nonpermissive temperature for 2 hours, *cut1* to *cut9*, *cut14*, *cut15*, *top2*, *nuc2* and *apc10* (Hirano et al., 1989; Samejima et al., 1993; Hirano et al., 1988; Kominami et al., 1998) were subjected to in situ hybridization with the oligo dT probe. All these mutants display the *cut* phenotype when they are cultured at the nonpermissive temperature. At the permissive temperature of 26°C, poly(A)$^+$ RNA was distributed throughout the cells in these mutants. In contrast, at the nonpermissive temperature, poly(A)$^+$ RNA accumulated in the nuclei of the cells exhibiting the *cut* phenotype in all the mutants (Fig. 2). Nuclear accumulation of poly(A)$^+$ RNA was not observed in cells without the *cut* phenotype, except for the case of *cut6*, at the nonpermissive temperature.

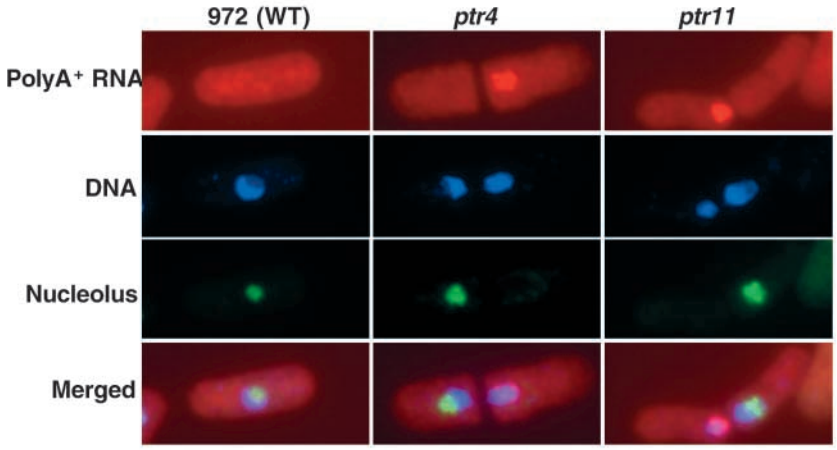

Fig. 3. Poly (A)$^+$ mRNA accumulated in the anucleolate nuclei in *ptr4-1* and *ptr11-1*. Cells cultured at 26°C were shifted to 37°C for 4 hours and then triple stained using the oligo dT probe, DAPI and anti-fibrillarin antibody (see Materials and Methods).

Poly(A)$^+$ RNA accumulated in anucleolate nuclei in *ptr11-1*

To analyze the precise localization of mRNA accumulated in the nuclei of *ptr11-1*, we did triple staining using the oligo dT probe, an antibody that recognizes a nucleolar protein fibrillarin (D77) (Aris and Blobel, 1988) and DAPI. In a *cut* phenotype cell, the nucleolus was distributed to one of the cleaved nuclei after aberrant cytokinesis, thereby producing the nucleus without the nucleolus (the anucleolate nucleus). Interestingly, as far as we examined, accumulation of poly(A)$^+$ RNA was detected only in the anucleolate nuclei that showed no staining with the D77 antibody (Fig. 3). In contrast, the divided nuclei containing the nucleolus did not accumulate poly(A)$^+$ RNA. These results are consistent with the hypothesis that the nucleolus is required for mRNA export from the nucleus in yeast (Schneiter et al., 1995; Tani et al., 1995).

The *ptr11-1* mutant contains granular materials in the cleaved nuclei

To learn about the alteration of the nuclear structure in *ptr11-1* at the ultrastructural level, we performed an electron microscopic analysis. The *ptr11-1* cells grown at 26°C were shifted to 37°C for 4 hours, then subjected to EM analysis. As shown in Fig. 4, electron-dense granular materials were observed in one of the cleaved nuclei that do not have the nucleolus. By contrast, the cleaved nuclei containing the nucleolus did not have such electron-dense materials. The wild-type 972 cells showed no electron-dense granular materials in the nuclei at 37°C (data not shown). Considering the results of the fluorescent in situ hybridization with the oligo dT probe, the granular materials found in the anucleolate nuclei are likely to be accumulated poly(A)$^+$ mRNA, although the electron microscopic in situ hybridization analysis using the oligo dT probe (Tani et al., 1995) is necessary for the conclusion.

