Characterization of the *ptr6*$^+$ Gene in Fission Yeast: A Possible Involvement of a Transcriptional Coactivator TAF in Nucleocytoplasmic Transport of mRNA

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ABSTRACT

Transport of mRNA from the nucleus to the cytoplasm is one of the important steps in gene expression in eukaryotic cells. To elucidate a mechanism of mRNA export, we identified a novel *ptr* [*p*oly(A)$^+$ RNA *tr*ansport] mutation, *ptr6*, which causes accumulation of mRNA in the nucleus and inhibition of growth at the nonpermissive temperature. The *ptr6*$^+$ gene was found to encode an essential protein of 393 amino acids, which shares significant homology in amino acid sequence with yTAFII67 of budding yeast *Saccharomyces cerevisiae* and human hTAFII55, a subunit of the general transcription factor complex TFIID. A Ptr6p-GFP fusion protein is localized in the nucleus, suggesting that Ptr6p functions there. Northern blot analysis using probes for 10 distinct mRNAs showed that the amount of *tbp*$^+$ mRNA encoding the TATA-binding protein is increased five- to sixfold, whereas amounts of others are rapidly decreased at the nonpermissive temperature in *ptr6-1*. *ptr6* has no defects in nuclear import of an NLS-GFP fusion protein. These results suggest that Ptr6p required for mRNA transport is a *Schizosaccharomyces pombe* homologue of yTAFII67 and hTAFII55. This is the first report suggesting that a TAF is involved in the nucleocytoplasmic transport of mRNA in addition to the transcription of the protein-coding genes.

IN a eukaryotic cell, the nucleus and the cytoplasm are spatially separated by nuclear membrane. Thus, translocation of proteins and RNAs across the nuclear membrane is an essential process for cellular functions. Although the process of mRNA export from the transcription site (nucleus) to the translation site (cytoplasm) is an important step for gene expression, related molecular mechanisms are not well understood compared to the case of nuclear protein import.

It was reported that transport of proteins and RNAs between the nucleus and the cytoplasm is a signal- and receptor-mediated process (Görlich and Mattaj 1996). The nuclear localization signal (NLS) is found in karyophilic proteins such as the SV40 large T antigen and nucleoplasmin. Import of these proteins into the nucleus is mediated by soluble and mobile receptors through the recognition of the NLS. On the other hand, the nuclear export signals (NES) are present in several proteins that shuttle between the nucleus and the cytoplasm (Gerace 1995). Some of these NES-containing proteins are RNA-binding proteins and could be involved in the nuclear export of their cognate cargo RNA. This was most convincingly shown for the human immunodeficiency virus type 1 (HIV-1) Rev protein (Fischer *et al.* 1994; Bogerd *et al.* 1995; Stutz *et al.* 1995) and the hnRNP A1 protein (Michael *et al.* 1995).

HIV Rev protein recognizes a specific HIV RNA sequence, the Rev response element (RRE), and mediates transport of the unspliced and partially spliced viral mRNA from the nucleus to the cytoplasm. Rev protein contains the short leucine-rich NES. By using the two-hybrid method, Rabp from humans (Bogerd *et al.* 1995) and Rip1p from yeast (Stutz *et al.* 1995) have been identified as factors interacting with the Rev NES. Recently, chromosomal region maintenance (Crm)1p has been suggested to function as an essential direct receptor for the leucine-rich NES in eukaryotic cells (Fornerod *et al.* 1997; Fukuda *et al.* 1997; Stade *et al.* 1997). The *crm1*$^+$ gene was originally identified by complementation of a cold-sensitive *S. pombe* strain showing abnormal chromosomal structures at the nonpermissive temperature (Adachi and Yanagida 1989). Crm1p shares significant homology with importin-β in amino acid sequence. It shuttles between the nucleus and the cytoplasm and mediates nuclear export of proteins containing NES by binding directly to the NES signal peptide. This process is dependent on the RanGTPase system.

On the other hand, hnRNP A1 shuttles rapidly between the nucleus and the cytoplasm and is associated with poly(A)$^+$ RNA in both compartments (Piñol-Roma and Dreyfuss 1991, 1992; Michael *et al.* 1995). The NES of hnRNP A1, called M9, is larger than those of HIV Rev, protein kinase inhibitor (PKI), and tran-

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scription factor IIIA (TFIIIA) and bears no sequence homology with the previously identified NES (for a review, see Görlich and Mattaj 1996). Interestingly, the M9 sequence also exhibits NLS activity. The nuclear import of M9-containing proteins does not involve use of the importin-mediated pathway. Recently, a 90-kD protein, called transportin, was identified as the nuclear import receptor for M9-bearing proteins (Pollard *et al.* 1996). From a kinetic competition experiment using injection of hnRNP A1 into *Xenopus laevis* oocytes, hnRNP A1, or other proteins containing an M9 sequence, was considered to play a role in mRNA export from the nucleus (Izaurralde *et al.* 1997).

Genetic approaches have been used to identify factors involved in nucleocytoplasmic transport of mRNA in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. In genetic screens using *in situ* hybridization with an oligo(dT) probe, temperature-sensitive mutants that accumulated poly(A)$^+$ RNA in the nucleus at the nonpermissive temperature were isolated and analyzed. In *S. cerevisiae*, 16 *m*RNA *tr*ansport (*mtr*) and 10 *r*ibonucleic *a*cid *t*rafficking (*rat*) mutants defective in mRNA export were identified (Amberg *et al.* 1992; Kadowaki *et al.* 1994). Among the genes that were mutated in these mutants, *MTR1/PRP20* was shown to be a homologue of RCC1 in mammalian cells and *pim1*$^+$ in *S. pombe* (Kadowaki *et al.* 1992, 1993). RCC1 protein functions as a guanine nucleotide exchange factor for the Ras-like guanosine triphosphatase (GTPase) Ran/TC4 in mammalian cells (Bischoff and Ponstingl 1991) and is required for nuclear RNA export (Cheng *et al.* 1995). *MTR13/NPL3* encodes an RNA-binding protein that shuttles in and out of the nucleus as hnRNP A1 and is suggested to mediate mRNA export (Lee *et al.* 1996). *RAT1* encodes an essential 116-kD protein with regions of homology to the protein encoded by *SEP1*, which is a nucleic acid-binding protein, a 5′→3′ exonuclease, and catalyzes DNA strand transfer reactions *in vitro* (Amberg *et al.* 1992). In addition, the *RAT7/NUP159* gene encodes an essential nucleoporin containing 22 XXFG and 3 XFXFG degenerate repeats (Gorsch *et al.* 1995). In *S. pombe*, four *p*oly(A)$^+$ RNA *tr*ansport (*ptr*) and one *r*ibonucleic *a*cid *e*xport (*rae*) mutants involved in mRNA export were identified. Wild-type genes for three, *ptr2*$^+$, *ptr3*$^+$, and *rae1*$^+$, have been cloned to date. Of those, the *ptr2*$^+$ gene was found to be identical to the *pim1*$^+$ gene, encoding a homologue of RCC1 (Azad *et al.* 1997). The *ptr3*$^+$ gene encodes an *S. pombe* homologue of the ubiquitin-activating enzyme E1 in *S. cerevisiae* (Azad *et al.* 1997). The Ptr3p essential for cell viability is localized in both the nucleus and the cytoplasm. The *rae1*$^+$ gene encodes a protein of 352 amino acids with four β-transducin/WD40 repeats (Brown *et al.* 1995).

