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Characterization of the *ptr5*$^+$ gene involved in nuclear mRNA export in fission yeast

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ABSTRACT

To analyze the mechanisms of mRNA export from the nucleus to the cytoplasm, we have isolated eleven mutants, *ptr* [poly(A)$^+$ RNA transport] *1* to *11*, which accumulate poly(A)$^+$ RNA in the nucleus at a non-permissive temperature in *Schizosaccharomyces pombe*. Of those, the *ptr5–1* mutant shows dots- or a ring-like accumulation of poly(A)$^+$ RNA at the nuclear periphery after shifting to the nonpermissive temperature. We cloned the *ptr5*$^+$ gene and found that it encodes a component of the nuclear pore complex (NPC), nucleoporin 85 (Nup85). The *ptr5–1* mutant shows no defects in protein transport, suggesting the specific involvement of Ptr5p/Nup85p in nuclear mRNA export in *S. pombe*. We identified Seh1p, a nucleoporin interacting with Nup85p, an mRNA-binding protein Mlo3p, and Sac3p, a component of the TREX-2 complex involved in coupling of nuclear mRNA export with transcription, as multi-copy suppressors for the *ptr5–1* mutation. In addition, we found that the *ptr5–1* mutation is synthetically lethal with a mutation of the mRNA export factor Rae1p, and that the double mutant exaggerates defective nuclear mRNA export, suggesting that Ptr5p/Nup85p is involved in nuclear mRNA export through Rae1p. Interestingly, the *ptr5–1* mutation also showed synthetic effects with several *prp* pre-mRNA splicing mutations, suggesting a functional linkage between the NPCs and the splicing apparatus in the yeast nucleus.

1. Introduction

Transport of mRNAs from the nucleus to the cytoplasm is an essential step for gene expression in eukaryotic cells. mRNAs, which carry protein information transcribed from DNA, transit the nuclear membrane through the nuclear pore complex (NPC) to the cytoplasm to be translated to proteins. In *Saccharomyces cerevisiae*, Mex67, a well-known essential mRNA export factor, binds to mRNA via interaction with Yra1 [1,2]. Recent studies revealed that the assembly of an mRNA export complex in the nucleus is performed coordinately with transcription and pre-mRNA splicing [3]. In addition, it has been shown that transcriptionally up-regulated genes are translocated to the NPCs by interactions between the chromatin remodeling complex and the NPCs [4,5]. The mRNA export process is post-transcriptionally regulated at various levels [6].

The nuclear pore complex (NPC), consisting of about 30 proteins called nucleoporins (Nups), mediates the trafficking of macromolecules across the nuclear membrane. Nups can be divided into three classes, transmembrane, scaffold and FG Nups. In *S. cerevisiae*, Nup85 is known as a structural component of the NPC [7]. Together with Nup84, Nup120, Nup133, Nup145C, Sec13 and Seh1, Nup85 forms the Nup84 complex in *S. cerevisiae* [7]. The Nup84 complex (Nup107–120 complex in *Schizosaccharomyces pombe*) is classified into scaffold nups and forms the outer and inner rings of the NPC. The mRNA export factor, Mex67, was originally discovered by a genetic screening using the Nup85 mutant in *S. cerevisiae* [1,8].

To identify factors involved in nuclear mRNA export, we screened a bank of *S. pombe* temperature-sensitive (*ts*$^-$) mutants using fluorescence *in situ* hybridization with a biotin-labeled oligo dT probe, and isolated *ptr* [poly(A)$^+$ RNA transport] *1* to *11* mutants that accumulate poly(A)$^+$RNA in the nucleus at a nonpermissive temperature [9–13]. In this study, we report the characterization of the *ptr5*$^+$ gene and suggest a functional linkage between the NPC and the splicing apparatus in fission yeast.

