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Possibility of Cytoplasmic pre-tRNA Splicing: the Yeast tRNA Splicing Endonuclease Mainly Localizes on the Mitochondria

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Pre-tRNA splicing has been believed to occur in the nucleus. In yeast, the tRNA splicing endonuclease that cleaves the exon-intron junctions of pre-tRNAs consists of Sen54p, Sen2p, Sen34p, and Sen15p and was thought to be an integral membrane protein of the inner nuclear envelope. Here we show that the majority of Sen2p, Sen54p, and the endonuclease activity are not localized in the nucleus, but on the mitochondrial surface. The endonuclease is peripherally associated with the cytosolic surface of the outer mitochondrial membrane. A Sen54p derivative artificially fixed on the mitochondria as an integral membrane protein can functionally replace the authentic Sen54p, whereas mutant proteins defective in mitochondrial localization are not fully active. *sen2* mutant cells accumulate unspliced pre-tRNAs in the cytosol under the restrictive conditions, and this export of the pre-tRNAs partly depends on Los1p, yeast exportin-t. It is difficult to explain these results from the view of tRNA splicing in the nucleus. We rather propose a new possibility that tRNA splicing occurs on the mitochondrial surface in yeast.

INTRODUCTION

RNAs transcribed in the nucleus are processed in various manners before they become mature. Among such processing on tRNAs, splicing is critical for the function of intron-containing tRNAs because all of the known tRNA introns interrupt the anticodon loop (Hopper and Martin, 1992; Trotta and Abelson, 1999; Wolin and Matera, 1999; Hopper and Phizicky, 2003). The splicing of tRNA introns is carried out by sequential action of three enzymes: tRNA splicing endonuclease (Peebles *et al.,* 1979; Rauhut *et al.,* 1990), tRNA ligase (Phizicky *et al.,* 1986), and 2′-phosphotransferase (Culver *et al.,* 1997). The yeast tRNA endonuclease that catalyzes the first step of tRNA splicing consists of four subunits, Sen54p, Sen2p, Sen34p, and Sen15p. Sen2p and Sen34p have catalytic centers that cleave a 5′-exon-intron junction and an intron-3′-exon junction, respectively (Trotta *et al.,* 1997). Both of the subunits show significant homology to homo-oligomeric archaeal tRNA endonuclease (Li *et al.,* 1998). In contrast to its soluble counterparts in archaea and *Xenopus* (DeRobertis *et al.,* 1981; Li *et al.,* 1998), the yeast enzyme has been regarded as an integral membrane protein, because the enzymatic activity is associated with membranes and its extraction requires detergents (Peebles *et al.,* 1983). Sen2p, which has a hydrophobic stretch suitable to traverse membranes, was thought to anchor the other subunits to the inner nuclear envelope (NE; Trotta *et al.,* 1997). tRNA ligase was also shown to be localized on the inner periphery of the NE (Clark and Abelson, 1987).

tRNA splicing has been believed to occur just before the export of mature tRNAs. The following observations suggested a "coupling model," in which the spliced tRNAs are directly handed to the nuclear export machinery (Peebles *et al.,* 1983; Sharma *et al.,* 1996). In yeast, mutants defective in mature tRNA export, like *rna1* and *los1*, concomitantly accumulate end-matured, unspliced pre-tRNAs in the nucleus (Hopper *et al.,* 1978, 1980). Rna1p is a Ran GAP homologue in yeast (Corbett *et al.,* 1995), and Los1p, a homologue of exportin-t, is an export carrier of tRNAs (Arts *et al.,* 1998; Hellmuth *et al.,* 1998; Kutay *et al.,* 1998). Defects in some nucleoporins also result in pre-tRNA accumulation (Sharma

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Abbreviations used: 5′-FOA, 5′-fluoroorotic acid; DAPI, 4′,6-diamidino-2-phenylindole; FISH, fluorescence in situ hybridization; IM, inner mitochondrial membrane; MSP, medium speed pellet; NE, nuclear envelope; NLS, nuclear localization signal; OM, outer mitochondrial membrane; ORF, open reading frame; PVP, polyvinylpyrrolidone; RS, aminoacyl-tRNA synthetase; Sen, splicing endonuclease.

et al., 1996). The nuclear localization of the accumulated pre-tRNAs in these mutants was demonstrated by fluorescence in situ hybridization (FISH; Sarkar and Hopper, 1998; Sarkar *et al.*, 1999; Grosshans *et al.*, 2000).

However, this coupling model has been challenged by several observations. In *Xenopus* oocytes, aminoacylation of functional tRNAs by aminoacyl-tRNA synthetase (RS) appears to take place in the nucleus before their export (Lund and Dahlberg, 1998). A similar system operates in yeast. In Δ*nup116* mutant cells, the mature tRNAs that accumulated in the nucleus were already aminoacylated (Sarkar *et al.*, 1999). Conversely, the blockade of a certain RS by a mutation or a specific inhibitor caused the accumulation of the corresponding mature tRNA, but not the pre-tRNA, in the nucleus (Grosshans *et al.*, 2000). A mutation within the nuclear localization signal (NLS) of TyrRS also caused the nuclear accumulation of tRNA-Tyr, without affecting its aminoacylation ability (Azad *et al.*, 2001). These results are against the direct coupling between the splicing and the export of tRNAs. Besides, under certain conditions, *los1* cells accumulate only pre-tRNAs, not mature tRNAs, in the nucleus (Feng and Hopper, 2002). Without tight coupling between the splicing and the export, we need to seek another mechanism to explain accumulation of unspliced pre-tRNAs in the export mutants.

In this situation, it is essential to know the exact place and timing in which the tRNA splicing occurs. Therefore, we decided to reexamine the localization of the tRNA splicing endonuclease in yeast. Unexpectedly, the endonuclease was present mainly on the mitochondrial surface, and several lines of evidence indicate that the mitochondrial pool of the enzyme has positive roles in tRNA splicing. On the basis of these results, we propose a new possibility that pre-tRNAs are spliced on the mitochondrial surface after their export out of the nucleus.

MATERIALS AND METHODS

Chemicals

Zymolyase, Nycodenz, and 5′-fluoroorotic acid (5′- FOA) were purchased from Seikagaku Corp. (Tokyo, Japan), Nycomed Pharma (Oslo, Norway), and BIO 101 Systems (Carlsbad, CA), respectively.

Rabbit polyclonal antibodies against Sen2p, Sen54p, Sec63p, Hht1p, Pom152p, and Nsp1p were raised using the corresponding recombinant proteins expressed in *Escherichia coli*. In the case of anti-Sen2p antibodies, the *SEN2* open reading frame (ORF; see below) was subcloned into pET21d (Novagen, Madison, WI) to express Sen2p-His_6. The fusion protein was purified from the inclusion body by preparative SDS-PAGE and electro-elution and used for immunization. For preparing antigen-agarose resin, the GST-Sen2p(1–219)-His_6 fusion protein was expressed in *E. coli*, purified with Ni-NTA agarose (Qiagen, Tokyo, Japan) under denaturing conditions, and immobilized on Affi-Gel 15 (Bio-Rad, Hercules, CA). A crude IgG fraction prepared by ammonium sulfate precipitation from anti-Sen2p antisera was applied to the Sen2p-agarose. Anti-Sen2p antibodies were eluted with 0.1 M glycine-HCl, pH 2.0, after extensive wash. The eluate was desalted, rechromatographed, adjusted to TBS + 1% BSA, and passed through yeast lysate-agarose, where yeast total lysate extracted with SDS was immobilized on Affi-Gel 15. Affinity-purified anti-Sen54p antibodies were prepared and purified essentially by similar procedures as above with the Sen54p(1–432)-His_6 fusion protein as an antigen. The affinity-purified anti-Sen2p and anti-Sen54p antibodies recognized single bands corresponding to their antigens in Western blotting in Figure 1, A and B and 4B. Rabbit antiprotein A antibodies were purchased from Biogenesis (Poole, Dorset, UK) and were passed through yeast lysate-agarose to remove contaminated antiyeast protein antibodies before use. An mAb against yeast Por2p and Alexa 488– and Alexa 546–labeled secondary antibodies were purchased from Molecular Probes (Eugene, OR). Rhodamine-labeled antibodies were from Boehringer Mannheim (Mannheim, Germany). Cy5-labeled secondary antibodies were from Amersham Biosciences (Tokyo, Japan).

Oligonucleotides were purchased from Amersham Biosciences. Probes for hybridization were as follows: pre-tRNA and mature tRNA-Ile^{UAU}, 5′-GTGGGGATTGAACCCACGACGGTCGCGTTATAAGCACGAAGCTCTAACCACTGAGCTACA-3′; pre-tRNA-Ile^{UAU}, 5′-CGTTGCTTTTAAAGGCCTGTTTGAAAGGTCTTTGGCACAGAAACTTCGGAAACCGAATGTTGCTAT-3′; pre-tRNA-Leu^{CAA}, 5′-TATTCCCACAGTTAACTGCGGTCAAGATAT-3′; pre-tRNA-Pro^{UGG}, 5′-CGCATGCTTTGTCTTCCTGTTTAATCAGGAAGTCGCCCAA-3′; U14snoRNA, 5′-AAGAAGAGCGGTCACCGAGAGTACTAACGATGGGTTCGTAAGCGTACTCCTACCGTGGAA-3′. For Northern hybridization, probes were enzymatically radiolabeled with γ-^{32}P. For FISH, either FITC or Cy5 was conjugated to the probes by the manufacturer.