In genetic screens based on synthetic lethality, several factors involved in mRNA export were identified in *S. cerevisiae*. Two *gle* mutations that were lethal in combination with a null allele of the gene encoding the nucleoporin Nup100p were isolated in a colony-sectoring assay (Murphy and Wente 1996; Murphy *et al.* 1996). Gle1p contains a sequence similar to the Rev NES and interacts with Rip1p and the nucleoporin Nup100p. It is localized predominantly at the nuclear pore complexes. On the other hand, Gle2p encodes a 40.5-kD polypeptide with a striking similarity to *S. pombe* Rae1p. In addition, Mex67p and Mtr10p were identified by a synthetic lethal screen with a null allele of *NUP85* (Segref *et al.* 1997; Senger *et al.* 1998). Mex67p is a nuclear pore-associated protein that contains an NES, binds to poly(A)$^+$ RNA, and is likely to participate directly in export of mRNA. Mtr10p functions as a nuclear import receptor for the mRNA-binding protein Npl3p/Mtr13p (Senger *et al.* 1998).

In attempts to elucidate the mechanism of mRNA transport from the nucleus to the cytoplasm, we isolated a novel temperature-sensitive mutant (*ptr6*) defective in mRNA transport in *S. pombe*, using *in situ* hybridization. We cloned and characterized the gene complementing *ptr6*. The *ptr6*$^+$ gene was found to encode a putative homologue of TBP-associated factor (TAF), a subunit of the transcription factor complex TFIID known as a transcriptional coactivator.

MATERIALS AND METHODS

Yeast strains, media, and genetic methods: The yeast strains used in this study are listed in Table 1. The complete media YPD or YE (Gutz *et al.* 1974; Sherman *et al.* 1986) and minimum medium (MM; Moreno *et al.* 1991) were used for standard cultures of *S. pombe*. The general genetic method used for *S. pombe* was as described (Gutz *et al.* 1974).

Isolation of a *ptr* mutant: The wild-type strain 972 was mutagenized with *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine to generate a bank of the ts$^-$ mutants that can grow at 26° but not at 37°, as described (Urushiyama *et al.* 1996). We screened a collection of 100 temperature-sensitive mutants by fluorescent *in situ* hybridization (FISH) with a biotin-labeled oligo(dT)$_{50}$ probe (Tani *et al.* 1995), and identified one that causes accumulation of mRNA in the nuclei at the nonpermissive temperature.

Cloning and sequencing of the *ptr6*$^+$ gene: The *ptr6*$^+$ gene was cloned by complementation of a temperature-sensitive growth defect of *ptr6-1* with an *S. pombe* genomic library con-

TABLE 1

Yeast strains

Strains	Genotype	Source
975	*h*$^+$	M. Yanagida
972	*h*$^-$	M. Yanagida
SU59-1D	*h*$^-$ *leu1-32*	S. Urushiyama
SB14	*h*$^-$ *leu1-32 ptr6-1*	This study
ST1	*h*$^-$ *ura4-D18 ptr6-1*	This study
S22	*h*$^-$ *leu1-32 ptr2-1*	A. Azad
UR443	*h*$^+$ *ura4-D18*	S. Urushiyama
UDP6	*h*$^+$/*h*$^-$ *leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M216/ade6-M210*	S. Urushiyama
CDC10	*h*$^-$ *leu1-32 cdc10-129*	P. Nurse

structed in pSS10 (Nakaseko *et al.* 1986). Transformation of *ptr6-1* was done using the lithium acetate method (Okazaki *et al.* 1990) and 16 ts$^+$ transformants were obtained after incubation at 37°. Cosmid DNAs isolated from ts$^+$ transformants were then subjected to restriction mapping analysis and were found to contain the same insert DNA. The recovered cosmid was retested for complementing ability for ts phenotype of *ptr6-1*. After several steps of subcloning into the pSP1 vector (Cottarel *et al.* 1993), the smallest fragment with complementing activity was identified. DNA sequencing was done using an automatic DNA sequencer (ABI PRISM 377 DNA sequencer; Perkin-Elmer, Foster City, CA).

To isolate *ptr6*$^+$ cDNA, total RNA prepared from the wild-type strain as described (Urushiyama *et al.* 1996) was subjected to reverse transcription with a primer 5′-CGC AGC GTA CAA ATG ACC AT-3′. The cDNA was then amplified by PCR using the above primer and primer 5′-CGT GCT GTT CAG CCT CCT CC-3′. The PCR products were cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced.

To identify the mutation site in the *ptr6-1* gene, we carried out a gap repair analysis (Guthrie and Fink 1991). Several linear pSP1 plasmids containing the gap within the *ptr6*$^+$ gene were prepared and introduced into the *ptr6-1* mutant. The gapped region was repaired using a chromosomal sequence as a template. Replicative plasmids that complement growth at 26° but not at 37° were isolated from the transformants. The sequence corresponding to the gapped region in the isolated plasmid was then determined.

Integration mapping: To determine if the cloned gene is a multicopy suppressor for *ptr6*, target integration mapping was done as described (Guthrie and Fink 1991). The 4.2-kb *Nco*I-*Bgl*II fragment containing the *ptr6*$^+$ gene was cloned into multicloning sites of pSKur4, which contains the *ura4*$^+$ marker in the *Hin*dIII site of the Bluescript SK$^+$ vector. The resulting plasmid was linearized at the *Nde*I site within the *ptr6*$^+$ gene and introduced into wild-type strain UR443. Stable Ura4$^+$ transformants were isolated and the correct integration of the plasmid into the *ptr6*$^+$ locus was verified by PCR, using primers corresponding to the *ura4*$^+$ gene and the flanking sequence of *ptr6*$^+$ outside of the used fragment. The obtained strain was mated with ST1 (*h*$^-$, *ura4*, *ptr6-1*), and tetrads analysis was performed.

Disruption of the *ptr6*$^+$ gene: To disrupt the *ptr6*$^+$ gene, the *Nru*I-*Nru*I fragment in the *ptr6*$^+$ open reading frame (ORF) was replaced with the *S. pombe ura4*$^+$ gene. The *Hin*dIII-*Bgl*II fragment containing the *ptr6::ura4* construct was isolated and introduced into the wild-type diploid strain UDP6. Stable Ura4$^+$ transformants were then isolated. The correct replacement of the disrupted gene with the *ptr6*$^+$ gene by homologous recombination was verified by PCR using primers corresponding to the *ura4*$^+$ gene and to the flanking sequence of the *ptr6*$^+$ gene outside of the fragment used for transformation. The heterozygous diploids were sporulated at 26°, and tetrads were dissected. Colonies grown at 26° were then replica plated to MM plates to examine for auxotrophy.

Analysis of localization of Ptr6p-GFP: To construct a gene expressing a Ptr6p-GFP fusion protein, the GFP-encoding fragment isolated from pEGFP-N1 (Clontech, Palo Alto, CA) was inserted in frame into a *Bst*EII site present in a 3′-end region of the *ptr6*$^+$ gene. The resulting plasmid was then introduced into the *ptr6-1* strain. The Ptr6p-GFP fusion protein rescued the growth defect of the *ptr6-1* strain at the nonpermissive temperature (37°). After staining with 4,6-diamidino-2-phenylindole (DAPI), *in vivo* localization of the GFP fusion protein was examined using a Zeiss (Thornwood, NY) Axioplan fluorescence microscope.