2. Materials and methods

2.1. Yeast strains

rae1–167 (*h*$^-$ *leu1–32 ura4-D18 rae1–167*) was provided by Dr. Ravi Dhar (NIH, Maryland) [14]. 972 (*h*$^-$) and HM123 (*h*$^-$ *leu1–32*) were provided by Dr. Mitsuhiro Yanagida. *ptr5–1* (*h*$^-$ *leu1–32 ptr5–1*) and *ptr5–1 rae1–167* (*h*$^-$ *ade6-M210 leu1–32 ura4-D18 ptr5–1 rae1–167*) were produced in this study.

2.2. Plasmids, media and genetic methods

pSP1 was provided by Dr. Tomohiro Matsumoto (Kyoto University, Japan). pR1GLFPA6 was provided by Dr. Minoru Yoshida

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(RIKEN, Japan) [15]. Complete YE or minimal MM medium was used for standard cultures of *S. pombe* strains. The genetic methods for *S. pombe* used in this study were described previously [16,17].

2.3. Fluorescence in situ hybridization and Immunofluorescence

Cells cultured at a nonpermissive temperature of 37 °C for the indicated times were fixed with 4% paraformaldehyde and subjected to *in situ* hybridization with a biotin-labeled oligo $(dT)_{50}$ probe [9]. The samples were stained with DAPI and observed with an Olympus AX70 fluorescence microscope equipped with a Photometrics Quantix cooled CCD camera.

2.4. Protein transport assay

Cells transformed with pR1GLFPA6, which expresses a GST-GFP protein tagged with the nuclear localization signal (NLS) and the leucine-rich nuclear export signal (NES) from the thiamine-repressible *nmt1* promoter [15], were grown at 26 °C in MM medium with thiamine. The expression of the GST-GFP fusion protein was then induced by incubation of the cells at 26 °C in MM medium without thiamine for 20 h. Cells were shifted to 37 °C for 2 h and treated with leptomycin B (LMB) to block the protein export from the nucleus.

3. Results and discussion

3.1. Isolation of the ptr5$^+$ gene

The *S. pombe ptr5–1* mutant was isolated as a mutant that is temperature-sensitive for growth (ts^-) and accumulates poly(A)$^+$ RNA in the nucleus at the nonpermissive temperature [10]. To analyze the kinetics of the nuclear accumulation of mRNA in the *ptr5–1* mutant, we examined the distribution of poly(A)$^+$ RNA by fluorescence *in situ* hybridization with the oligo dT probe after shifting to 37 °C for 15, 30, 60, 120, 180 and 240 min (Fig. 1A). The *ptr5–1* mutant rapidly accumulated poly(A)$^+$ RNA in the nucleus after shifting to 37 °C. We could detect the nuclear accumulation of poly(A)$^+$ RNA even after 15 min at 37 °C. Further incubation of cells at the nonpermissive temperature induced dots or a ring-like accumulation of poly(A)$^+$ RNA in the nucleus and concomitant decrease of the cytoplasmic signals of poly(A)$^+$ RNA, thereby suggesting that the *ptr5–1* mutant has a defect in nuclear mRNA export (Fig. 1A).

To clone the *ptr5*$^+$ gene, we transformed the *ptr5–1* mutant with an *S. pombe* pSP1 genomic library. After transformation, we incubated cells at 37 °C and isolated six transformants that grew at the nonpermissive temperature. Plasmids that rescued the ts^- phenotype of the *ptr5–1* mutant were then isolated from the transformants. Restriction mapping revealed two types of plasmid clones represented by #4 and #20. For the #4 clone, we identified a single ORF (SPBC17G9.04c) responsible for complementation of the ts^- growth and defective mRNA export of the *ptr5–1* mutant after several steps of subcloning (Fig. 1B). The ORF was found to encode Nucleoporin 85 (Nup85), which is an essential component of the Nup107–120 subcomplex in the nuclear pore complex (NPC) [18,19]. Sequence analysis revealed that the *ptr5–1* mutant has a single nucleotide substitution, G–T, at position + 496 (where + 1 is the first nucleotide of the initiation codon), resulting in the replacement of 166E with K, in the *nup85*$^+$ gene. We, therefore, conclude that *nup85*$^+$ is the gene responsible for the *ptr5–1* mutation.