Plasmids

The multicopy vectors pYO324 (2μ*TRP1*) and pYO326 (2μ*URA3*) were a gift from Dr. Y. Ohya, University of Tokyo (Qadota *et al.*, 1992). A 2.47-kb *Sac*I-*Spe*I fragment including the *SEN2* gene was amplified by PCR and was subcloned into pRS316, pRS314, and pYO326 to yield pTYSC017, pCOSC05, and pYU042, respectively. A 4.13-kb *Sac*I-*Kpn*I fragment including the *SEN54* gene was amplified by PCR and was subcloned into pRS316, pRS314, and pYO324, yielding pTYSC155, p314–54, and pTYSC161, respectively. To construct $FLAG_3$ or protein A fusion plasmids, a 0.58-kb fragment containing *ADH1* terminator and *HIS3* gene from *Candida glabrata* (Sakumoto *et al.*, 1999) were inserted into pBluescript SK(−) in this order. A 90-base pair DNA fragment encoding three tandem FLAG epitopes (DYKDDDDKRP) or a 710-base pair fragment encoding the IgG-binding domain of protein A from pRIT2T were inserted in the plasmid to yield pTYE247 and pTYE248, respectively. To construct p70N-54 with *TOM70N-SEN54*, the *SEN54* promoter region (0.53 kb), a *TOM70* fragment encoding the first 61 amino acids (0.19 kb) and the *SEN54* ORF (1.53 kb) were amplified, conjugated with appropriate restriction sites by PCR, and assembled on pBluescript SK(−) in this order. A 0.54-kb *Mlu*I-*Bgl*II fragment from p314–54, containing the 5′ portion of *SEN54* gene, was replaced with that of the above plasmid to yield p70N-54. The *sen54*Δ*200-232* and *sen54*Δ*275-313* deletion mutants were constructed by oligonucleotide-directed mutagenesis of p314–54, using the oligonucleotides, 5′-GAAACCACTAAACAGCGATTCTTGATTGCTGGGTTTTAG-3′ and 5′-ATTGTAAACGAAAACTTGGAATTTTTTATTCTTTGGTACAGC-3′ to yield p314-Δ200 and p314-Δ275, respectively. The 0.54-kb *Mlu*I-*Bgl*II fragment of the two plasmids was replaced with that of p70N-54 to yield p70N-Δ200 and p70N-Δ275. All of the PCR fragments were confirmed by DNA sequencing.

Yeast Strains

The yeast strains used in this study are listed in Table 1. Standard yeast genetic techniques for the construction of these strains were as described (Guthrie and Fink, 1991). Wild-type diploid and haploid strains were W303 or its derivatives. The diploid strains TYSC257 (*SEN54*/Δ*sen54::HIS3*) and YUSC025 (*SEN2*/Δ*sen2::LEU2*) and the Δ*los1::URA3* haploid strains were constructed by one-step gene replacement (Guthrie and Fink, 1991). For construction of haploid strains with $FLAG_3$- or protein A-tagged genes, DNA fragments harboring tag sequences, *ADH1* terminator and *CgHIS3*, were amplified with 60-base pair tabs homologous to target genes from pTYE247 and pTYE248, respectively, and integrated into the 3′-end of *SEN2* or *SEN54* loci of a wild-type haploid, TYSC188, as de-

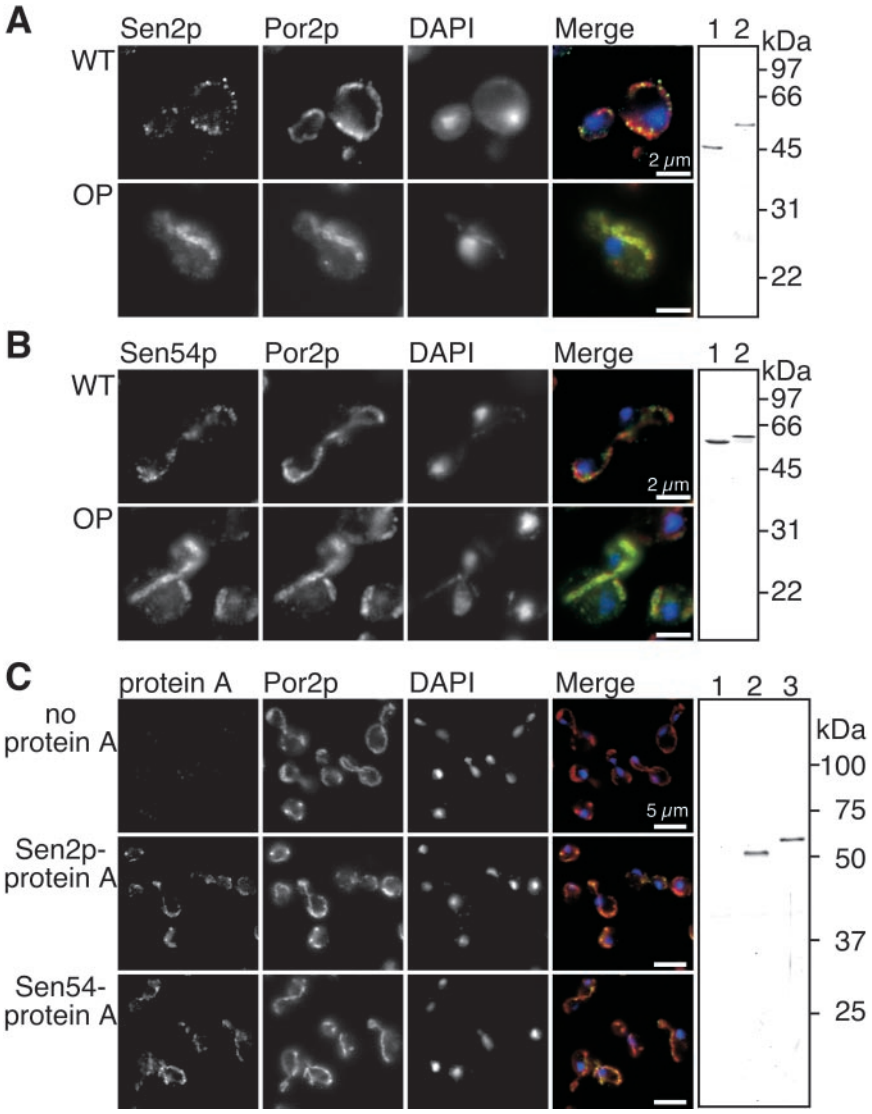

Figure 1. Sen2p and Sen54p are localized to mitochondria. (A) Sen2p in a wild-type strain, TYSC188 (WT), and an overproducer with pYU042 (OP) was visualized by immunofluorescence with affinity-purified anti-Sen2p antibodies (1st column). Mitochondria and the nucleus were visualized with an anti-Por2p mAb (2nd column) and DAPI (3rd column). Three images, green for Sen2p, red for Por2p, and blue for DAPI, are merged in the 4th column. (B) Sen54p in TYSC188 (WT) and TYSC188/pTYSC161 (OP) was visualized with affinity-purified anti-Sen54p antibodies as in A. The Western blotting in the right most panels demonstrates the specificity of these antibodies. Lane 1, wild-type cells; lane 2, cells only expressing Sen2p-$FLAG_3$ (A) or Sen54p-$FLAG_3$ (B). The gel mobility change of the single bands indicates that the antibodies recognize only their target proteins. (C) *SEN2-proteinA* (Sen2p-protein A) and *SEN54-protein A* (Sen54p-protein A) strains and their parental strain TYSC188 (no protein A) were subjected to immunofluorescence with anti-protein A antibodies as in A. The Western blotting in the right most panels demonstrates expression of the protein A fusion proteins. Lane 1, wild-type cells; lane 2, cells only expressing Sen2p-protein A; lane 3, cells only expressing Sen54p-protein A.

scribed (Schneider *et al.*, 1996). To construct the *sen2*-3 strain, *SEN2* on pTYSC017 was mutagenized to *sen2*-3 by oligonucleotide-directed mutagenesis with the oligonucleotide, 5′-TATTATATAAGAGAGAGCCACCATTTCAAC-3′. A haploid strain whose chromosomal disruption of *SEN2* was complemented by this *sen2*-3 plasmid was constructed with YUSC025 by tetrad dissection. For the new *sen2* ts selection, pCOSC05 was mutagenized with hydroxylamine, and ts alleles were isolated by the plasmid shuffling method with the haploid COSC04–2 (Δ*sen2::LEU2*/pTYSC017) as the recipient. Yeast clones that lost pTYSC017 and showed temperature-sensitive growth were selected on 5′-FOA medium. Strains with various mutant *sen54* genes were also constructed by plasmid shuffling with a haploid TYSC322 (Δ*sen54::HIS3*/pTYSC155), which was derived from TYSC257 by tetrad dissection.

Cell Biological Techniques

Immunofluorescence of yeast cells was done as described (Guthrie and Fink, 1991). Fluorescent images were acquired with an Olympus IX70 microscope equipped with a MicroMax cooled CCD camera (Roper Scientific, Tucson, AZ), and were analyzed by IP Lab (Scanalytics, Inc., Billerica, MA).

For the overall cell fractionation, yeast cells grown in appropriate media were converted into spheroplasts and were disrupted by agitation with glass beads for 30 s in lysis buffer (20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 10 mM spermidine-HCl, 0.5 mM DTT, 200 mM $(NH_4)_2SO_4$, 10% sucrose, and protease inhibitor cocktail). After the cell debris was removed, the lysate was centrifuged at 10,000 × *g* for 20 min to obtain the medium speed pellet (MSP) fraction. The MSP fraction was subjected to a 20–80% sucrose gradient and was centrifuged at 100,000 × *g* for 36 h at 4°C with a swing-out rotor (RPS27–2, Hitachi, Tokyo, Japan). The gradient was recovered from the bottom. The recovery of organelle markers was assayed by quantitative Western blotting with specific antibodies.