Analysis of nuclear protein import: To analyze nuclear protein import, we used the method of Shulga *et al.* (1996) developed in *S. cerevisiae* with modifications for usage in *S. pombe*. To construct a gene expressing a reporter protein, the double-stranded oligonucleotide encoding the nucleoplasmin bipartite basic NLS, KRPAAIKKAGQAKKKK (Dingwall and Laskey 1991), was inserted in frame into the 5′-end of the GFP gene in pEGFP-N1 (Clontech). The fragment encoding NLS-GFP was then isolated and inserted downstream of the *nmt1* promoter in pREP3 (Maundrell 1993). This plasmid was introduced into the wild-type strain SU59-1D, the *ptr6-1* strain, or *ptr2-1* strain that has a defect in nuclear protein import (Azad *et al.* 1997) as a control. For protein import assay, transformants were incubated at 26° and grown to early log phase in MM without thiamine, and then each culture was preincubated at 37° for 1 hr. After centrifugation at room temperature in a microcentrifuge, the cell pellets were washed with dH_2O and suspended in 10 mm sodium-azide and 10 mm 2-deoxy-d-glucose in glucose-free MM. The mixture was incubated for 1 hr at 37° to allow for equilibration of the NLS-GFP fusion protein between the nucleus and the cytoplasm. The cells were pelleted again and suspended in MM prewarmed at 37°. The suspension was then incubated for 30 min at 37°. The import was initiated at time 0 by suspending cells in MM. Localization of the NLS-GFP fusion proteins was examined using a Zeiss AxioplanII fluorescence microscope.

RNA preparation and Northern blot analysis: Total RNA from *S. pombe* was prepared by the glass bead method as described (Urushiyama *et al.* 1996). RNA was quantified by absorbance at 260 nm and visualization on an agarose gel stained with ethidium bromide. For Northern blot analysis, 20 μg of each total RNA was subjected to electrophoresis in a formaldehyde/agarose gel, followed by blotting onto a nylon membrane (Gene Screen; New England Nuclear, Boston). The RNA was fixed to the membrane by UV crosslinking. The membranes were prehybridized in 6× SSC, 50 mm sodium phosphate, pH 7.5, 2 mm EDTA, 0.1% SDS, 5× Denhardt's solution, and 0.2 mg/ml boiled salmon sperm DNA at 42° for several hours. Hybridization was performed overnight in the same solution with ^{32}P-labeled oligonucleotide probes at 42°. The membranes were washed three times in 6× SSC at room temperature, then soaked in a solution containing 6× SSC and 0.1% SDS at 50° for 5 min. The filters were exposed to a Fuji imaging plate, and a Bioimaging Analyzer BAS1000 (Fuji Photo Film Co., Kanagawa, Japan) was used for analysis.

Sequences of oligonucleotides used as probes are listed in Table 2. To determine if the *ptr6* mutant was defective in pre-mRNA splicing, a probe complementary to the third exon of TATA-binding protein (TBP) pre-mRNA was used. A ^{32}P-labeled oligo(dT)$_{30}$ probe was used to quantify the amount of the total poly(A)$^+$ RNA.

Fluorescence-activated cell sorting (FACS) analysis: FACS analysis was performed as described (Alfa *et al.* 1993). A total of 2×10^6 exponentially growing cells cultured at 26° or shifted to 37° for 2 or 4 hr were fixed in 70% ethanol at 4° overnight. After washing with 1 ml of 50 mm sodium citrate, the cells were resuspended in 1 ml of a solution containing 50 mm sodium citrate, 0.1 mg/ml RNase A, and 2 μg/ml propidium iodide and were subjected to an analysis with a Becton-Dickinson (San Jose, CA) FACScan. The *cdc10-129* strain that arrests in the G1 phase at 37° (Nurse *et al.* 1976) was used as a control.

Nucleotide sequence accession number: The nucleotide sequence reported here has been submitted to the DDBJ data base. The assigned accession no. is AB016928.

RESULTS

Isolation of a novel mutant defective in mRNA transport: From a bank of ts$^-$ mutants generated by ethylmethane sulfonate, we initially isolated four mRNA

TABLE 2

Oligonucleotides used as probes in Northern blot analysis

TBP	5′-	GAG	CTT	GGA	GTC	ATC	CTC	GG	-3′		
PTR6	5′-	GTC	GTC	CCG	ATC	GAT	GCT	GGT	ACC	ACG	AAG	-3′
ACT1	5′-	CAG	AGT	CCA	AGA	CGA	TAC	CAG	TGG	TAC	GAC	-3′
RNA1	5′-	TCT	GAC	AAC	CAT	CTA	GCA	GCT	TCG	GTT	CCA	-3′
RAE1	5′-	TGA	CCC	AGT	TGC	CAG	AAT	AGG	TGA	AGT	ACC	-3′
PIM1	5′-	GAA	GTC	GCA	TCT	GCG	TCG	GTT	CAG	CTG	TAC	-3′
TAF72	5′-	GAT	CAC	AGC	TCC	AAA	GTT	GTG	CAG	TCT	GGT	-3′
PRP2	5′-	CGA	GCC	TGT	CTG	CTA	GCA	CCT	GGT	TGT	AAG	-3′
CDC2	5′-	CAC	GAA	TAG	CTG	TGC	TAG	GAA	CTC	CCT	CAG	-3′
RPB1	5′-	GCG	TTG	TCC	ACT	TTC	ATC	CAT	GGT	CTC	AGG	-3′

transport mutants (*ptr1–4*; Azad *et al.* 1997) and later another additional mutant named *ptr5* (T. Akimatsu, T. Tani and Y. Ohshima, unpublished results) by screening by *in situ* hybridization. To identify novel mRNA transport mutants, we screened a collection of ts$^-$ mutants obtained by mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (Urushiyama *et al.* 1996). Of the 100 mutants screened, we identified 1 ts$^-$ mutant (ts376) that accumulates poly(A)$^+$ RNA in the nuclei at the nonpermissive temperature (Figure 1). This mutant showed nuclear signals in >90% of the cells 4 hr after shifting to the nonpermissive temperature. The intensities of the nuclear signals were weaker than those of the previously identified *ptr* mutants.

This mutant was then backcrossed three times with a wild-type strain to remove extra mutations. Tetrad analysis showed 2:2 segregation of the temperature-sensitive and wild-type phenotypes in all cases, which means that the ts$^-$ phenotype is due to a single mutation. Cosegregation of the mRNA export defect with the ts$^-$ phenotype was also observed, suggesting that the ts$^-$ growth phenotype is linked with a mutation responsible for blocking mRNA transport. This mutation is recessive, as the heterozygous diploid with the wild-type alleles grew well at the nonpermissive temperature.

To determine if the isolated mutant is a novel mRNA transport mutant, complementation analyses were done with the previously identified mRNA transport mutants, *ptr1–5*. Heterozygous diploids in all combinations grew at 37°, thereby demonstrating that the isolated mutant belongs to a new complementation group that we named *ptr6*.

Growth characteristics of *ptr6*: To examine the growth characteristics of *ptr6*, the growth rates and viabilities of the wild-type strain and the *ptr6-1* mutant were compared over a 12-hr period (Figure 2). The growth rate and viability of the *ptr6-1* mutant were almost the same as those of the wild-type strain at the permissive temperature (26°). In contrast, *ptr6* ceased growing and rapidly lost viability after shifting to the nonpermissive temperature (37°).