The mutant with the collapsed nucleolus is defective in mRNA export

In relation to the hypothesis that the nucleolus is involved in export of mRNA from the nucleus, we analyzed mRNA export in mutants that show an altered nucleolar structure at the nonpermissive temperature. Fission yeast mutant *nuc1-632* has a mutation in the gene encoding the large subunit of RNA polymerase I (Hirano et al., 1989). At the nonpermissive temperature, this mutant shows the collapsed nucleolus and abnormal nuclear structure; the chromatin region becomes a hollow bowl-like structure (the ring phenotype) due to the loss of the active RNA polymerase I. As expected, a strong nuclear accumulation of poly(A)$^+$ RNA was detected in *nuc1-632* at the non-permissive temperature (Fig. 5). The accumulated poly(A)$^+$ RNA co-localized with the fibrillarin-rich nucleolar region, which redistributed to the center of the nucleus.

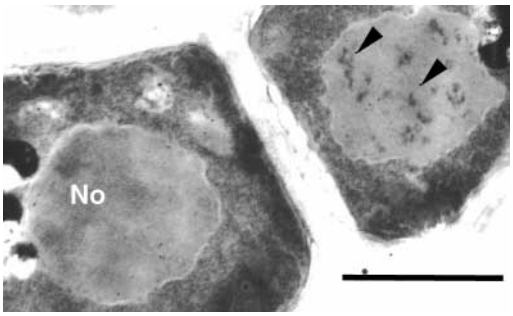

Fig. 4. Electron microscopic analysis of *ptr11-1*. The *ptr11-1* cells were cultured at 37°C for 4 hours, fixed and embedded in Quetol-651. Thin sections were examined under an electron microscope. Part of a *cut* phenotype cell with the cleaved nuclei is shown. Arrowheads denote electron-dense materials. No, the nucleolus. Bar, 1 μm.

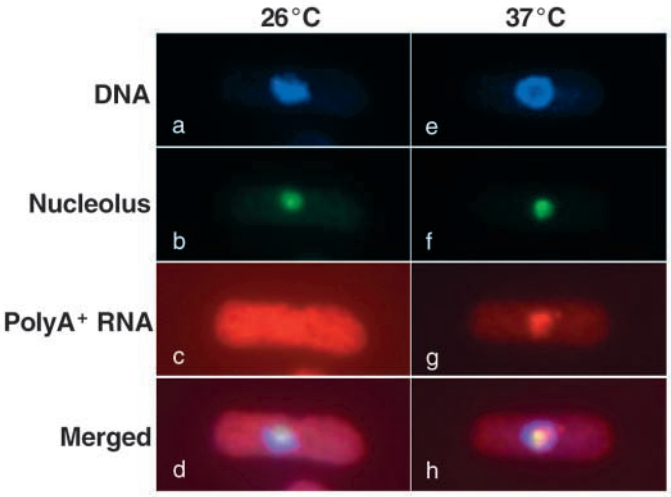

Fig. 5. In situ hybridization of poly(A)$^+$ RNA in *nuc1-632*. The mutant cells were cultured at 26°C to the midlog phase and shifted to 37°C for 4 hours. The cells were then subjected to triple staining analysis using the oligo dT probe, DAPI and anti-fibrillarin antibody.

In some yeast mutants, snRNAs are reported to be aberrantly polyadenylated (Elela and Ares, 1998; Van Hoof et al., 2000). To exclude the possibility that nuclear signals detected in *nuc1* and *ptr11-1* result from such polyadenylated snRNAs, we performed northern blot analyses using oligonucleotide probes for U1 to U6 snRNAs in these mutants. As a result, no such aberrantly polyadenylated snRNAs were detected in these mutants, suggesting that observed nuclear signals reflect accumulation of poly(A)$^+$ mRNA (data not shown).