Mitochondria were prepared as described (Lewin *et al.*, 1990). Briefly, yeast cells grown in a medium with lactate as a carbon source were converted into spheroplasts and disrupted in 5 mM MES-KOH, pH 6.0, 0.5 mM EDTA and 0.6 M sorbitol with a Dounce homogenizer. After the cell debris was removed, the organelle fraction was recovered by centrifugation at 27,000 × *g* for 10 min. The pellet was suspended in

Table 1. Yeast strains

Strain	Genotype	Reference
TYSC188	*MAT***a** *GAL2 ADE2 ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11, 15 *can1*-100 (W303-1A derivative)	This study
TYSC254	*MAT***a** *GAL2 ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11, 15 *can1*-100 Δ*sen2*::*LEU2*/pTYSC168 [*CEN6-ARSH4 URA3 sen2*-3]	This study
TYSC257	*MAT***a**/α *GAL2*/+ *ade2*-1/- *ura3*-1/- *leu2*-3, 112/- *trp1*-1/- *his3*-11, 15/- *can1*-100/- *PEP4*/Δ*pep4*::*ADE2 SEN54*/Δ*sen54*::*HIS3*	This study
TYSC269	*MAT***a** *GAL2 ADE2 ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11, 15 *can1*-100 Δ*los1*::*URA3*	This study
TYSC321	*MAT***a** *GAL2 ade2*-1 *ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11, 15 *can1*-100 Δ*sen2*::*LEU2* Δ*los1*::*URA3*/pCOSC05 [*CEN6-ARSH4 TRP1 sen2*-41]	This study
TYSC322	*MAT*α *GAL2 ade2*-1 *ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11, 15 *can1*-100 Δ*sen54*::*HIS3*/pTYSC155 [*CEN6-ARSH4 URA3 SEN54*]	This study
TYSC330	*MAT***a** *GAL2 ADE2 ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11, 15 *can1*-100 *SEN2-FLAG3*::*CgHIS3*	This study
TYSC331	*MAT***a** *GAL2 ADE2 ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11, 15 *can1*-100 *SEN2-protein A*::*CgHIS3*	This study
TYSC332	*MAT***a** *GAL2 ADE2 ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11, 15 *can1*-100 *SEN54-FLAG3*::*CgHIS3*	This study
TYSC333	*MAT***a** *GAL2 ADE2 ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11, 15 *can1*-100 *SEN54-protein A*::*CgHIS3*	This study
TYSC360	*MAT*α *GAL2 ade2*-1 *ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11,15 *can1*-100 Δ*sen54*::*HIS3*/p314-54 [*CEN6-ARSH4 TRP1 SEN54*]	This study
TYSC361	*MAT*α *GAL2 ade2*-1 *ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11,15 *can1*-100 Δ*sen54*::*HIS3*/p314-Δ200 [*CEN6-ARSH4 TRP1 sen54*Δ200-232]	This study
TYSC364	*MAT*α *GAL2 ade2*-1 *ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11,15 *can1*-100 Δ*sen54*::*HIS3*/p70N-54 [*CEN6-ARSH4 TRP1 SEN54p*::*TOM70*(1-61)-*SEN54*]	This study
TYSC365	*MAT*α *GAL2 ade2*-1 *ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11,15 *can1*-100 Δ*sen54*::*HIS3*/p70N-Δ200 [*CEN6-ARSH4 TRP1 SEN54p*::*TOM70*(1-61)-*SEN54*Δ200-232]	This study
YUSC025	*MAT***a**/α *GAL2*/+ *ade2*-1/- *ura3*-1/- *leu2*-3, 112/- *trp1*-1/- *his3*-11, 15/- *can1*-100/- *PEP4*/Δ*pep4*::*ADE2 SEN2*/Δ*sen2*::*LEU2*	This study
COSC04-2	*MAT***a** *GAL2 ADE2 ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11, 15 *can1*-100 Δ*sen2*::*LEU2*/pTYSC017 [*CEN6-ARSH4 URS3 SEN2*]	This study
COSC05	*MAT***a** *GAL2 ADE2 ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11, 15 *can1*-100 Δ*sen2*::*LEU2*/pCOSC05 [*CEN6-ARSH4 TRP1 SEN2*]	This study
COSC06	*MAT***a** *GAL2 ADE2 ura3*-1 *leu2*-3, 112 *trp1*-1 *his3*-11, 15 *can1*-100 Δ*sen2*::*LEU2*/pCOSC05 [*CEN6-ARSH4 TRP1 sen2*-41]	This study

a buffer with 0.24 M sucrose, subjected to a gradient consisting of layers of 17, 25, and 37% Nycodenz and then centrifuged at 100,000 × *g* for 2 h. Fractions were recovered from the bottom.

Submitochondrial fractionation was carried out as described (Jascur, 1991). Intact mitochondria suspended in a buffer with 0.6 M sorbitol were diluted by 10-fold with 20 mM HEPES-KOH, pH 7.4, and were sonicated to produce mitochondrial membrane vesicles. After the removal of the unlysed mitochondria by centrifugation at 30,000 × *g* for 20 min, the supernatant was centrifuged at 100,000 × *g* for 1 h to collect the vesicles. The pellet was suspended and subjected to a 0.85–1.6 M linear sucrose gradient. After centrifugation at 100,000 × *g* for 20 h, the gradient was collected from the bottom.

Yeast nuclei were prepared by PVP-sucrose gradient centrifugation as described (Rout and Kilmartin, 1990). Briefly, yeast cells grown in YPD were converted to spheroplasts. The spheroplasts were suspended as 1×10^9 spheroplasts/ml in PVP solution (20 mM K-phosphate, pH 6.5, 0.5 mM $MgCl_2$, 8%wt/vol polyvinylpyrrolidone [PVP-40] supplemented with protease inhibitors cocktail). After addition of final 0.02%wt/vol Triton X-100, the suspension was homogenized with a Dounce homogenizer and then mixed with an equal volume of 0.6 M sucrose in PVP solution. Crude membranes were recovered after centrifugation at 10,000 × *g* for 10 min, and suspended in 1.7 M sucrose in PVP Solution. The suspension was loaded onto a 2.01M/2.10 M/2.30 M sucrose discontinuous gradient in PVP solution and centrifuged at 100,000 × *g* for 4 h. Nuclei banded at 2.10 M/2.30 M sucrose interface were recovered.

Northern Analysis

Total small RNAs were prepared from yeast cells by the hot phenol method with GTE (100 mM Tris-HCl, pH, 7.6, 10 mM EDTA, 4 M guanidine thiocyanate) as a lysis buffer (Guthrie and Fink, 1991). Total RNA samples were separated on 10% polyacrylamide gel with 7 M urea, transferred to Hybond N^+ (Amersham Biosciences) by electric blotting, and then hybridized with appropriate oligonucleotide probes terminally labeled with [γ-^{32}P]ATP. The radioactivity on the membranes was detected with Imaging Plate (Fuji Film, Tokyo, Japan) and Storm 860 Image Analyzer (Molecular Dynamics, Sunnyvale, CA).

tRNA Splicing Endonuclease Assay

For analyzing the tRNA endonuclease activity, cell extracts were prepared as described (Winey and Culbertson, 1988). Endonuclease assays were performed with an end-matured, unspliced form of pre-tRNA-Phe^{GAA} labeled with [α-^{32}P]ATP as a substrate in 10 μl of reaction mixture (40 mM Tris-HCl, pH 8.0, 10% wt/vol glycerol, 0.5 mM EDTA, 20 mM $(NH_4)_2SO_4$, 4 mM spermidine-HCl, 0.2% wt/vol Triton X-100) at 30°C (Peebles *et al.*, 1983). Products were analyzed on a 10% polyacrylamide gel with 6 M urea. The radioactivity in the gel was detected as above.

FISH Analysis

FISH was performed essentially as described (Sarkar and Hopper, 1998) with some modifications. Hybridization was performed in 4× SSC, 10% dextran sulfate, 0.2% BSA, 0.5 mg/ml salmon sperm DNA, 0.25 mg/ml *E. coli* tRNA, 0.5 U/μl RNasein, and 0.1 pmol/μl an FITC- or Cy5-labeled probe at 42°C. Stringent washes were done four times in 4× SSC for 15 min at 42°C. After the final wash, DNA was stained with 0.3 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in 4× SSC for 5 min. Fluorescent images were acquired with an Olympus IX70 microscope equipped with a MicroMax cooled CCD

camera (Roper Scientific), and were analyzed by IP Lab software (Scanalytics, Inc.).

RESULTS

The tRNA Splicing Endonuclease Is Localized to Mitochondria

We first examined the localization of Sen2p by immunofluorescence with its specific antibodies. Although Sen2p was thought to be an integral membrane protein of the inner NE (Peebles *et al.*, 1983), most of the Sen2p signals were distributed as discrete dots along tubular structures that were distinct from the nucleus (Figure 1A, WT). These structures were identified as mitochondria by costaining with a mAb against Por2p, a mitochondrial marker. Only minor, if any, signals were detected in other regions of the cell, including the nucleus. Similar images were obtained when Sen54p, another subunit of the Sen complex, was detected with its antibodies despite the fact that Sen54p has a typical NLS-like sequence, ^{402}KKKR^{406}K (Figure 1B, WT). The mitochondrial signal by the antibodies is specific to Sen54p, because a deletion mutation of its mitochondrial localization domain altered the signal distribution (Figure 5C; see below). The punctate signals of Sen2p and Sen54p on the mitochondria partially result from the fact that only a small number of Sen complexes (~100 molecules) exist in a cell (Trotta *et al.*, 1997). It seems that these few molecules are not enough for uniform signals on the mitochondria. In fact, when overproduced from a multicopy plasmid, Sen2p and Sen54p were more evenly distributed along the mitochondria, and their signals were enhanced (Figure 1, A and B, OP). By Western blotting, the Sen2p overproducer and Sen54p overproducer expressed 13- to 17-fold Sen2p and 10- to 16-fold Sen54p, respectively. Preimmune sera of the anti-Sen2p or anti-Sen54p antibodies did not produce such mitochondrial staining in the immunofluorescence (our unpublished results). These observations were not specific to our strain background. We obtained essentially the same results with several wild-type strains, including 20-B12 that was used in Peebles *et al.* (1983) (our unpublished results).

To confirm the localization of Sen2p and Sen54p on mitochondria, we monitored localization of Sen2p-protein A and Sen54p-protein A fusion proteins that were expressed from chromosomes with their authentic promoters. These strains grew normally and expressed only full-size fusion proteins (Figure 1C, right most panel), indicating full functions of the fusion proteins. Anti-protein A antibodies again revealed colocalization of these protein A fusions with Por2p (Figure 1C). No signal was detected in their parental strain TYSC188 (Figure 1C, no-protein A) and in experiments without anti-protein A antibodies (our unpublished results). We confirmed that protein A fusions are not recognized by the anti-Por2p antibody (mouse IgG1) and the secondary antibodies (from goat) under these conditions. These results indicate that Sen2p and Sen54p are localized mainly to the mitochondria and also suggest that the two subunits can be present on the mitochondria by themselves.