Cloning of the *ptr6*$^+$ gene: To clone the *ptr6*$^+$ gene,

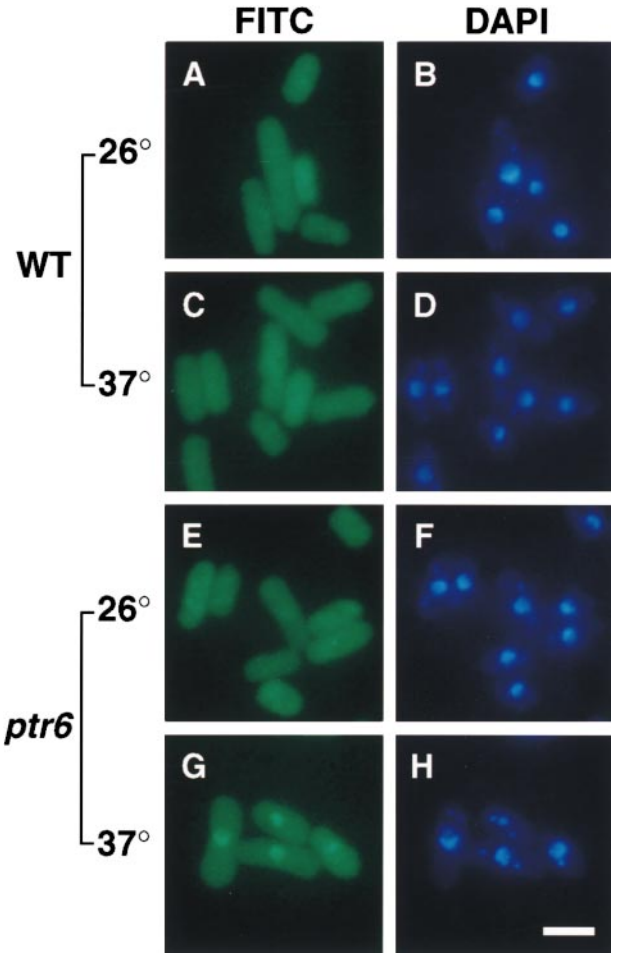

Figure 1.—Accumulation of poly(A)$^+$ RNA in the nucleus in *ptr6* after a shift to the nonpermissive temperature. Wild-type and *ptr6-1* cells were grown to the midlog phase at 26° and shifted to 37° for 4 hr. The cells were fixed and analyzed by *in situ* hybridization with a biotin-labeled oligo(dT)$_{50}$ probe. Hybridized signals were detected by FITC-conjugated avidin. A, C, E, and G show the poly(A)$^+$ RNA distribution, and B, D, F, and H show the cells stained by DAPI in the corresponding fields. Bar, 10 μm.

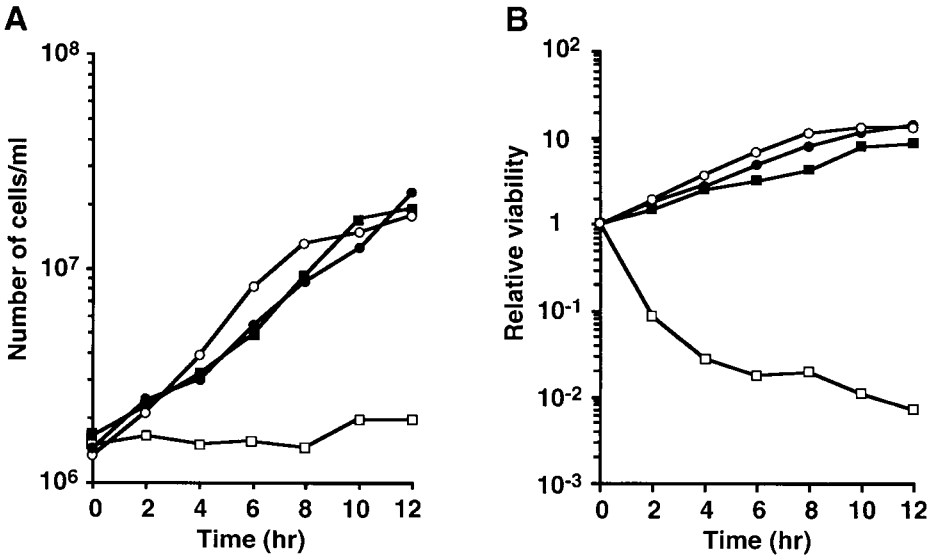

Figure 2.—Growth characteristics of *ptr6-1* mutant at the permissive and nonpermissive temperatures. Wild-type strain and *ptr6-1* were cultured in YPD medium at 26° up to a midlog phase, and then either maintained at 26° or transferred to 37°. (A) Growth curves. The total cell number was measured using a hemacytometer at the indicated times after shifting. (B) Relative viabilities. The results were obtained from the number of colonies grown at 26° after culturing at 26° or shifting to 37° for the indicated times. The symbols indicate the strains as follows: ●, wild type maintained at 26°; ○, wild type shifted to 37°; ■, *ptr6-1* maintained at 26°; □, *ptr6-1* shifted to 37°.

we transformed the *ptr6-1* mutant with an *S. pombe* wild-type genomic library and isolated 16 cosmid clones that complemented the temperature-sensitive growth of *ptr6*. Restriction mapping of those clones revealed that they contained the same insert DNA of ~30 kb in length. After several steps of subcloning, the complementing activity was localized in a 4.2-kb *Nco*I-*Bgl*II fragment. The restriction map is shown in Figure 3A. We sequenced this fragment and found a single complete ORF. We also found parts of two other ORFs at both ends of the fragment: one partial ORF encodes a carboxy-terminal region of a putative homologue of *Bacillus stearothermophilus* GldAp (glycerol dehydrogenase; Mallinder *et al.* 1992) and the other encodes an amino-terminal region of a putative homologue of *S. cerevisiae* Msh1p involved in mismatch repair of mitochondrial DNA (Reenan and Kolodner 1992a,b). As digestion of the fragment with *Hin*dIII or *Bcl*I, which disrupts the complete ORF, abolished the complementing activity, we conclude that this ORF is responsible for the rescue activity for *ptr6-1*.