Intron-dependent accumulation of TBP mRNA in *ptr11* and *nuc1*

We next examined the export of a specific mRNA in *ptr11* and *nuc1* cells. To that end, we introduced a plasmid containing the genomic TBP gene fused with the *nmt1* promoter into *ptr11* and *nuc1* cells. Localization of the transcripts was then visualized by in situ hybridization using Cy3-labeled oligonucleotide probe specific for TBP transcripts from the introduced plasmid. As shown in Fig. 6A, nuclear signals were detected with the TBP probe both in *ptr11-1* and *nuc1* at the nonpermissive temperature. At the permissive temperature, the hybridized signals were observed throughout the cells, indicating that the TBP transcripts were exported to the cytoplasm (Fig. 6A).

It has been suggested that mRNA export is coupled with pre-mRNA splicing in eukaryotic cells (reviewed in Reed and Hurt, 2002). In *S. pombe*, about 57% of the genomic genes have no introns (Wood et al., 2002). We asked if the presence of an intron in the gene would affect mRNA export in *ptr11* and *nuc1*. We constructed the plasmid containing the intron-less TBP cDNA instead of the genomic TBP gene that has two introns, and introduced it into the mutants. Efficient expression of the TBP transcripts from the plasmid was confirmed by northern blot analysis (data not shown). Interestingly, in the case of the TBP transcript from the cDNA, hybridized signals were detected throughout cells at the nonpermissive temperature of 37°C (Fig. 6B), indicating that the