To further establish the mitochondrial localization of the tRNA splicing endonuclease activity in wild-type cells, we performed subcellular fractionations. Although the endonuclease was shown to be associated with membranes, the distribution of its activity was not compared with various organelle markers (Peebles *et al.*, 1983). By differential centrifugation, Sen2p and Sen54p in wild-type cells were mainly recovered in an MSP fraction, a pellet from a 10,000 × *g* centrifugation. When the MSP was resolved on a 20–80% wt/vol sucrose density gradient, Sen2p and Sen54p were distributed with a major peak around fraction 11 and a minor peak around fraction 17 (Figure 2A). These patterns resemble those of the mitochondrial markers, Tim23p and Tom40p (Vestweber *et al.*, 1989; Emtage and Jensen, 1993), but are clearly different from those of the nuclear markers, histone H3 (Hht1p), Nsp1p and Pom152p (Hurt, 1990; Wozniak *et al.*, 1994; Kumar *et al.*, 2002), and an ER marker, Sec63p (Feldheim *et al.*, 1992; Figure 2A; our unpublished results). We then assayed the tRNA endonuclease activity in each fraction with end-matured pre-tRNA-Phe^{GAA} as a substrate. The endonuclease activity that cleaves the pre-tRNA into two exons and an intron also showed a distribution similar to that of the mitochondrial markers, with a major peak around fraction 11 with a shoulder around fraction 17 (Figure 2, A and B). Cofractionation of the endonuclease with the mitochondria was also observed in different gradient systems with Nycodenz (our unpublished results).

To confirm that the detected endonuclease activity arose from the Sen complex, we carried out a similar fractionation with *sen2*-3 mutant cells. The *sen2*-3 cells cleave the intron-3′-exon junction normally, but cleave the 5′-exon-intron junction inefficiently, resulting in the accumulation of a 5′-exon-intron 2/3 molecule (Ho *et al.*, 1990). The pre-tRNA cleavage activity of *sen2*-3 cells, mainly detected in the MSP fraction, now processed the pre-tRNA to the 2/3 molecule and the 3′-exon (Figure 2B). All of the fractions throughout the gradient of *sen2*-3 membranes processed the pre-tRNA into the same molecules (Figure 2B, lower). Therefore, the endonuclease activity we detected indeed came from the Sen complex, indicating that the majority of the tRNA endonuclease activity is associated with the mitochondria.

Because resolution of the overall fractionation is not sufficient for complete separation of mitochondria and the nucleus, we performed organelle-specific fractionations. First, we prepared intact mitochondria from wild-type cells by Nycodenz gradient centrifugation (Lewin *et al.*, 1990). Sen2p and Sen54p were enriched in the mitochondrial fraction with purity similar to that of the mitochondrial markers, Tom70p (Lithgow *et al.*, 1994) and Tim23p, whereas the nuclear markers, Hht1p and Nsp1p, peaked in different fractions (Figure 2C, left). Then, we prepared nuclei by PVP-sucrose density gradient centrifugation (Rout and Kilmartin, 1990). Hhtp and Nsp1p were enriched in the nuclear fraction more than 10-fold. In this fraction, Sen2p and Sen54p were somewhat contaminated but their levels were similar to those of mitochondrial markers (Figure 2C, right). These results again support the idea that the majority of tRNA splicing endonuclease is localized to the mitochondria and no significant pool of the enzyme exists in the nucleus.

Next, we investigated the localization of Sen2p and Sen54p within the mitochondria. Mitochondrial membrane vesicles were prepared and separated into outer mitochondrial membrane (OM) vesicles and inner mitochondrial membrane (IM) vesicles with a sucrose density gradient. The sedimentation patterns of Sen2p (Figure 3A) and Sen54p (our unpublished results) resembled that of Tom70p (OM),

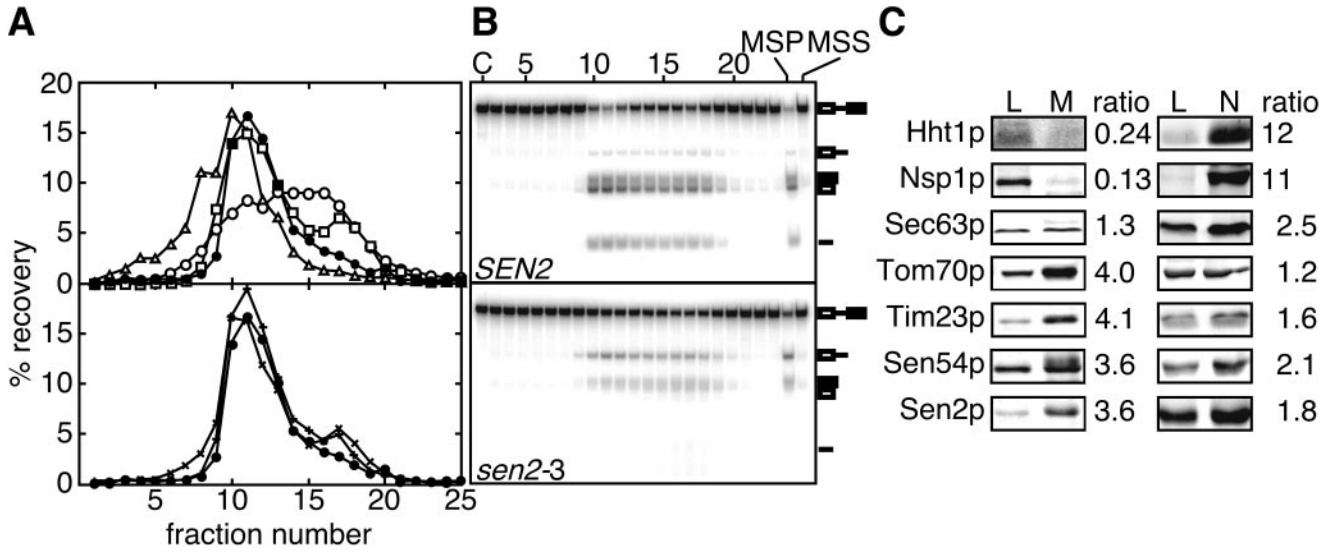

Figure 2. Sen2p, Sen54p, and tRNA splicing endonuclease activity are cofractionated with mitochondria but not with the nucleus. (A) The MSP fraction prepared from wild-type cells was separated on a 20–80% wt/vol sucrose density gradient and was collected from the bottom. In the top panel, the recoveries of Nsp1p (△), Tim23p (□), and Sec63p (○) were compared with that of the endonuclease activity (●). In the lower panel, the recoveries of Sen2p (pluses), Sen54p (crosses), and the enzyme activity (●) were compared. (B) Fractionations similar to A were performed for *SEN2* cells (top) and *sen2*-3 cells (bottom). The endonuclease activities were assayed, and the products were analyzed by urea-PAGE. To visualize the products of the minor peak fractions, the gel was overexposed. The expected products are schematically represented on the right. (C) Mitochondria and nuclei were prepared from wild-type cells (TYSC188) with Nycodenz gradient and PVP-sucrose gradient centrifugation, respectively. Ten micrograms of total lysate and the purified mitochondria in the mitochondrial preparation (left column; lane L and lane M, respectively) were subjected to Western blotting with the antibodies listed on the left. Similar Western blotting analysis was done for samples of the nuclear preparation (right column; lane L for lysate and lane N for nuclei). Signal ratios (M vs. L, or N vs. L) are listed on the right of each panel.

but differed from that of Tim23p (IM). The contaminating NE, represented by Nsp1p, was purified away from the OM. Therefore, the Sen complex is associated with the mitochondrial OM. We then treated intact mitochondria with proteinase K to see whether these subunits were exposed on the cytosolic surface of the mitochondria. The two subunits were degraded by proteinase K (Figure 3B). Tim23p, an IM protein with a domain exposed to the intermembrane space, was protected from the digestion, whereas an OM protein, Tom70p, was degraded, indicating that the OM was intact. All of the proteins were digested by the protease in the presence of Triton X-100. This implies that Sen2p and Sen54p are exposed to the cytosol.

The endonuclease was regarded as an integral membrane protein, because the release of the activity from membranes requires detergent and Sen2p has a hydrophobic stretch (Peebles *et al.*, 1983; Trotta *et al.*, 1997). We then analyzed the membrane interactions of Sen2p and Sen54p. They were released from the mitochondrial membrane with 0.2 M Na_2CO_3, which can extract peripheral membrane proteins and with Triton X-100 in the presence of 1 M NaCl. The integral membrane proteins Tom70p and Tim23p were solubilized only in the presence of the detergent (Figure 3C). These results indicate that Sen2p and Sen54p are peripheral membrane proteins. The crystal structure of a soluble archaeal tRNA endonuclease, a homologue of Sen2p, also suggests that the putative transmembrane domain of Sen2p (225–243) is embedded in the interior of the protein (Li *et al.*, 1998). Finally, we tested whether the endonuclease activity itself was extracted with 0.2 M Na_2CO_3, which seemed harsh for the enzyme. In fact, some of the activity was solubilized under these conditions (Figure 3D), whereas the total activity was reduced to 67% of its original value. Therefore, we concluded that the yeast tRNA endonuclease is peripherally associated with the cytosolic surface of the mitochondrial OM.