3.2. Distribution of NPCs in the ptr5–1 cells

NPCs are usually distributed uniformly in the nuclear envelope. It was reported that the Nup107–120 complex is involved in the structural maintenance and distribution of NPCs [19]. In *S. cerevisiae*, the

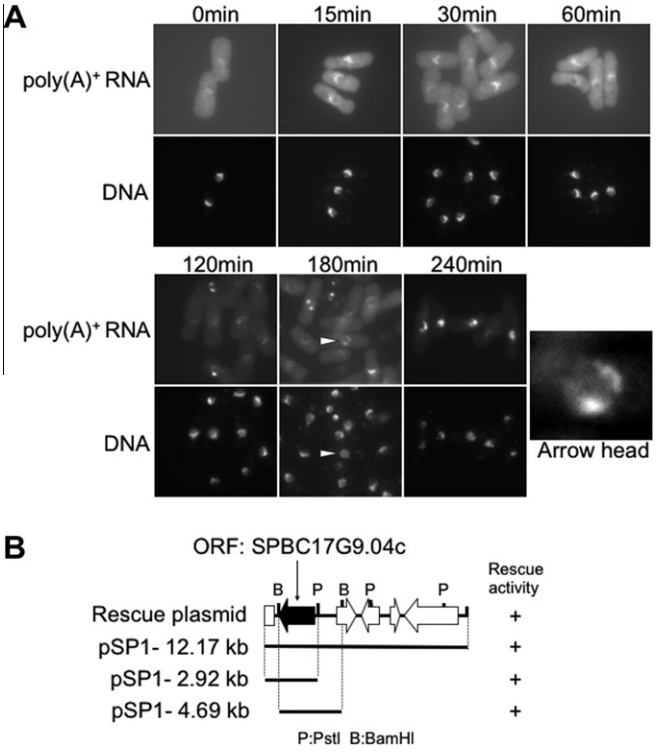

Fig. 1. (A) The *ptr5–1* mutant rapidly accumulates poly(A)$^+$ RNA in the nucleus after the shift to a nonpermissive temperature. Wild-type and *ptr5–1* cells were grown at 26 °C and then shifted to 37 °C for 0 min to 240 min. Cells were fixed and analyzed by *in situ* hybridization with a biotin-labeled oligo $(dT)_{50}$ probe. Poly(A)$^+$ RNA and DNA were stained with FITC-avidin and DAPI, respectively. The arrowhead in the 180 min panel shows a cell with the ring-like poly(A)$^+$ signal. The panel on the right, denoted as "Arrow head", shows an enlarged image of the ring-like poly(A)$^+$ signal at the periphery of the nucleus. (B) Restriction maps of the plasmid clone that rescues the *ptr5–1* mutation. The subcloned plasmid pSP-4.69 kb contains a single ORF (SPBC17G9.04) as a gene responsible for the *ptr5–1* mutation.

nup85 mutant shows clustering of NPCs [8]. To evaluate the effect of the *ptr5–1* mutation on the distribution of NPCs, we performed immunostaining of the *ptr5–1* mutant using an antibody against NPC components. In contrast to the case for *S. cerevisiae*, no cells showed an abnormal distribution of NPCs compared to that in wild-type cells at the nonpermissive temperature, at which the *ptr5–1* cells showed growth inhibition and the accumulation of poly(A)$^+$ RNA (data not shown).

3.3. Protein transport in the ptr5–1 mutant

To examine whether protein transport is also inhibited in the *ptr5–1* mutant, we carried out a protein transport assay using a GST-GFP protein tagged with the NLS and NES [15]. The fusion protein was expressed in wild-type and *ptr5–1* cells, and its localization was observed (Fig. 2). In both the *ptr5–1* and wild-type cells, the fusion protein distributed throughout the cells at 26 °C and 37 °C. Addition of leptomycin B (LMB), which blocks the nuclear export of proteins with the NES, induced the rapid accumulation of the fusion protein in the nucleus, suggesting that the fusion protein can shuttle between the cytoplasm and nucleus at both temperatures. This result means that there are no defects in the import and export of a protein with the NLS and NES in *ptr5–1* cells, suggesting that Ptr5p/Nup85p is specifically involved in nuclear mRNA export in *S. pombe*.