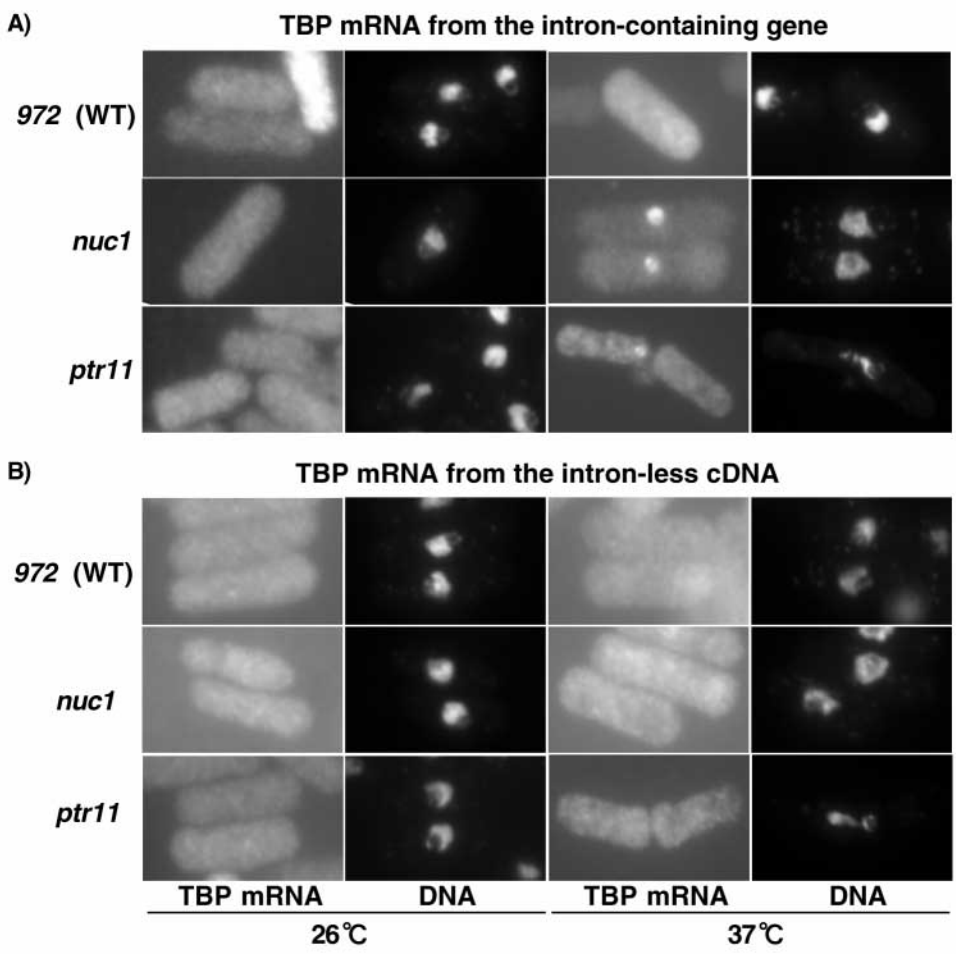

Fig. 6. Cellular distribution of TBP mRNA transcribed from the intron-containing gene (A) or intron-less cDNA (B) in *ptr11-1* and *nuc1*. Wild-type 972, *ptr11-1* or *nuc1* cells harboring the TBP-expressing plasmid was cultured at 26°C without thiamine for 16 hours, then maintained at 26°C or shifted to 37°C for 6 hours. The cells were subsequently subjected to in situ hybridization with the Cy3-labeled TBP probe specific for the mRNA expressed from the plasmid. The first and third columns show the intracellular distribution of the TBP mRNA and the second and fourth columns show cells stained with DAPI in the corresponding fields.

intron-less TBP transcripts were transported to the cytoplasm in the *cut* phenotype cells.

Nucleolar distribution of the export factor Mex67p in the heat shocked *S. pombe* cells

The results presented above suggest that part of mRNAs are exported through transient association with the nucleolus in *S. pombe*. If that is the case, then a portion of an export factor is expected to be present in the nucleolus in *S. pombe*. We therefore examined distribution of Mex67p in *S. pombe* cells. Mex67p and its metazoan orthologue Tap are known as essential export factors for mRNA and play a key role in a mRNA export pathway (for reviews, see Lei and Silver, 2002; Reed and Hurt, 2002; Cullen, 2003). The *S. pombe* homologue for Mex67p (spMex67p) was also shown to be involved in mRNA export in *S. pombe* (Yoon et al., 2000).

We introduced the pREP81-Gmex67 plasmid that expresses spMex67p fused with GFP from the weakest *nmt1* promoter (Yoon et al., 2000) into the wild-type *S. pombe* cells, and observed the in vivo location of the expressed fusion protein. The fusion protein was shown to be functional in *S. pombe* (Yoon et al., 2000). As shown in Fig. 7, spMex67p-GFP was localized at the nuclear periphery and diffusely in the nucleus at 30°C as previously reported (Yoon et al., 2000). It is noteworthy that spMex67p-GFP is apparently distributed in the nucleolus in addition to the chromatin region in the nucleus at the steady state (Fig. 7, insets in left panels).

It has been previously shown that a severe heat shock at 42° causes fragmentation of the nucleolus and block of mRNA transport from the nucleus, resulting in the nucleolar accumulation of bulk poly(A)$^+$ mRNA in *S. pombe* (Tani et al., 1995). Interestingly, we found that the severe heat shock of the *S. pombe* cells at 42°C impedes the localization of spMex67p-GFP to the nuclear periphery and results in its accumulation in the nonchromatin region, namely, the nucleolus (Fig. 7A, middle and right panels). By contrast, cellular distribution of Cut15p, the *S. pombe* homologue for importin α that is involved in protein import and localizes at the nuclear periphery, was not changed under the same heat shock conditions, suggesting that the nucleolar accumulation under the heat shock stress is specific for spMex67p (Fig. 7B).

Discussion

By screening of the *S. pombe ts* mutant bank with in situ hybridization, we identified seven mutants defective in transport of poly(A)$^+$ mRNA from the nucleus to the cytoplasm at the nonpermissive temperature. The complementation analysis revealed that those mutants can be classified into five novel *ptr* complementation groups named *ptr7* to *ptr11*.

mRNA export is defective in the anucleolate nuclei

In addition to defects in mRNA export, *ptr11* showed the *cut* phenotype; septation and/or cytokinesis occurs in the absence of sister-chromatid separation at the nonpermissive temperature. Sequence analysis of the *ptr11*$^+$ gene revealed it to be identical to the *top2*$^+$ gene encoding DNA topoisomerase II, the mutation of which is known to cause the *cut* phenotype