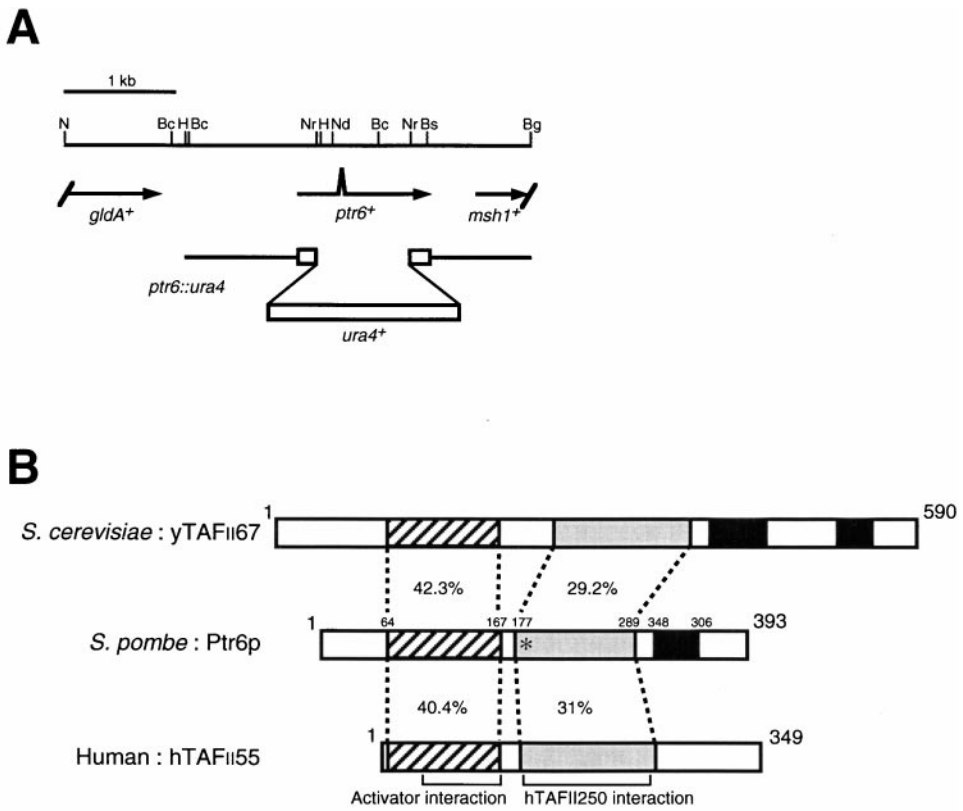

Figure 3.—(A) Restriction map of the 4.2-kb *Nco*I-*Bgl*II fragment that rescues the *ptr6* mutation. The arrows show the ORFs for the *ptr6*$^+$, *gldA*$^+$, and *msh1*$^+$ genes. Also shown is the direction of transcription. The kinked line represents an intron region. Restriction sites are abbreviated as follows: Bc, *Bcl*I; Bg, *Bgl*II; Bs, *Bst*EII; H, *Hin*dIII; N, *Nco*I; Nd, *Nde*I; Nr, *Nru*I. The *Nru*I-*Nru*I fragment within the *ptr6*$^+$ gene was replaced by the *ura4*$^+$ gene for gene disruption. (B) Schematic representation of the structures of Ptr6p, yTAFII67, and hTAFII55. The numbers indicate amino acid positions. Hatched or stippled boxes denote the regions highly conserved among organisms. Solid boxes represent regions containing stretches of acidic amino acids. The amino acid identities between domains are shown in percentages. The asterisk indicates the site of the mutation in *ptr6-1*.

To obtain evidence that the identified gene is not an extragenic multicopy suppressor for the *ptr6-1*, we did integration mapping as described in materials and methods. Integration of the cloned gene linked with an *ura4* marker into the authentic locus was confirmed by PCR (data not shown). One of the stable integrants was then mated with the *ptr6-1* strain (ST1), and 20 tetrads were dissected. All of the ts^- spores were found to be uracil auxotrophs and all ts^+ spores were uracil prototrophs, demonstrating that the cloned gene complementing the *ptr6* mutation is genetically linked to the $ptr6^+$ locus. Thus, we concluded that the isolated gene is not an extragenic suppressor but the authentic $ptr6^+$ gene.

We found a possible intron within the amino-terminal region of the $ptr6^+$ gene. To determine the intron region precisely, partial cDNA containing the predicted intron region was isolated using RT-PCR, and sequence analysis revealed that the $ptr6^+$ gene has an intron of 41 nucleotides. The ORF of the $ptr6^+$ gene is 1220 nucleotides in length and encodes a predicted protein of 393 amino acids.

The $ptr6^+$ gene encodes a homologue of yTAFII67 of *S. cerevisiae* and human hTAFII55: A comparison of the amino acid sequence of Ptr6p with those in the Swiss Prot database by using the BLAST program (Altschul *et al.* 1990) revealed that the predicted amino acid sequence of Ptr6p shares a significant homology with those of yTAFII67 of budding yeast *S. cerevisiae* (Moqtaderi *et al.* 1996b) and human hTAFII55 (Chiang and Roeder 1995; Lavigne *et al.* 1996). TAFs are subunits of the general transcription factor complex named TFIID, a central component of the eukaryotic RNA polymerase II transcription apparatus consisting of TBP and a set of TAFs (for reviews, see Burley 1996; Tansey and Herr 1997; Lee and Young 1998).

The overall amino acid identity and similarity between Ptr6p and yTAFII67 are 32 and 54%, and those between Ptr6p and hTAFII55 are 22 and 36%, respectively. The amino-terminal region (from amino acids 64 to 167) and the central region (from amino acids 177 to 289) of the Ptr6p are highly conserved among the three organisms (Figure 3B). In the case of hTAFII55, it was reported that these two regions are functional domains for the protein-protein interactions (Chiang and Roeder 1995; also see discussion). The same conserved regions in Ptr6p and yTAFII67 may also be functioning as domains for protein interaction in yeast. Ptr6p and yTAFII67 contain a stretch of acidic amino acids (aspartic acid and glutamic acid) in the carboxy-terminal region, which is lacking in hTAFII55. The acidic stretches of yTAFII67 are longer than those of Ptr6p. In addition, yTAFII67 contains a stretch of basic amino acids (lysine and arginine) in the amino-terminal region, although there is no such stretch in Ptr6p and hTAFII55.

To identify a mutation site in *ptr6-1*, a gap repair experiment was done. We constructed several gapped plasmids carrying the leu^+ marker and introduced them into *ptr6-1*. Plasmids containing the gap between *Hin*dIII and *Bcl*I sites in the $ptr6^+$ gene could not complement the ts^- growth phenotype of *ptr6-1* at 37°, indicating that the mutation site is present within the gapped region. The repaired plasmid was isolated from the transformant and sequenced. *ptr6-1* has a single mutation site: G at nucleotide position 586 was changed to A, resulting in replacement of glycine at amino acid position 182 to glutamic acid. The mutation is located at a conserved residue within the conserved region (Figure 3B).

***ptr6*$^+$ is an essential gene:** To determine whether the $ptr6^+$ gene is required for growth in *S. pombe*, we carried out one-step gene disruption. The *Nru*I-*Nru*I fragment in the middle of the $ptr6^+$ gene was replaced with the $ura4^+$ gene to make a null mutation of *ptr6* (Figure 3A). Approximately 70% of the $ptr6^+$ ORF (from amino acid 53 to 331) was deleted in the disrupted gene. A *Hin*dIII-*Bgl*II fragment carrying the disrupted $ptr6^+$ gene was then used to transform wild-type diploid strain UDP6 lacking the $ura4^+$ alleles. We verified disruption of one of the $ptr6^+$ alleles in the diploid cells by PCR (data not shown). Tetrad analysis of 38 asci showed two viable and two inviable spores in all the cases (data not shown). All the viable spores were ura^-. These results suggest that the $ptr6^+$ gene is essential for cell viability in *S. pombe*.

Ptr6p is localized in the nucleus: To determine the subcellular localization of Ptr6p, we constructed a gene encoding GFP-tagged Ptr6p and introduced it into the *ptr6-1* mutant. The chimeric gene was capable of complementing temperature sensitivity of *ptr6-1*, demonstrating that the fusion protein is functional. The Ptr6p-GFP was localized in the nucleus at both 26° and 37°, suggesting that Ptr6p is a nuclear protein (Figure 4). The transformants harboring a plasmid with the untagged $ptr6^+$ gene gave no fluorescence (data not shown).