3.4. Genetic interactions of Ptr5p with factors involved in mRNA export

To investigate roles of Ptr5p/Nup85p in mRNA export, we attempted to identify factors interacting with Ptr5p/Nup85p by

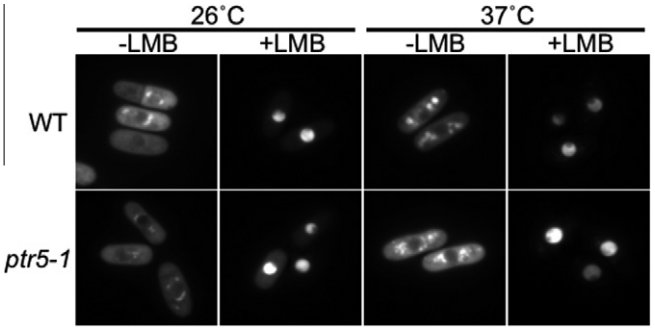

Fig. 2. The NLS-NES-GST-GFP fusion protein was expressed in the *ptr5–1* mutant and its localization was analyzed by fluorescence microscopy. Treatment with leptmycin B (LMB) blocked the export of the fusion protein. The localization of the fusion protein in the *ptr5–1* mutant was identical to that in wild-type cells after shifting to 37 °C for 1.5 h, suggesting that the *ptr5–1* mutant has no defects in protein import and export mediated by the NLS and NES.

performing a genetic analysis of *ptr5*$^+$/*nup85*$^+$ and the genes involved in nuclear mRNA export, that is, *rae1*$^+$, *mex67*$^+$, *mlo3*$^+$ and *sac3*$^+$. Rae1p is an essential mRNA export factor in *S. pombe*, located at the nuclear rim [20,21]. It forms a complex with the non-essential mRNA export factor Mex67p and mRNA-binding protein Mlo3p in *S. pombe* [22]. Sac3p is a component of the TREX-2 complex that links transcription with nuclear mRNA export and coordinates the gene relocation of transcriptionally activated genes to the NPC [23,24].

As shown in Fig. 3A, over-expression of Mlo3p and Sac3p suppressed the *ts*$^-$ phenotype of the *ptr5–1* mutant, although over-expression of Rae1p and Mex67p did not. However, the *ptr5–1 rae1–167* double mutant showed lethality and accumulated poly(A)$^+$ RNA in the nucleus at 28 °C, at which *ptr5–1* and *rae1–167* single mutants are viable and show no defects in nuclear mRNA export (Fig. 3B and C). These synthetic effects suggest that Ptr5p/Nup85p links genetically with Rae1p in the mRNA export pathway. It is possible that the mRNA export complex including Rae1p, Mlo3p and Sac3p traverses NPCs through interaction with Nup85p.

We also analyzed another plasmid clone (#20) that complemented *ts*$^-$ growth of the *ptr5–1* mutant. Subcloning and sequence analysis revealed that the *seh1*$^+$ gene, which encodes a nucleoporin interacting with Nup85 [19], is responsible for the suppressor activity of the #20 clone. There are no mutations in the *seh1*$^+$ gene in the *ptr5–1* mutant, indicating that Seh1p is a multi-copy suppressor for the *ptr5–1* mutation. Over-expression of Seh1p from the multicopy plasmid suppressed the *ts*$^-$ growth and also the accumulation of poly(A)$^+$ RNA at 36 °C (data not shown). However, disruption of the *seh1*$^+$ gene did not affect cell growth and nuclear mRNA export, demonstrating that *seh1*$^+$ is a non-essential gene (data not shown). As the Nup107–120 complex contains a protein named Sec13p that is highly homologous with Seh1p, it might substitute for Seh1p in cells with the *seh1*$^+$ deletion (Δ*seh1*). Conversely, the double mutant containing Δ*seh1* and the *ptr5–1* mutation showed synthetic lethality (data not shown), suggesting that the association of Seh1p with mutated Ptr5p/Nup85 is required for proper functioning of the NPCs.