A Sen54p Fusion Protein Artificially Fixed on Mitochondria Is Functional

Although the above results indicate that the majority of Sen2p, Sen54p and the tRNA endonuclease activity are associated with the mitochondria, it is still possible that the endonuclease shuttles between the mitochondria and the nucleus dynamically and carries out the tRNA splicing in the nucleus. We thus investigated this possibility. The localization of Sen2p and Sen54p was not altered in several nuclear transport mutants (our unpublished results). However, it is difficult to rule out the nuclear-cytoplasmic shuttling by the analysis of transport mutants, because some proteins utilize multiple receptors for their import and others do not even require the Ran gradient across the NE for their transport (Rout *et al.*, 1997; Takizawa *et al.*, 1999). Therefore, we adopted a different approach. We constructed a fusion gene encoding Tom70N-Sen54p, in which the entire Sen54p is fused to the C termini of the first 61 residues of Tom70p (Tom70N). This region of Tom70p is sufficient to anchor a nonmitochondrial protein to the mitochondrial OM

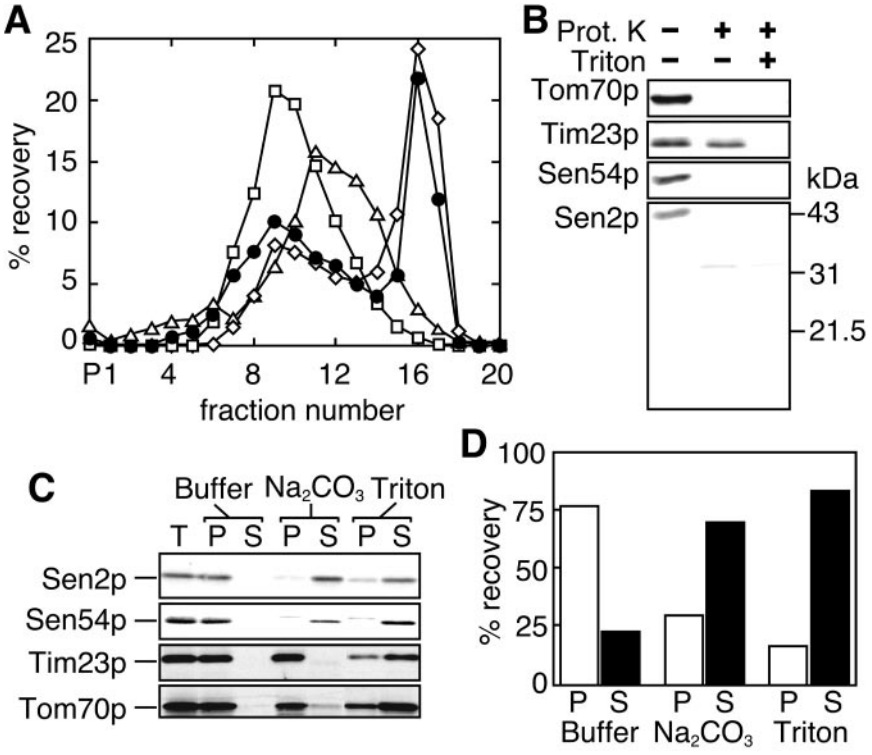

Figure 3. Sen2p and Sen54p are peripherally associated with the mitochondrial surface. (A) Mitochondrial vesicles prepared from the Nycodenz-purified mitochondria were separated by a sucrose density gradient. Recoveries of marker proteins and Sen2p were quantified by Western blotting. ◇, Tom70p; □, Tim23p; △, Nsp1p; ●, Sen2p. The fraction P represents the pellet of the gradient. (B) The intact mitochondria were treated with 0.1 mg/ml proteinase K at 4°C for 20 min in the absence or presence of 0.2%wt/vol Triton X-100 and were analyzed by Western blotting. (C) The mitochondria (T) were extracted with lysis buffer (Buffer), 0.2 M Na_2CO_3 (Na_2CO_3) or lysis buffer with 1% Triton X-100 + 1 M NaCl (Triton) and were separated into a pellet (P) and a supernatant (S) by centrifugation at 100,000 × *g* for 30 min. Each protein was detected with specific antibodies. (D) The mitochondria were extracted as in C. The supernatants were passed through gel filtration columns to exchange the buffers to lysis buffer with 1% Triton X-100. The pellets were suspended in the same buffer. The endonuclease activity in each fraction was assayed and is represented by the recovery.

as an integral membrane protein (Nakai *et al.*, 1989), and thus Tom70N-Sen54p is expected to behave as an integral membrane protein. It has been demonstrated that the NLS for soluble proteins cannot deliver integral membrane proteins into the nucleus (Soullman and Worman, 1995). Therefore, this fusion protein is not expected to shuttle between the mitochondria and the nucleus and should be sequestered from the nucleus.

We put the fusion ORF under the *SEN54* promoter on a low copy plasmid and tested whether the fusion gene could replace the authentic *SEN54* by tetrad analysis. Because *SEN54* is essential for yeast growth, a diploid strain with one disrupted copy of *SEN54* on its chromosomes produced two viable (*SEN54*) and two dead spores (Δ*sen54*) (Figure 4A, Vec). However, the diploid strain harboring a low-copy plasmid with *TOM70N-SEN54* produced three or four viable spores, resembling dissections with the authentic *SEN54* on a plasmid (Figure 4A, 54 and 70N-54). No growth defects were observed in the viable spores (our unpublished results). Genetic markers indicated that all of the viable haploids with a disrupted copy of *SEN54* on their chromosome had the *TOM70N-SEN54* fusion gene on the plasmid. These results suggest that the fusion gene can functionally replace *SEN54*.

Western blotting revealed that only a full-size Tom70N-Sen54p, but no degradation product, was present in total cell extracts (Figure 4B). Tom70N-Sen54p, like Sen54p, was colocalized with the mitochondrial marker, Por2p (Figure 4B). We then analyzed the interaction of the fusion protein with membranes. In contrast to Sen54p, Tom70N-Sen54p was not extracted from the crude membranes with Na_2CO_3, indicating that it behaves as an integral membrane protein (Figure 4C). We next examined the pre-tRNA accumulation in vivo and the endonuclease activity in vitro in the *TOM70N-SEN54* cells. Northern blotting demonstrated that the amounts of unspliced precursors of tRNA-Ile^{UAU}, tRNA-Leu^{CAA}, and tRNA-Pro^{UGG} in the *TOM70N-SEN54* cells were similar to those of the wild-type cells (Figure 4D). We then prepared crude extracts from the *TOM70N-SEN54* and wild-type cells and assayed the tRNA endonuclease activity in vitro with pre-tRNA-Phe^{GAA} as a substrate. There was essentially no difference in the endonuclease activity between the two strains (Figure 4E). Therefore, Sen54p can function even when it is artificially fixed on the mitochondria, providing evidence against the nuclear-cytoplasmic shuttling of the Sen complex.

sen54 *Mutants Defective in Mitochondrial Localization Are Not Fully Functional*

Next, we asked whether the mitochondrial localization of Sen54p is required for its function. First, we identified the mitochondrial localization signal of Sen54p by deletion analyses. Its region including amino acids 200–313 was sufficient for targeting GFP to the mitochondria (unpublished results). Then, we constructed *TRP1* low-copy plasmids harboring *SEN54* derivatives with partial deletions of this region: *sen54*Δ200-232 (p314-Δ200) and *sen54*Δ275-313 (p314-Δ275) (Figure 5A). We introduced these plasmids into a haploid strain whose chromosomal *SEN54* disruption is complemented by the authentic *SEN54* on a *URA3* plasmid. Cells losing the *URA3* plasmid were selected on a 5′-FOA plate. Cells with p314-Δ275 could not grow on the 5′-FOA plate,

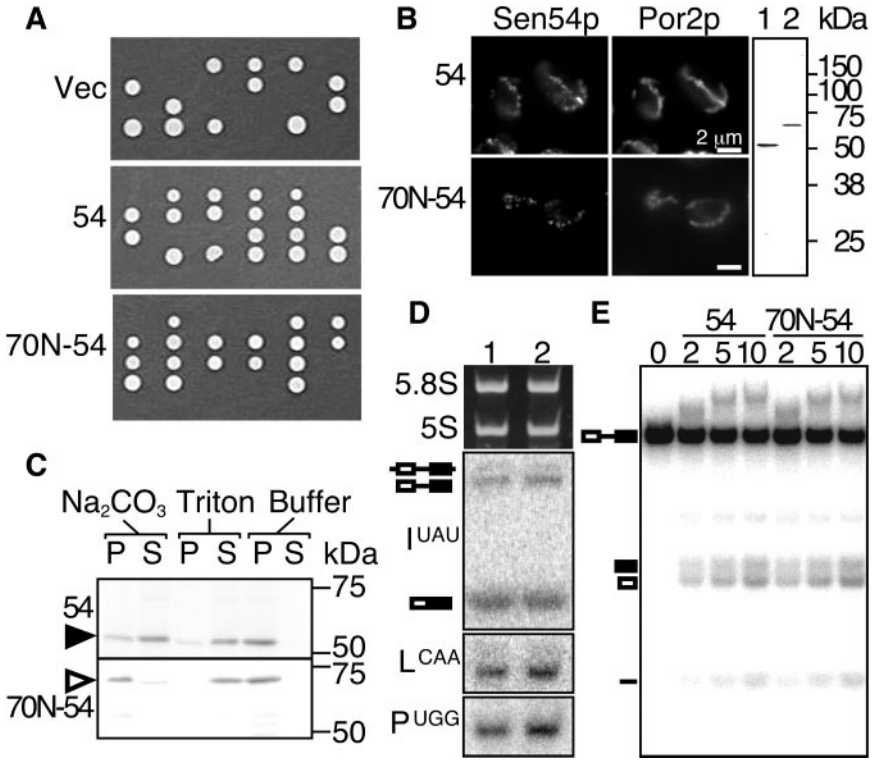

Figure 4. The *TOM70N-SEN54* fusion gene can replace the authentic *SEN54*. (A) A low-copy plasmid with either no insert (Vec; pRS314), *SEN54* (54; p314–54), or *TOM70N-SEN54* (70N-54; p70N-54) was introduced into a *SEN54*/Δ*sen54::HIS3* diploid, and the resulting diploids were sporulated. Their tetrads were dissected and cultured on YPD at 23°C. (B) The localization of Tom70N-Sen54p was examined by immunofluorescence (Sen54p; left). The mitochondria were visualized with an anti-Por2p antibody (Por2p; middle). The right panel shows Western blotting of total extracts prepared from the wild-type cells (lane 1) and the *TOM70N-SEN54* cells (lane 2). (C) Crude membranes were prepared from the wild-type cells (54) and the *TOM70N-SEN54* cells (70N-54). The membranes were extracted as in Figure 3C. Closed arrowhead, authentic Sen54p; open arrowhead, Tom70N-Sen54p. (D) Total RNAs were prepared from the strains expressing either *SEN54* (lane1) or *TOM70N-SEN54* (lane2). One microgram of each was analyzed by Northern blotting with probes recognizing unspliced and mature tRNA-Ile^{UAU} (I^{UAU}), pre-tRNA-Leu^{CAA} (L^{CAA}), or pre-tRNA-Pro^{UGG} (P^{UGG}). On the top panel, the 5.8S and 5S rRNAs on the gel before transfer were visualized by ethidium bromide staining as controls for loading. (E) Cell extracts were prepared from the *SEN54* (54) and *TOM70N-SEN54* (70N-54) cells, and their endonuclease activity was assayed at 30°C for 10 min with pre-tRNA-Phe^{GAA} as a substrate. The indicated amounts (μg protein) of each total extract were added to 10-μl reaction mixtures. Expected products are represented as in Figure 2B.

indicating that *sen54Δ275-313* cannot complement Δ*sen54* (Figure 5B, top).