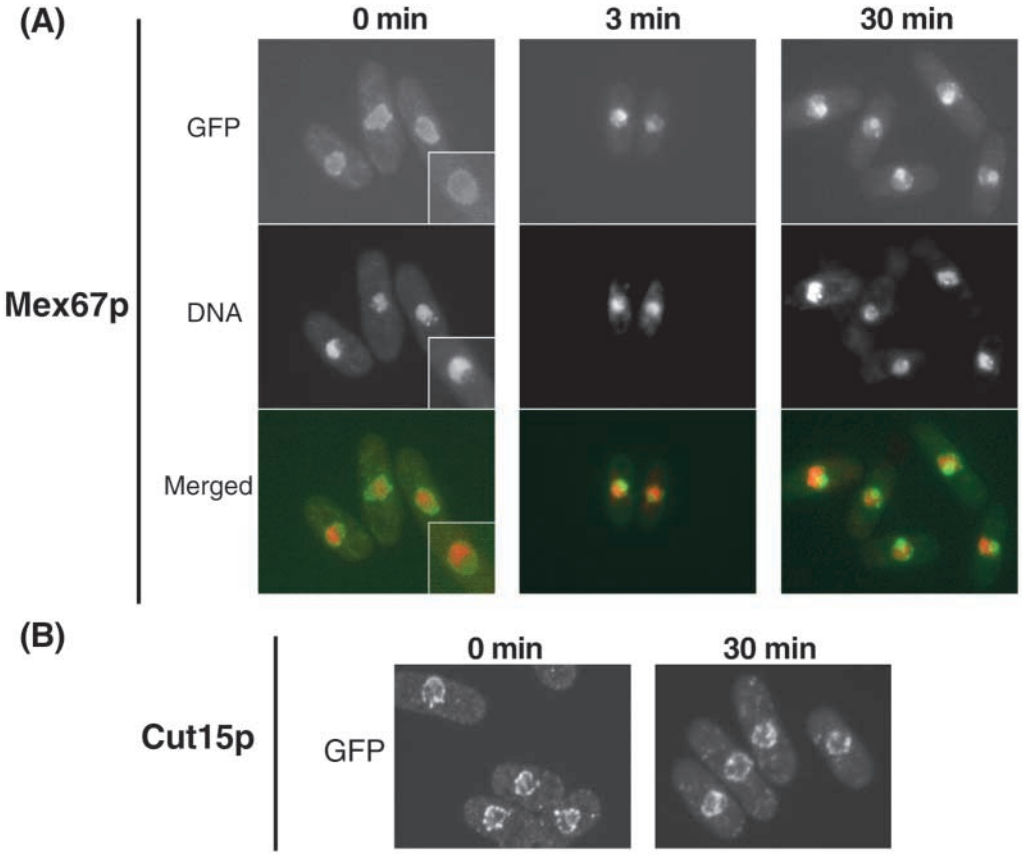

Fig. 7. spMex67p-GFP accumulates in the nucleolar region in the heat-shocked cells. (A) The wild-type *S. pombe* cells expressing spMex67p-GFP from the weakest *nmt1* promoter were grown at 30°C and either maintained at 30°C (left panels) or shifted to 42°C for 3 or 30 minutes (middle and right panels). After staining with Hoechst 33342, the distribution of spMex67p-GFP was observed with a fluorescence microscope. Insets in the left panels show enlarged pictures of the nucleus. In merged images, red denotes DNA and green denotes spMex67p-GFP. (B) Wild-type cells expressing Cut15p-GFP using the native *cut15* promoter were grown at 30°C (left panel) or heat shocked at 42°C for 30 min (right panel). Intracellular distribution of Cut15p-GFP was not changed after the heat shock treatment, whereas spMex67p-GFP accumulates rapidly in the nucleolar region under the same conditions.

at the nonpermissive temperature. We reported that the previously isolated mutant, *ptr4*, also exhibits the *cut* phenotype at the nonpermissive temperature in addition to the mRNA export defect (Azad et al., 1997). *ptr4-1* is allelic with *cut1*, and a mutant of Cut2p cooperating with Cut1p in sister chromatid separation was also shown to be defective in mRNA export at the nonpermissive temperature (Azad et al., 2003). In addition to these mutants, we found that 11 other mutants (*cut3* to *cut9*, *cut14*, *cut15*, *apc10* and *nuc2*) known to exhibit the *cut* phenotype accumulate poly(A)$^+$ mRNA in the aberrantly cleaved nuclei at the nonpermissive temperature (Fig. 2). Genes responsible for these mutations code for various proteins involved in coordinated mitosis and have diverse activities. It is unlikely that all the products from these genes themselves play direct roles in mRNA export. A block of mRNA export, if anything, seems to couple with representation of the *cut* phenotype.