***ptr6* has no defects in nuclear protein import:** The *ptr2*/*pim1* and *ptr3* mutants cause defects in nuclear protein import as well as in mRNA export (Azad *et al.* 1997). To determine if *ptr6* has defects in nuclear pro-

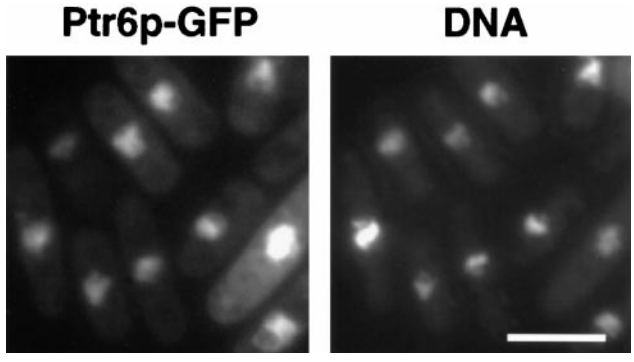

Figure 4.—Intracellular localization of Ptr6p. Cells transformed with the gene encoding the Ptr6p tagged with GFP were analyzed using a fluorescence microscope. DNA shows cells stained with DAPI in the corresponding field. Bar, 10 μm.

tein import, we examined localization of a NLS-GFP fusion protein in the wild type, *ptr2-1*, and *ptr6-1* (Figure 5). At the permissive temperature (26°), nuclear accumulation of the NLS-GFP fusion protein was observed in wild-type, *ptr2-1*, and *ptr6-1* strains, indicating that the NLS used in this study is functional in *S. pombe* (Figure 5, A, D, and G). Cells expressing the NLS-GFP fusion protein were preincubated at the nonpermissive temperature of 37° for 1 hr and then treated with azide and deoxyglucose at 37° for 1 hr, resulting in the poisoning of the energy metabolism and the leakage of the NLS-GFP fusion protein into the cytoplasm (Figure 5, B, E, and H). After washing and resuspending the cells in glucose-containing MM, the cells were incubated for 30 min at 37° to analyze the reimport of the NLS-GFP fusion protein into the nucleus (Figure 5, C, F, and I). Protein import of the NLS-GFP fusion protein was inhibited in *ptr2-1* cells at 37° (Figure 5I), whereas no defect in nuclear protein import was observed in the *ptr6-1* and wild-type cells (Figure 5, C and F). The defect in protein import was not observed, even when the *ptr6-1* cells were preincubated at 37° for 6 hr (data not shown).

***ptr6* has defects in transcription:** To determine if *ptr6* has defects in pre-mRNA splicing, we isolated total RNA from *ptr6-1* cells cultured at 26° or shifted to 37° for 2 or 4 hr, and carried out Northern blot analysis of TBP mRNA. The TBP gene of *S. pombe* contains three introns (Hoffmann *et al.* 1990). We used the oligonucleotide probe complementary to the third exon sequence of TBP pre-mRNA (Table 2). As shown in Figure 6A, *ptr6* showed no accumulation of TBP pre-mRNA at both the permissive and nonpermissive temperatures and neither did the wild-type cells. Therefore, *ptr6* has no defects in pre-mRNA splicing. Interestingly, the amount of TBP mRNA in *ptr6* at the nonpermissive temperature was increased ~5 to 6 times compared to those of wild type and *ptr6* at the permissive temperature. The increase of TBP mRNA at the nonpermissive temperature in *ptr6* was rescued completely with pSP1-N/Bg containing the *ptr6*$^+$ gene (Figure 6A).

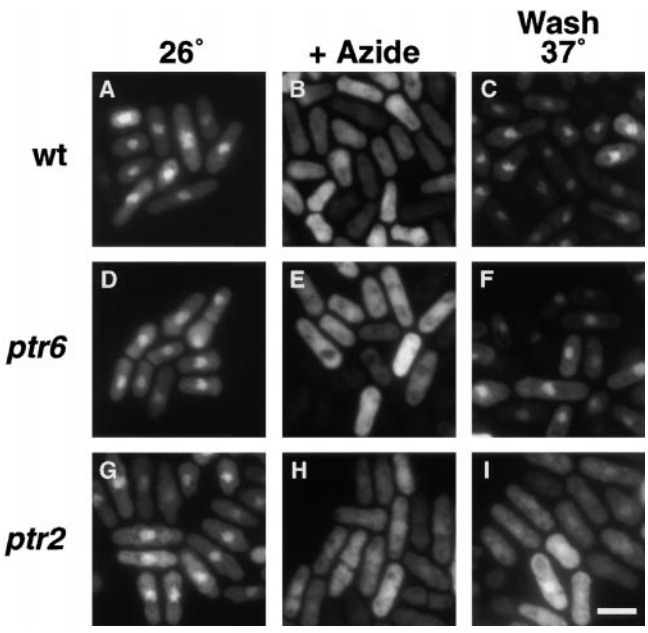

Figure 5.—Analysis of nuclear protein import. Wild type (A–C), *ptr6-1* (D–F), and *ptr2-1* (G–I) expressing the NLS-GFP fusion protein were cultured at 26° (A, D, and G). Cells were preincubated for 1 hr at 37° and then metabolically poisoned to allow nucleocytoplasmic equilibration of the NLS-GFP fusion protein by using azide and deoxyglucose (B, E, and H). After recovery from the drug treatment in glucose-containing MM medium for 30 min at 37°, the reimport of the NLS-GFP fusion protein into the nucleus was analyzed (C, F, and I). Bar, 10 μm.

Homology search showed that Ptr6p is a putative homologue of a TFIID subunit, hTAFII55. Also, Northern blot analysis using the TBP probe revealed abnormal increases in TBP mRNA at the nonpermissive temperature. Therefore, we asked if *ptr6* is abnormal in the transcription of genes in general. Total RNA of *ptr6-1* cells cultured at 26° or shifted to 37° for 2 hr or 4 hr were subjected to Northern blot analysis using probes for *tbp*$^+$, *ptr6*$^+$, *act1*$^+$, *rna1*$^+$, *rae1*$^+$, *pim1*$^+$, *taf72*$^+$, *prp2*$^+$, *cdc2*$^+$, and *rpb1*$^+$ mRNAs. Act1p (actin1) is a constitutively expressed protein. Rna1p, Rae1p, and Pim1p/Ptr2p are essential factors involved in mRNA transport from the nucleus to the cytoplasm in *S. pombe* (Melchior *et al.* 1993; Brown *et al.* 1995; Azad *et al.* 1997). Taf72p is an *S. pombe* homologue of yTAFII90 in *S. cerevisiae* (Yamamoto *et al.* 1997). Prp2p is a homologue of the mammalian splicing factor U2AF^{65} (Potashkin *et al.* 1993). Cdc2p is a factor involved in cell-cycle progression (Rhind *et al.* 1997) and Rpb1p is the largest subunit of RNA polymerase II (Azuma *et al.* 1991). Intriguingly, in contrast to the drastic increase of *tbp*$^+$ mRNA in *ptr6* at the nonpermissive temperature, the amounts of *ptr6*$^+$, *act1*$^+$, *rna1*$^+$, *rae1*$^+$, *pim1*$^+$, *taf72*$^+$, *prp2*$^+$, *cdc2*$^+$, and *rpb1*$^+$ mRNAs were decreased rapidly at the same temperature (Figure 6A). The decrease of *ptr6*$^+$ mRNA at the nonpermissive temperature was also rescued with pSP1-N/Bg. The increase of *ptr6*$^+$ mRNA in pSP1-N/Bg transformants at both the permissive and nonpermissive temperatures is due to overexpression of *ptr6*$^+$ mRNA from the gene in the multicopy plasmid. These results suggest that *ptr6* is defective in the regulation of transcription of many genes at the nonpermissive temperature.

To determine if *ptr6* has defects in transcription of genes driven by RNA polymerase II in general, we evaluated the amount of the total poly(A)$^+$ RNA by hybridization with a ^{32}P-labeled oligo(dT)$_{30}$ probe. The results are shown in Figure 6B. The quantity of total poly(A)$^+$ RNA in the wild-type cells was maintained constant at the nonpermissive temperature; however, the amount of the total poly(A)$^+$ RNA in *ptr6* was remarkably decreased after shifting to the nonpermissive temperature.