On the other hand, *sen54Δ200-232* cells grew on the 5′-FOA plate and showed a ts phenotype. At 37°C, *sen54Δ200-232* cells accumulated unspliced pre-tRNAs (our unpublished results). We observed similar but weak splicing defects in another partial deletion of the *SEN54* mitochondria targeting domain, *sen54Δ233–246* (our unpublished results). Sen54Δ200-232p was no longer localized to the mitochondria, but was distributed throughout the cytoplasm even at the permissive temperature (Figure 5C). There are some Sen54p signals in the nuclear regions of the mutant cells. The deletion mutation might result in exposure of the NLS-like sequence, ^{402}KKKR^{406}K, in Sen54p through alteration of its overall structure. If the growth defect comes from abnormal localization of Sen54p, regaining the mitochondrial localization by introducing an ectopic localization signal will suppress the defect. Fusion of the *TOM70N* to the 5′-terminus of the *sen54Δ200-232* ORF resulted in the recovery of the growth at 37°C and of the mitochondrial localization of the mutant protein (Figure 5, B, bottom, and C). The in vitro endonuclease activity at 30°C of *sen54Δ200-232* extracts was reduced to 29% of the wild-type extracts, but the activity of *TOM70N-sen54Δ200-232* extracts was recovered to 68%, indicating that the mitochondrial localization of Sen54p correlates with the total endonuclease activity.

Mutations in a localization signal of a protein are expected not to affect the total activity of the protein if its signal domain and catalytic domain are separated. However, in the case of yeast tRNA endonuclease, a multi-subunit enzyme that has more than two subunits with independent localization information, failure in mitochondrial targeting of Sen54p alone will cause inefficient complex assembly on the surface of mitochondria, leading to a decrease in the total endonuclease activity. Indeed, Figure 1, A and B, demonstrate that Sen54p and Sen2p can be targeted to the mitochondria independently. Furthermore, the localization of Sen2p was not affected by *sen54Δ200-232* mutation (Figure 5D), indicating that only a part of Sen54p was colocalized with Sen2p. Tom70N-Sen54Δ275-313p was not functional (Figure 5B), implying that the 275-313 region has a role other than in mitochondrial localization. These results indicate that the defects of the *sen54Δ200-232* mutant come from its mislocalization from the mitochondria and that the mitochondrial localization of Sen54p is required for the assembly and/or enzymatic activity of tRNA endonuclease.

Endonuclease-deficient Cells Accumulate pre-tRNAs in the Cytosol

If the tRNA splicing endonuclease functions on the mitochondrial surface, the unspliced pre-tRNAs must be exported from the nucleus. We thus analyzed the localization of unspliced pre-tRNAs in endonuclease deficient mutants by FISH. We focused on Sen2p, one of the catalytic subunits of the enzyme. Because the *sen2*-3 mutation is leaky and not conditional (Ho *et al.*, 1990), we screened new *sen2* ts alleles and obtained one allele, *sen2*-41, that showed a clear ts growth (Figure 6A). Northern blotting showed that *sen2*-41 cells accumulated end-matured unspliced forms of tRNA-

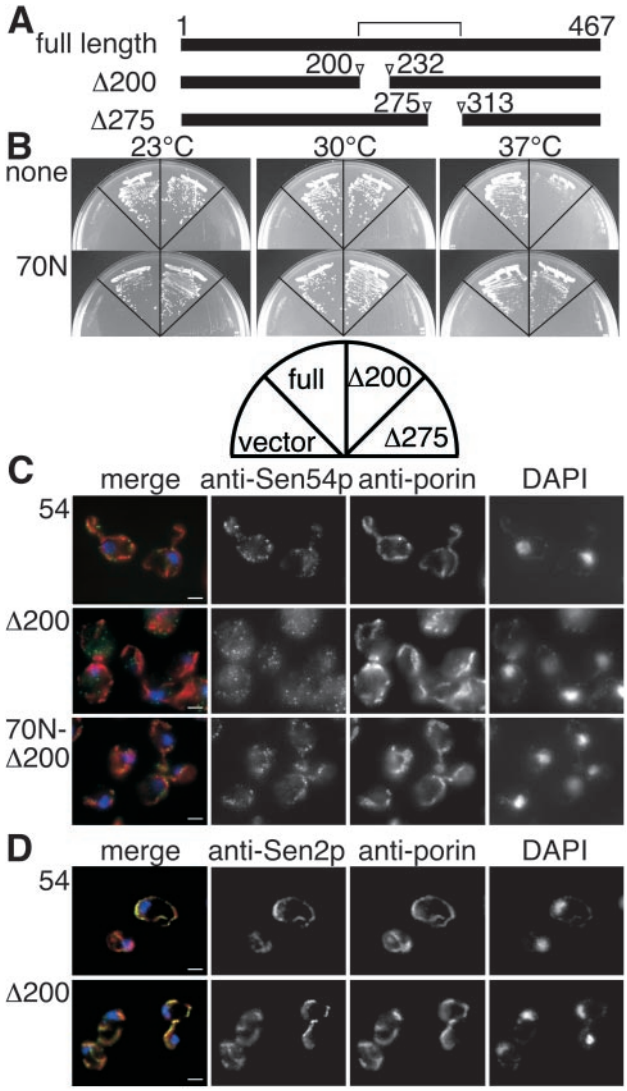

Figure 5. Partial deletion of the Sen54p mitochondrial localization signal impaired tRNA splicing. (A) A schematic diagram of *sen54* mutants with partial deletion in the mitochondrial localization signal (residues 200–313). The blank area between the bars indicates the deleted region in each mutant. (B) A *TRP1* low-copy plasmid harboring the wild-type *SEN54* gene or the mutant *sen54* genes with the deletion of Δ200-232 or Δ275-313 was introduced into a haploid strain whose chromosomal disruption of *SEN54* was complemented with a *URA3* plasmid with wild-type *SEN54* (none). Mutant genes fused with a *TOM70N* region at their N-termini were also introduced in the same recipient (70N). Yeast cells dependent on the *SEN54* genes on the *TRP1* plasmids were selected on 5′-FOA plates, and their growth was compared at the indicated temperatures. (C) The localization of Sen54p in the wild-type (54), *sen54*Δ*200-232* (Δ200), and *TOM70N-sen54*Δ*200-232* (70N-Δ200) cells at 30°C was visualized by immunofluorescence as in Figure 1A. (D) The localization of Sen2p in the wild-type (54) and *sen54*Δ*200-232* (Δ200) cells was visualized as above.

Ile^{UAU}, tRNA-Leu^{CAA}, and tRNA-Pro^{UGG} during a 4-h incubation at 37°C (Figure 6B). 5′-exon-intron 2/3 molecules and introns were apparently not accumulated in this mutant

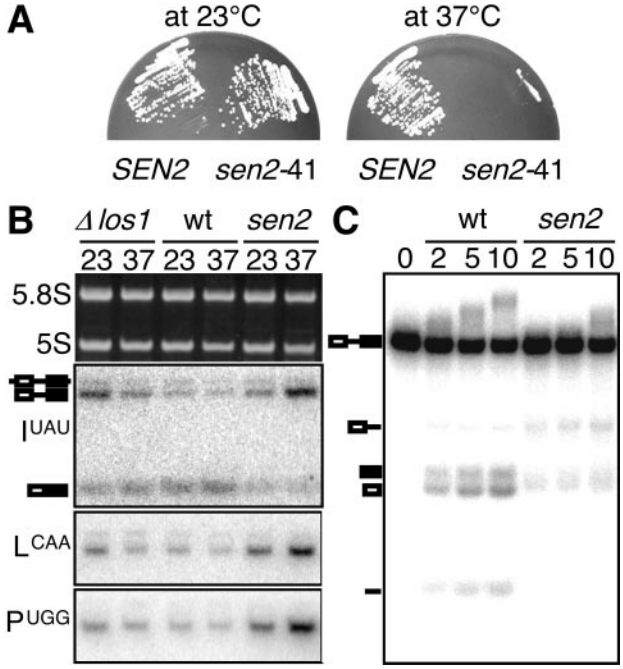

Figure 6. *sen2*-41, a new temperature-sensitive allele of *SEN2*. (A) The growth of strains with a chromosomal disruption of *SEN2*, complemented by either wild-type *SEN2* or *sen2*-41 on a low-copy plasmid, was compared on YPD at the indicated temperatures. (B) Wild-type, Δ*los1* mutant, and *sen2*-41 mutant strains were cultured at 23°C (23) and were shifted to 37°C (37) for 4 h. Pre-tRNAs in 1 μg of total RNAs from each culture were detected with specific probes, as in Figure 4D. (C) The tRNA endonuclease activity in the wild-type (wt) and *sen2*-41 cells (*sen2*) was assayed as in Figure 4E.

(our unpublished results). Even at 23°C, the mutant cells contained higher amounts of the pre-tRNAs than wild-type cells. Next, we tested the tRNA endonuclease activity in vitro. The *sen2*-41 extract prepared from the mutant cells grown at 23°C had significantly lower endonuclease activity, when tested at 30°C (Figure 6C). The extract was also inactive at both 23 and 37°C (our unpublished results). A reaction product corresponding to a 5′-exon-intron 2/3 molecule was detected in the gel. Therefore, the mutant has a primary defect in the cleavage of the 5′-exon-intron junction of pre-tRNAs in vitro.