3.5. Linkage between the mRNA export pathway and pre-mRNA splicing

Several lines of evidence suggested that nuclear mRNA export is coupled with pre-mRNA splicing in mammalian cells [25–27]. To evaluate functional relationships between the mRNA export and pre-mRNA splicing in fission yeast, we constructed double mutants with *ptr5–1* and one of the *prp* splicing mutations [28]. As a result, double mutants with *ptr5–1* and *prp1–4, prp3–2, prp8–1, prp10–1*

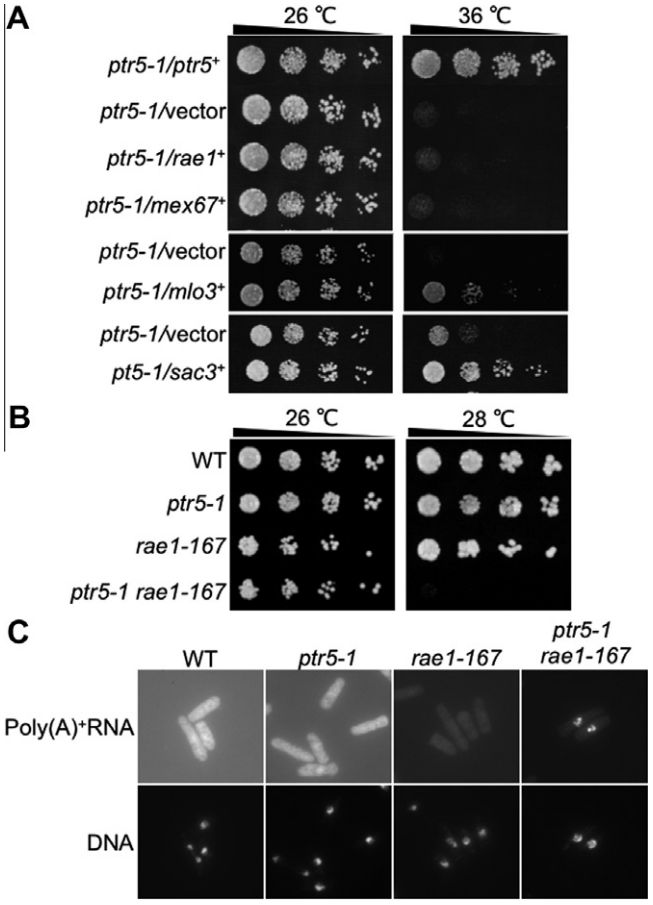

Fig. 3. (A) Over-expression of Mlo3p and Sac3p suppressed the *ts*$^-$ growth of the *ptr5–1* mutant. The *ptr5–1* mutant was transformed with pREP41, pREP41-*HArae1*$^+$, pREP41-*HA*mex67$^+$, pREP41-*HAmlo3*$^+$ or pSP1-*sac3*$^+$. Transformants ware incubated at 26 or 36 °C on MM plates. (B) The *ptr5–1 rae1–167* double mutant shows synthetic effects on mRNA export. (C) The *ptr5–1 rae1–167* double mutation exaggerates defective nuclear mRNA export. Wild-type, *ptr5–1*, *rae1–167*, or *ptr5–1 rae1–167* cells were grown to the midlog phase at 26 °C, and then shifted to 28 °C for 2 h. The cells were fixed and analyzed by *in situ* hybridization with the biotin-labeled oligo $(dT)_{50}$ probe. mRNA signals were detected by FITC-conjugated avidin. DNA was stained with DAPI.

or *prp13–1* exhibited synthetic effects on growth (Fig. 4). Double mutants containing *ptr5–1* and *prp1–4, prp3–2, prp8–1* or *prp13–1* showed synthetic lethality, whereas the double mutant with *ptr5–1* and *prp10–1* showed synthetic growth.