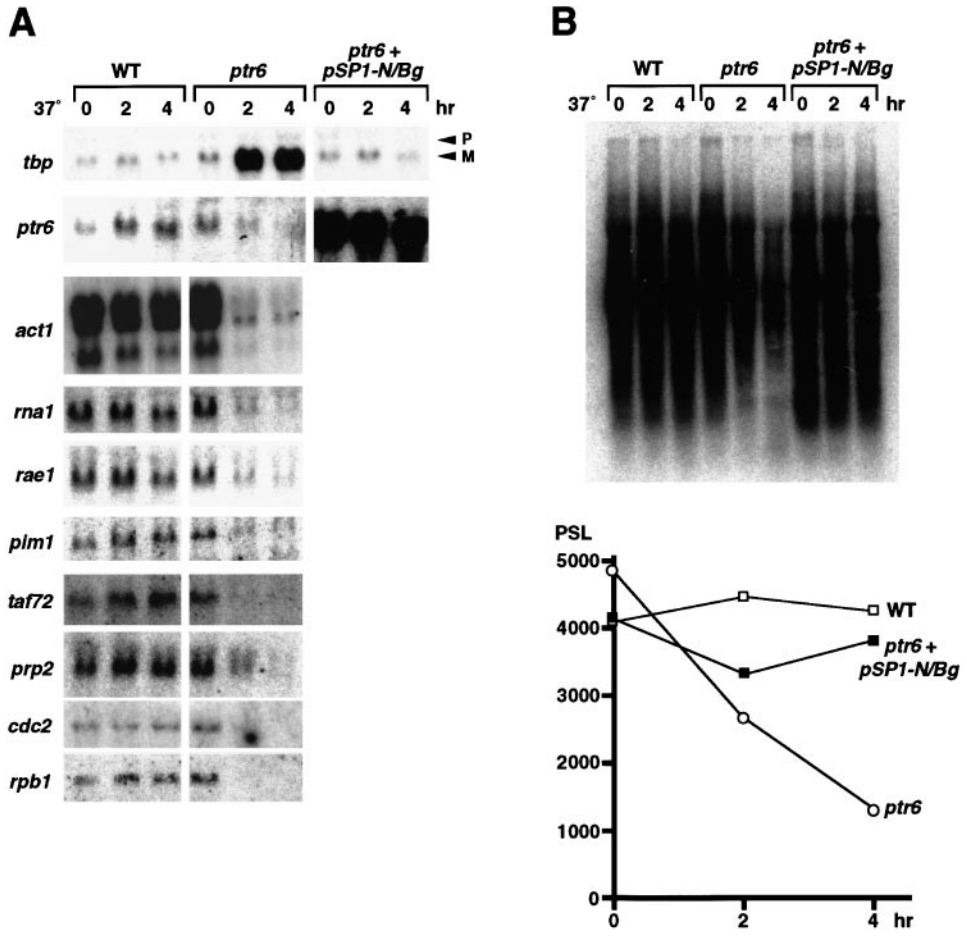

Figure 6.—(A) Northern blot analysis of specific mRNAs. Total RNA (20 μg) prepared from the wild type, *ptr6-1*, or *ptr6-1* transformed with pSP1-N/Bg that had been incubated at the permissive temperature (26°) or shifted to the nonpermissive temperature (37°) for 2 or 4 hr was subjected to Northern blot hybridization. Arrowheads on the right indicate the unspliced precursor (P) and the mature TBP mRNA (M). (B) Analysis of total poly(A)$^+$ RNA. Wild type, *ptr6-1*, and *ptr6-1* containing pSP1-N/Bg were maintained for the indicated times at 37°. Total RNA (20 μg) was then analyzed by Northern blot hybridization with the ^{32}P-oligo(dT)$_{30}$. Intensity of the smeared band in each lane was quantitated using a Bioimaging Analyzer BAS1000 (Fuji Photo Film Co.) and is shown at the bottom using photo stimulated luminescence (PSL) units of BAS1000.

Taken together, we conclude that the transcription of most genes by RNA polymerase II in *ptr6* is repressed abnormally at the nonpermissive temperature, supporting the hypothesis that the *ptr6*$^+$ gene encodes the *S. pombe* homologue of hTAFII55.

FACS analysis: It has been reported that mutations in yTAFII145 and yTAFII90 result in specific arrests in G1 and G2/M, respectively, in *S. cerevisiae* (Apone *et al.* 1996; Walker *et al.* 1997). To examine if *ptr6* also has a cell-cycle defect at the nonpermissive temperature, we performed FACS analysis. In contrast to the cell division cycle mutant *cdc10-129* arresting in the G1 phase, the *ptr6-1* mutant grown in a rich medium appears to have no specific defects in the cell cycle at the nonpermissive temperature, although slight delay in DNA synthesis was observed 4 hr after shifting to the nonpermissive temperature (Figure 7). In addition, the *ptr6-1* mutant showed no major morphological abnormality as was observed in the *cdc* mutant, even after shifting to the nonpermissive temperature for 12 hr (data not shown).

DISCUSSION

We identified one new *S. pombe ptr* mutant defective in the transport of poly(A)$^+$ RNA from the nucleus to the cytoplasm by *in situ* hybridization with the oligo(dT) probe and classified it as *ptr6*. The *ptr6* mutant shows nuclear accumulation of mRNA, severe growth defect, and defects in gene transcription at the nonpermissive temperature.

Ptr6p is a homologue of TAFs: The *ptr6*$^+$ gene en-

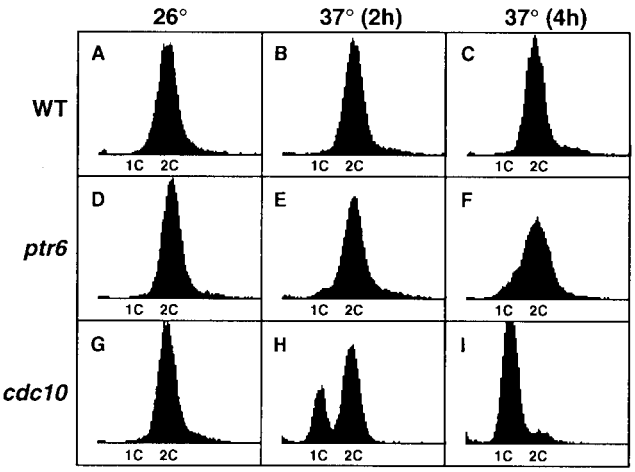

Figure 7.—FACS analysis of wild-type (A–C), *ptr6-1* (D–F), and *cdc10-129* (G–I) cells following a shift to the nonpermissive temperature. The DNA content and relative cell number were plotted along with *x* and *y* axes, respectively. The positions of 1C and 2C DNA content are indicated.

codes a putative homologue of *S. cerevisiae* yTAFII67 and human hTAFII55. yTAFII67 was identified as a homologue of human hTAFII55 by a computer search of the complete *S. cerevisiae* genome and cloned by PCR amplification. yTAFII67 is reported to be essential for cell viability (Moqtaderi *et al.* 1996b). On the other hand, hTAFII55 interacts with multiple transcription activators through the amino-terminal domain (between amino acids 38 and 113) and also interacts with the largest subunit (hTAFII250) of human TFIID, which has been implicated in the regulation of cell-cycle progression (Hisatake *et al.* 1993; Ruppert *et al.* 1993; Wang and Tjian 1994) through the central region (between amino acids 139 and 249; Chiang and Roeder 1995; Lavigne *et al.* 1996; Figure 3B).