Using the *sen2*-41 mutant, we analyzed the localization of tRNA-Ile^{UAU} by FISH with two probes: one recognizing both the precursor and mature forms of the tRNA and the other against its intron. First, we monitored the time course of pre-tRNA accumulation after shift to the restrictive temperature. As reported previously (Sarkar and Hopper, 1998; Grosshans *et al.*, 2000), in wild-type cells, unspliced pre-tRNA-Ile^{UAU} was detected mainly in the nucleus at 23°C. Its localization was not changed by up to 4-h incubation at 37°C (Figure 7A, wt, left column). We usually saw a transient decrease in the signal intensity within 1 h after the shift. This transient decrease was also detected by Northern blotting (Figure 7B). In the *sen2*-41 cells, the unspliced pre-tRNA-Ile^{UAU} localization at 23°C rather resembled that of the wild-type cells. The pre-tRNA signal decreased transiently within 1 h after the shift to 37°C, as was the case of the

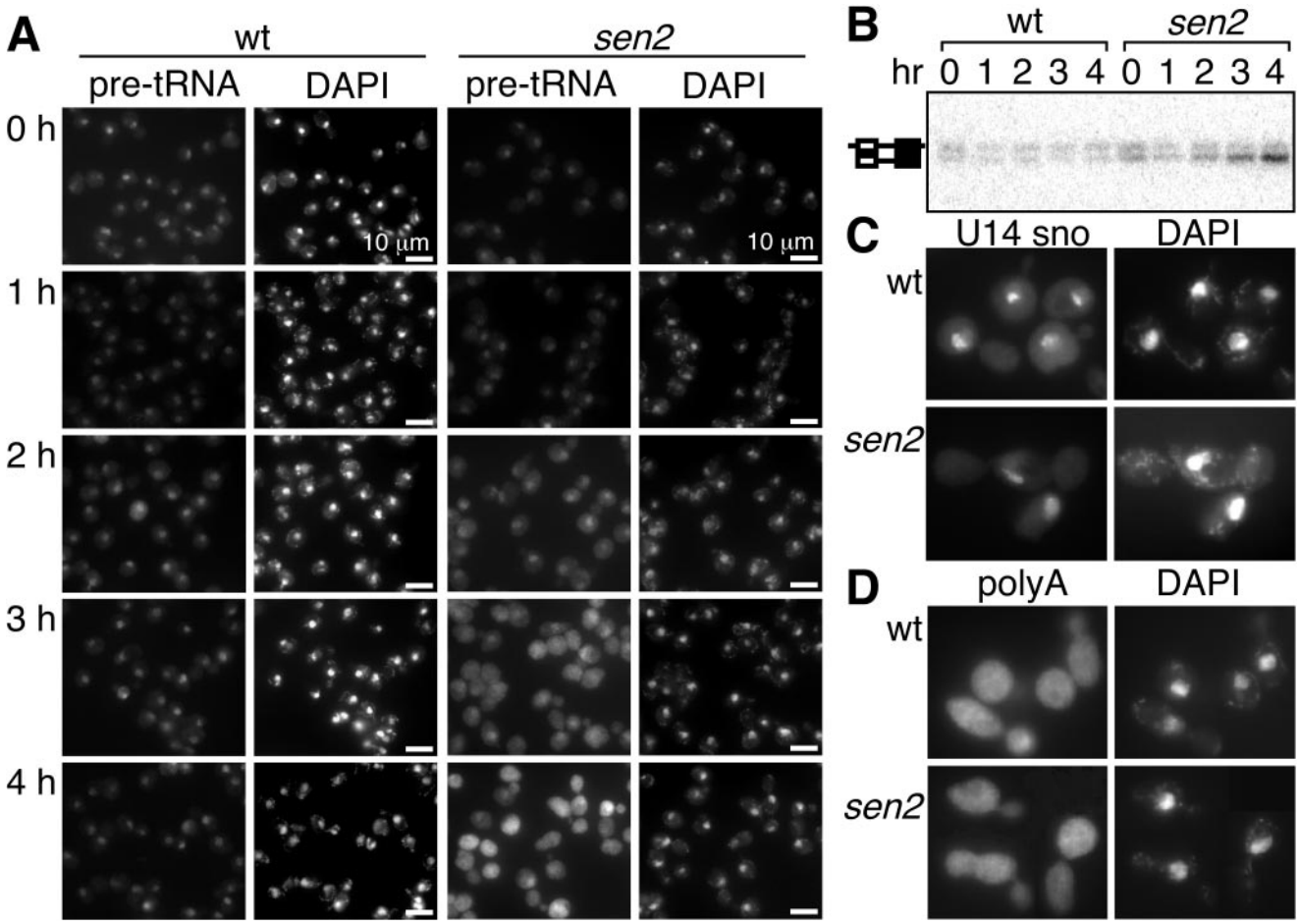

Figure 7. *sen2*-41 accumulates unspliced pre-tRNAs in the cytosol. (A) Time course of unspliced pre-tRNA accumulation in *sen2*-41 cells was monitored by FISH. Wild-type cells (1st and 2nd columns) and *sen2*-41 cells (3rd and 4th columns) were cultured at 23°C, shifted to 37°C and then harvested at 0, 1, 2, 3, and 4 h after the shift. pre-tRNA-Ile^{UAU} was detected with an intron-specific probe (pre-tRNA), and the position of nucleus was visualized by DAPI staining (DAPI). Bars, 10 μm. (B) Amounts of precursor forms of tRNA-Ile^{UAU} were monitored by Northern blotting. Total RNA samples were prepared from the wild-type and *sen2*-41 cells in a similar time course experiment as in A. One microgram of the total RNA was analyzed by Northern blotting with the intron-specific probe. The expected precursor forms are schematically represented on the left. (C) The localization of U14 snoRNA was visualized by FISH. Wild-type (wt) and *sen2*-41 (*sen2*) cells were fixed after 3-h exposure at 37°C and stained with a probe against U14 snoRNA (U14 sno) to visualize the nucleolus. DNA was stained with DAPI (DAPI). (D) The localization of mRNA in *sen2*-41 cells was visualized by FISH with oligo-dT$_{50}$ probe as in C.

wild-type cells. The pre-tRNA then accumulated gradually from 2 h after the shift (Figure 7A, *sen2*, left column). The increase in the cytosolic signal was more prominent than that in the nucleus during this period. Within 3 h, most of the cells accumulated similar levels of the pre-tRNA, in both the cytosol and the nucleus. The increase of the cytosolic pre-tRNA in the mutant cells monitored by FISH correlates well with the total accumulation of the pre-tRNA monitored by Northern blotting (Figure 7B). Experiments with intron-specific probes for tRNA-Trp^{CCA}, tRNA-Leu^{CAA}, and tRNA-Pro^{UGG} gave similar results (our unpublished results). These results indicate that the unspliced pre-tRNAs are exported to and accumulated in the cytosol in the absence of the splicing endonuclease activity. The fact that an extremely high accumulation of the pre-tRNA in the nucleus was not observed argues against the possibility that the cytosolic signal is a mere leakage from the nucleus where high amounts of pre-tRNAs accumulate because of the lack of the splicing activity. The mutation did not affect the localization of total tRNA-Ile^{UAU} (Figure 8, n and t), mature tRNA-Trp^{CCA}, mature tRNA-Leu^{CAA}, and the intron-less tRNAs, tRNA-Gly^{GCC}, and tRNA-Glu^{UUC} (our unpublished results). The integrity of the NE and intranuclear structures, like the nucleolus, seems to be intact in the *sen2*-41 cells, because the locations of U14 snoRNA (Figure 7C), U6 snRNA (our unpublished results), and mRNA (Figure 7D) in the mutant cells were indistinguishable from those in the wild-type cells.

Next, we analyzed the localization of tRNA-Ile^{UAU} in a Δ*los1 sen2*-41 double mutant to assess the effects of a mutation in the tRNA export machinery. Δ*los1* cells, which lack the yeast exportin-t homologue Los1p, accumulate both the

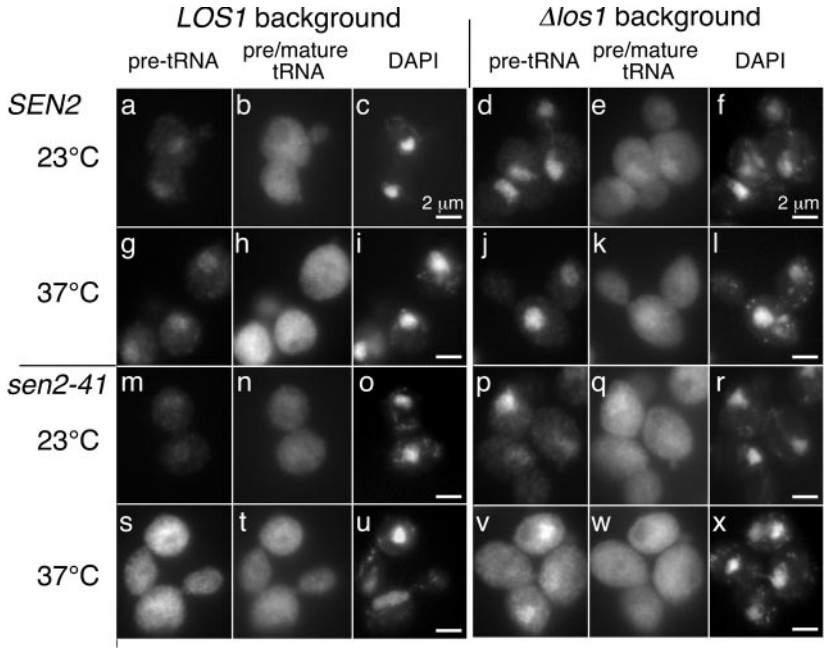

Figure 8. Δ*los1 sen2* double mutant accumulates a part of pre-tRNAs in the nucleus. Unspliced precursor (1st and 4th columns) and all (2nd and 5th columns) forms of tRNA-Ile^{UAU} in wild-type cells (a–c, g–i), *sen2*-41 cells (m–o, s–u), Δ*los1* cells (d–f, j–l), and Δ*los1 sen2*-41 double mutant cells (p–r, v–x) were visualized by FISH. DNA was detected with DAPI (3rd and 6th columns). Each strain was cultured at 23°C (23°C), and then its aliquot was shifted to 37°C for 3 h (37°C). Bars, 2 μm.

pre-tRNA and the mature tRNA in the nucleus as expected (Sarkar and Hopper, 1998; Figure 8, d–f and j–l). The nuclear pool of the pre-tRNA in the Δ*los1* cells was much larger than that in the *sen2*-41 cells. In the Δ*los1 sen2*-41 cells, the pre-tRNA accumulated in the nucleus, like the Δ*los1* cells, but a considerable amount of the pre-tRNA was present in the cytosol, like the *sen2*-41 cells (Figure 8, v–x). Similar results were obtained with intron-specific probes against tRNA-Trp^{CCA}, tRNA-Leu^{CAA}, and tRNA-Pro^{UGG} (our unpublished results). These results further indicate that the mere accumulation of the pre-tRNAs in the nucleus does not result in the leakage of the pre-tRNAs to the cytosol. The simplest interpretation of the experiments described above is that the pre-tRNAs are actively exported to the cytosol, in a manner that is partly dependent on Los1p. The results are consistent with the idea that tRNA splicing occurs on the mitochondria.