As 45.5% of genes contain intron sequences in *S. pombe*, the splicing reaction and its linkage with subsequent mRNA export are more essential than in *S. cerevisiae*, in which only 4% of genes contain an intron. Our results suggest that multiple pre-mRNA splicing factors functionally interact with the NPC component, Ptr5p/Nup85p, in *S. pombe*. It is noteworthy that the *ptr5–1* mutation could suppress the *ts*$^-$ phenotype of the *prp10–1* mutation, leading to synthetic growth (Fig. 4). An epistasis map (E-MAP) analysis demonstrated that positive genetic interaction, such as synthetic growth, can identify pairs of genes whose products physically interact and/or function in the same pathway [29,30]. It is, therefore, likely that Ptr5p/Nup85p associates with the splicing factor Prp10p in fission yeast. Prp10p/Sap155p (U2 snRNP), Prp1p/Zer1p and Prp13/U4snRNA (U4/U6.U5 tri-snRNP), Prp3p/Cwf2p (Prp19 complex), and Prp8p/Cdc28p (RNA helicase) are involved in the splicing reaction prior to the first step of the reaction [31]. Pre-mRNA splicing is known to occur cotranscriptionally [32,33], and transcription occurs near the NPCs in yeast [34–36]. Taken together with our results, it is possible that NPCs provide a platform to coordinate transcription, pre-mRNA splicing and

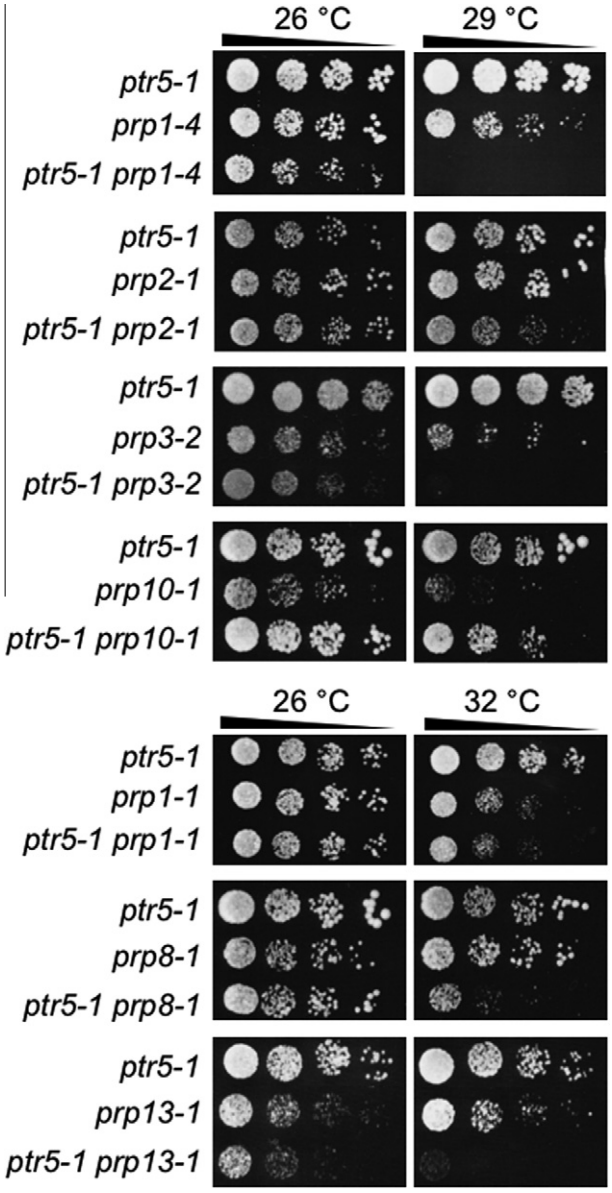

Fig. 4. The double mutants with the *ptr5–1* and *prp* mutations showed synthetic effects on growth. *ptr5–1* and the double mutant cells were serially diluted and spotted onto YEALU plates, and then incubated at 26 °C, 29 °C or 32 °C.

assembly of the export complex in fission yeast. Further analysis on the molecular mechanism of the linkage between Ptr5p/Nup85p and the splicing machinery is now underway.

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