In the *cut* phenotype cells, only the anucleolate nuclei produced by the aberrant cytokinesis accumulated poly(A)$^+$ mRNA. The other half of the cleaved nuclei containing the nucleolus did not accumulate poly(A)$^+$ mRNA. Interestingly, transcripts from the intron-containing TBP gene accumulated in the anucleolate nuclei, whereas no nuclear accumulation was observed for transcripts from the intron-less cDNA (Fig. 6). As we detected no defects in pre-mRNA splicing in the *ptr11* cells shifted to 37°C on northern blot (data not shown), it is likely that the spliced TBP mRNA accumulated in the nuclei. These results lead us to speculate that transcripts from the intron-containing genes are exported to the cytoplasm through transient association with the nucleolus in *S. pombe*. Supporting this scenario, the *S. pombe* mutant *nuc1*, exhibiting the collapsed nucleolus, trapped poly(A)$^+$ RNA and transcripts from the intron-containing TBP gene in the nucleolar region at the nonpermissive temperature (Figs 5, 6). In fission yeast, export pathways for the intron-containing and intron-less mRNAs might be different from each other, at least in part.

The partially distinct export processes for intron-containing and intron-less mRNAs have been suggested in mammalian cells (for a review, see Reed and Hurt, 2002). For the transcripts from the intron-containing genes, the splicing factor UAP56 (Sub2p in yeast) associates with intron-containing transcripts at an early step of the spliceosome assembly. UAP56 is then thought to recruit the export factor Aly (Yra1p in yeast) to the spliced mRNA as a component of the exon junction complex (EJC), which is formed 20-24 nucleotides upstream of the exon-exon junction in a splicing-dependent manner (Luo et al., 2001; Le Hir et al., 2001; Reichert et al., 2002). Subsequently, Aly interacts directly with TAP (Mex67p in yeast), which has an ability to bind with the NPC components (Stutz et al., 2000). By contrast, for naturally intron-less mRNAs such as the histone H2a mRNA, specific sequences that facilitate the nuclear export have been identified (Liu and Mertz, 1995; Huang and Carmichael, 1997). It was recently shown that SR proteins SRp20 and 9G8 interact specifically with a 22-nt element in the histone H2a mRNA and promote its export from the nucleus (Huang and Steiz, 2001). Those factors were later found to serve as adapter proteins for TAP-dependent mRNA export (Huang et al., 2003). As there are no apparent homologues for SRp20 and 9G8 in *S. pombe*, further studies are necessary to elucidate the mechanism by which the intron-less TBP mRNA is exported to the cytoplasm in the anucleolate cells.

Involvement of the nucleolus in mRNA export

The nucleolus is widely known as a subnuclear organelle for transcription and processing of rRNAs and their subsequent assembly into the pre-ribosome. However, recent studies indicate that the nucleolus has many activities unrelated to ribosome biogenesis (reviewed in Pederson, 1998; Olson et al., 2000). It was reported that the nucleolus plays roles in sequestering regulatory molecules for the cell cycle (Visintin et al., 1999), modifying small nuclear RNAs (Ganot et al., 1999; Mouaikel et al., 2002; Gerbi and Lange, 2002), assembly of small RNPs (Politz et al., 2000), and controlling aging (Guarente, 1997), in addition to pre-ribosome biogenesis.