DISCUSSION

Peebles *et al.* (1983) suggested that the yeast tRNA splicing endonuclease was an integral membrane protein of the inner NE. However, the biochemical and cytological analyses in the present study revealed that the yeast endonuclease mainly localizes to the mitochondria, and only a small amount, if any, of the enzyme exists in the nucleus. In *Xenopus* oocytes, the tRNA endonuclease activity was mainly detected in the nucleoplasm (DeRobertis *et al.*, 1981) and is thought to act in the nucleus in vivo (Melton *et al.*, 1980). This raises the possibility that the primary location of this enzyme differs in different organisms and/or cell types. The present mutant analysis in yeast further suggests that the mitochondrial localization of the tRNA endonuclease has a positive role in tRNA splicing. Finally, we obtained pieces of evidence that support pre-tRNA export from the nucleus. These results apparently do not fit the currently accepted model that tRNA splicing occurs in the nucleus and only mature tRNAs are exported to the cytoplasm.

Although we demonstrated that large portions of Sen2p, Sen54p, and the endonuclease activity are localized on the mitochondria, there are still two explanations for these observations from the view of nuclear pre-tRNA splicing. One possibility is nuclear-cytoplasmic shuttling of the endonuclease. However, the Sen54p derivatives inserted in the OM are fully functional. Disintegration of integral membrane proteins from membranes would require special machinery that does not seem to function in the usual nuclear-cytoplasmic transport. Therefore, a large part of Tom70N-Sen54p should exist and function on the OM. This fact suggests that the above possibility is less likely. The other explanation is that a minor nuclear pool of the endonuclease is responsible for the tRNA splicing. The Sen complex may assemble on the mitochondrial surface and be stored there until it leaves for the nucleus. It may also be possible that the mitochondrial Sen complex has another function instead of tRNA splicing. There are many examples where one protein localizes at two distinct cellular compartments and each portion of the enzyme has different functions (Danpure, 1995). Even in the case of RNA processing, RNase P and RNase MRP share most of their protein subunits but fill distinct roles of pre-tRNA end-processing in the nucleus and 5.8S rRNA processing in the mitochondria, respectively (Chamberlain *et al.*, 1998; Gold *et al.*, 1989). The Sen complex might be another example of this case. In fact, it is logically difficult to prove that no tRNA endonuclease exists in the nucleus. At least, our various fractionation and immunofluorescence analyses did not reveal a significant pool of the enzyme in the nucleus. In *TOM70N-SEN54* cells, a larger amount of the Sen54p moiety should be trapped on the mitochondria than in wild-type cells. Therefore, the nuclear pool of the enzyme

should be quite small. Because only ~100 molecules of the endonuclease exist in a yeast cell (Trotta *et al.,* 1997), these observations suggest that, for tRNA splicing in the nucleus, only several molecules of the enzyme in a nucleus would have to provide the entire function in wild-type cells that grow normally. On the other hand, alteration of the mitochondrial localization of Sen54p by the partial deletion of its localization signal compromises normal growth and tRNA splicing activity. Regain of mitochondrial localization by addition of an unrelated targeting signal is enough to restore the phenotypes. Therefore, these observations favor the idea that the mitochondrial pool of Sen54p contributes to the tRNA splicing in yeast cells.

In the presence of our new observations, how can we explain the nuclear accumulation of end-matured, unspliced pre-tRNAs in the mutants defective in tRNA export? As mentioned before, several observations do not support "splicing-export coupling model" based on the nuclear splicing. The existence of a proofreading step by RS indicates that splicing, proofreading, and export occur sequentially in this order (Lund and Dahlberg, 1998; Sarkar *et al.,* 1999; Grosshans *et al.,* 2000). Because excess amounts of mature tRNAs do not inhibit tRNA splicing in vitro, product inhibition by mature tRNAs accumulated in the nucleus is unlikely (Peebles *et al.,* 1979). In fact, when the aminoacylation of a certain tRNA is blocked, only its mature form, but not its precursor, is accumulated in the nucleus, indicating that such product inhibition does not occur even in vivo (Azad *et al.,* 2001). The discovery of intranuclear translation predicts that the nuclear aminoacyl-tRNAs have a vital role in this process, indicating that there is an exchangeable pool of mature tRNAs in the nucleus before export (Iborra *et al.,* 2001). Finally, we showed that *sen2*-41 cells accumulate pre-tRNAs in the cytosol and that this was not due merely to leakage from the nucleus. The nuclear splicing model predicted that endonuclease-deficient mutants would accumulate unspliced pre-tRNAs in the nucleus; otherwise, *sen2*-41 should be a special mutant that compromised nuclear tethering of unspliced pre-tRNAs. A *SEN54*-depletion strain also accumulated pre-tRNAs in the cytosol (Tanaka, Endo, and Yoshihisa, unpublished results), suggesting that the cytosolic accumulation of pre-tRNAs is a general phenotype in *sen* mutants. Because the amount of the endonuclease in the nucleus seems to be very small, if the endonuclease plays a role in retaining pre-tRNAs in the nucleus, it would have to act catalytically. Therefore, several conditions are required to understand ours and other's observations from the view of the nuclear splicing.

On the contrary, if we accept that the endonuclease on the mitochondria is physiologically active, these observations can be easily explained in the following manner. The pre-tRNAs are exported to the cytoplasm for their splicing on the mitochondria, and this export is the rate-limiting step for their splicing; therefore, the export mutations prevent the pre-tRNAs from access to the endonuclease. Several parallel pathways for tRNA export have been reported (Hellmuth *et al.,* 1998; Azad *et al.,* 2001; Feng and Hopper, 2002). These pathways may be divided into two classes. One is specific for fully matured and functional tRNAs, and is governed by RSs, eEF-1α, etc. The other can export the pre-tRNAs and partly depends on Los1p. Indeed, the *los1* mutation causes the nuclear accumulation of the mature form of intron-containing tRNAs, but not that of some intronless tRNAs (Grosshans *et al.,* 2000). Although we have not definitively proven that pre-tRNAs are exported to the cytosol to gain access to the mitochondrial endonuclease, the mitochondrial splicing model should be considered as a probable working hypothesis.

If pre-tRNAs are cleaved on the mitochondrial surface, then a question may arise as to the fate of the resulting tRNA exons. The exons may come back to the nucleus to be ligated by the yeast tRNA ligase (Rlg1p) localized there (Clark and Abelson, 1987), and the ligated tRNAs may be exported again to the cytosol. Alternatively, Rlg1p may shuttle between the nucleus and the cytosol to mediate tRNA ligation in the cytosol. Rüegsegger *et al.* reported that the yeast *HAC1* pre-mRNA is spliced in the cytosol by a nonconventional mechanism similar to that of the pre-tRNAs. *HAC1* exons are indeed joined by Rlg1p, suggesting that a fraction of Rlg1p functions in the cytosol (Sidrauski *et al.,* 1996; Rüegsegger *et al.,* 2001). In any case, if intranuclear translation occurs in yeast as in the case of mammalian cells, a fraction of the mature tRNAs must be retained in or sent back to the nucleus.

Another fact that may not be consistent with the mitochondrial splicing model is that splicing and end-processing of tRNAs are not completely ordered to each other (O'Connor and Peebles, 1991). Because the RNA subunit of RNase P, the *RPR1* gene product, was found to be associated with the nucleolus (Bertrand *et al.,* 1998), end-immature spliced pre-tRNAs must go back to this nuclear compartment for their end-processing. As mentioned above, our data suggest existence of some mechanism that operates for nuclear import of tRNA molecules. Such mechanism may support the pathway where the splicing precedes the end-maturation. At least, we mainly detected end-mature precursors of tRNA–Ile^{UAU}, tRNA-Leu^{CAA}, and tRNA-Pro^{UGG} in *sen2-41* mutant cells, indicating that the splicing defects do not affect the end-processing in the mutant. On the other hand, end-immature spliced pre-tRNAs were accumulated in mutants of Sm-like proteins (Lsm proteins) and a La homologue in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Intine *at al*., 2002; Kufel *et al.,* 2002). Especially, a La derivative that is not efficiently retained in the nucleus causes accumulation of end-immature spliced pre-tRNAs, whereas this mutant La interacts with these end-immature species (Intine *et al.,* 2002). Because Lsm and La proteins are thought to interact with early processing precursors of tRNAs, the end-immature spliced pre-tRNAs accumulated in these mutants might escape from the normal processing pathway to be delivered to the cytoplasm. Or the aberrant retention of the end-immature species in the cytosol may cause inefficient end-processing in the splicing-first pathway. Most of these results, including ours, were obtained through Northern blotting. Therefore, further investigation with pulse-chase experiments will be necessary to reveal exact fates of incompletely processed species in tRNA biogenesis.

In summary, accumulated findings including ours suggest that, in yeast, the tRNA splicing endonuclease on the mitochondria has positive roles in tRNA biogenesis, although its physiological meaning is still obscure. The tRNA traffic in eukaryotic cells should be carefully reexamined in the view of the unexpected finding that the enzyme is localized on

mitochondria. The tRNAs themselves, but not the processing enzymes, may dynamically shuttle between the nucleus and the cytoplasm during and after their maturation.

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