These previously identified TAFs were shown to associate with TBP by immunoprecipitation assay. In the case of Ptr6p, the possible association with TBP was not determined. However, there are lines of evidence suggesting that Ptr6p is a TAF. First, Ptr6p has significant homologies with yTAFII67 and hTAFII55 in overall amino acid sequence and contains several conserved domains present in yTAFII67 and hTAFII55 (Figure 3B). Second, Ptr6p localized in the nucleus (Figure 4). Third, the *ptr6* mutant has defects in the transcription of the tested genes at the nonpermissive temperature (Figure 6). Finally, the antibody against Ptr6p was found to coimmunoprecipitate a homologue of the *S. cerevisiae* yTAFII145 (T. Miyake and T. Kokubo, personal communication). From these results, we believe that the Ptr6p is the *S. pombe* homologue of yTAFII67 and hTAFII55.

What is the function of Ptr6p in *S. pombe*? The transcription factor complex TFIID is composed of TBP and a set of TAFs. Although essential roles of TBP in all eukaryotic transcription have been extensively analyzed *in vivo* and *in vitro* (Hernandez 1993; Struhl 1995), the functions of the TAFs are less clear.

Information on the functions of TAFs has been mostly obtained from *in vitro* studies on partially purified mammalian and Drosophila factors (Goodrich and Tjian 1994). These *in vitro* studies suggested that TAFs are dispensable for basal transcription but are required as transcriptional coactivators for the response to regulatory proteins or activators (Tjian and Maniatis 1994) and that specific TAFs may act as molecular bridges between particular activators and the general transcription machinery (Chen *et al.* 1994; Sauer *et al.* 1995).

To analyze the *in vivo* function of a specific TAF, several temperature-sensitive TAF mutants of *S. cerevisiae* have been constructed using reverse genetics. Of those, the yTAFII145 ts mutant shows a cell-cycle arrest in the G1 phase at the nonpermissive temperature (Walker *et al.* 1996, 1997; Shen and Green 1997). The yTAFII145 is a homologue of the mammalian hTAFII250 implicated in cell-cycle regulation (Hisatake *et al.* 1993; Ruppert *et al.* 1993; Wang and Tjian 1994). yTAFII145 was shown to be required for transcription of G1/S cyclin genes and thereby involved in the transcriptional regulation of the cell-cycle progression (Walker *et al.* 1997). The yTAFII90 ts mutant also shows the G2/M phase cell-cycle arrest (Apone *et al.* 1996), suggesting a linkage between TAFII functions and cell-cycle progression. On the other hand, studies have also shown that despite inactivation or depletion of several yTAFIIs, a variety of yeast genes driven by diverse activators were transcribed normally (Apone *et al.* 1996; Moqtaderi *et al.* 1996a; Walker *et al.* 1996). A dot blot assay showed no significant decrease in the synthesis of total poly(A)$^+$ RNA following temperature-sensitive inactivation of yTAFII145, TSM1, or yTAFII90 (Walker *et al.* 1997). These studies suggested that multiple yTAFIIs are dispensable for transcription of the vast majority of yeast mRNA-encoding genes. However, gene disruption experiments in budding yeast revealed that most TAFs, except for yTAFII30, are essential for cell viability (Reese *et al.* 1994; Poon *et al.* 1995; Moqtaderi *et al.* 1996b; Lee and Young 1998). Taken together, it was suggested that yTAFs play a role in the transcription of specific genes involved in cell-cycle progression.

In contrast, the *ptr6* mutant does not display the specific defects in cell-cycle progression in the FACS and morphological analyses, suggesting that Ptr6p does not function in cell-cycle progression primarily. We examined the possibility that Ptr6p functions in the transcription of the genes involved in mRNA transport. Northern blot hybridization showed that transcription of the genes involved in mRNA transport (*rna1*$^+$, *rae1*$^+$, and *pim1*$^+$) was inhibited at the nonpermissive temperature in *ptr6* (Figure 6). However, transcription of the *act1*$^+$, *taf72*$^+$, *prp2*$^+$, *cdc2*$^+$, and *rpb1*$^+$ genes unrelated to the mRNA transport was also dramatically decreased, suggesting that Ptr6p is not a TAF specifically required for the transcription of the genes involved in mRNA export. In addition, the amount of the total poly(A)$^+$ RNA was decreased at the nonpermissive temperature in *ptr6*. These results suggest that, in the *ptr6* mutant, most or all protein-coding genes are not transcribed normally at the nonpermissive temperature. Therefore, in contrast to previously reported yTAFII145 and yTAFII90, the Ptr6p may be a TAF required generally for transcription of the genes. The slight delay in DNA synthesis in *ptr6* at the nonpermissive temperature is likely to be a secondary effect of the drastic decrease of gene transcription, considering that many labile proteins are involved in that process. Rapid repression of the gene transcription at the nonpermissive temperature may also account for the weak nuclear signals in *ptr6* by *in situ* hybridization compared to those in previously identified *ptr* mutants.

Interestingly, Northern blot analysis revealed that the amount of TBP mRNA was increased five- to sixfold in contrast to the other analyzed mRNAs at the nonpermissive temperature. One explanation for this phenome-

non is that production of TBP might be increased to compensate for reduction of the Ptr6p / hTAFII55 activity and to maintain basal transcription activity.

Relationship between mRNA transport and transcription: Severe defects in gene transcription were detected 2 hr after shifting to the nonpermissive temperature in *ptr6*. In contrast, a significant accumulation of mRNA in the nucleus requires a shifting time exceeding 2 hr. Thus, block of mRNA export might possibly be a secondary effect of the reduced transcription of genes required for mRNA transport, such as the *rna1*$^+$, *rae1*$^+$, and *pim1*$^+$ genes. However, though the *pim1*$^+$ gene is also essential for protein import, defects in protein import were not seen in *ptr6*. In addition, despite the decreased transcription levels of the *prp2*$^+$ gene, the *ptr6* mutant did not show defects in pre-mRNA splicing. Moreover, the inhibition of protein synthesis by cycloheximide in *S. pombe* did not block mRNA export, suggesting that labile proteins are not involved in the pathway of mRNA transport (Tani *et al.* 1995). Therefore, it is likely that defects in poly(A)$^+$ RNA transport in the *ptr6* mutant are not simply caused by defects in gene transcription leading to reduction of proteins required for mRNA transport, but rather are a direct consequence of the inactivation of Ptr6p. There is a precedent that a transcription factor is directly involved in mRNA export. It has been shown that the TFIIIA, an RNA polymerase III transcription factor, binds to 5S rRNA and mediates its nuclear export (Fridell *et al.* 1996). TFIIIA contains a leucine-rich sequence functioning as an effective NES in both human cells and frog oocytes. Similarly, Ptr6p might also have dual roles in mRNA export and gene transcription, although no typical leucine-rich NES was found. To our knowledge, this is the first report suggesting that a TAF is involved in poly(A)$^+$ RNA transport besides transcription.

Interestingly, hTAFII55 was recently shown to bind strongly with the 160-kD subunit of human cleavage-polyadenylation specificity factor (CPSF) by the immunoprecipitation assay (Dantonel *et al.* 1997). CPSF is a complex that plays a role in 3′ processing of precursor mRNA by binding to the AAUAAA signal sequence and coordinating interactions with other polyadenylation factors (Keller 1995). Several lines of evidence suggested a link between the polyadenylation (3′ end formation) and the export of RNA from the nucleus (Eckner *et al.* 1991; Huang and Carmichael 1996; Kessler *et al.* 1997). Therefore, if Ptr6p, an *S. pombe* homologue of hTAFII55, also interacts with CPSF in *S. pombe*, Ptr6p may play an important role in coordinated interplay between mRNA export and the 3′ end processing. Further investigations of the functions of Ptr6p will shed light on the connection among mRNA transport, 3′ end processing, and gene transcription.

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