Possible involvement of the nucleolus in mRNA export has also been proposed from observations made in yeasts and higher eukaryotic cells. Ealier studies in higher eukaryotic cells showed that inactivation of the HeLa and BSC-1 nucleoli by UV irradiation inhibits the export of nonribosomal RNAs from the nucleus to the cytoplasm (Sidebottom and Harris, 1969; Deák, 1973). c-myc mRNA was reported to localize in the nucleolus of mammalian cells (Bond and Wold, 1993; Bains et al., 1997). In addition, the HIV and HTLV-I proteins Rev and Rex, involved in viral mRNA export, show a predominantly nucleolar localization (Siomi et al., 1988; Cullen et al., 1988; Kubota et al., 1989). Furthermore, a mutation in the nucleolar protein Mtr3p was shown to cause nucleolar accumulation of poly(A)$^+$ RNA in *S. cerevisiae* (Kadowaki et al., 1995) and several mRNA transport mutants in *S. cerevisiae* accumulate poly(A)$^+$ RNA in the fragmented nucleoli at the nonpermissive temperature, suggesting a possible role of the nucleolus in mRNA export (Schneiter et al., 1995). Similar nucleolar accumulation of poly(A)$^+$ RNA was observed in heat-shocked cells not only in *S. pombe* (Tani et al., 1995) but also in *S. cerevisiae* (Liu et al., 1996; Saavedra et al., 1996).

Interestingly, we found that a severe heat shock at 42°C causes very rapid redistribution of spMex67p to the nucleolus in *S. pombe* (Fig. 7). spMex67p is present at the nuclear periphery and diffusely in the nucleus at a normal state (30°C). Redistribution of spMex67p to the nucleolus was detected even three minutes after the shift to 42°C (Fig. 7A). In the heat-shocked cells, nuclear accumulation of poly(A)$^+$ mRNA was first observed at 5 minutes after heat shock (Tani et al., 1995), suggesting that nucleolar accumulation of spMex67p is preceding to the block of mRNA export.

At present, a precise mechanism that causes redistribution of spMex67p under the heat shock stress is unknown. Using photobleaching techniques, it has been shown that the splicing factor SF2/ASF and the rRNA processing protein fibrillarin rapidly associate and dissociate with their corresponding nuclear compartments, that is, the nuclear speckles and the nucleolus (Phair and Misteli, 2000). spMex67p might also reiterate rapid association and dissociation with the nucleolus to transport a subset of mRNAs to the cytoplasm, and disorganization (fragmentation) of the nucleolus caused by heat shock might prevent the dissociation of spMex67p from the nucleolus, resulting in the accumulation of spMex67p and poly(A)$^+$ mRNA in the nucleolus. Distribution of spMex67p in the nucleolus at a steady state (Fig. 7, left panels) supports this

idea. A subset of mRNAs might be exported to the cytoplasm through transient association with the nucleolus in *S. pombe.*

Our results obtained in this study provide another line of evidence that the nucleolus plays a role in mRNA export in yeast. Recently, directed proteomic analysis of the human nucleolus was reported (Andersen et al., 2002). Using a combination of mass spectrometry and sequence database searches, 271 nucleolar proteins were identified. Interestingly, all the identified proteins do not necessarily have functions associated with the known roles of the nucleolus, that is, pre-ribosome assembly. It is noteworthy that an essential export factor Aly/REF (Zhou et al., 2000) was identified to be a nucleolar component in that proteomic analysis (Andersen et al., 2002). As mentioned above, Aly/REF is a component of EJC, and it has been thought that EJC is involved in mRNA export in addition to the nonsense-mediated decay and the cytoplasmic localization of mRNA. The presence of Aly/REF in the nucleolus implies a possible involvement of the nucleolus in the export of spliced mRNAs.

In addition to Aly/REF, hnRNPA1 required for the export of a subset of mRNAs (Izaurralde et al., 1997) was identified in the isolated nucleolus (Saavedra et al., 1996). Aly/REF and hnRNPA1 are thought to constitute the mRNP complex for nuclear export. In yeast, the nucleolus might function as a subnuclear structure for assembly or maturation of the export competent mRNP complex for some subsets of mRNA species, such as spliced TBP mRNA, like the nucleolar assembly of SRP (Politz et al., 2000). Further study will continue to elucidate molecular details of nucleolar functions in mRNA export.

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