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Yeast Nucleoporin Mutants Are Defective in pre-tRNA Splicing

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We have screened nucleoporin mutants for the inhibition of tRNA splicing, which has previously been proposed to be coupled to transport. Strains mutant for Nup49p or Nup116p, or genetically depleted of Nup145p, strongly accumulated unspliced pre-tRNAs. Splicing was inhibited for all 10 families of intron-containing pre-tRNA, but no effects on 5′ or 3′ end processing were detected. Strains mutant for Nup133p or Nsp1p accumulated lower levels of several unspliced pre-tRNAs. In contrast, no accumulation of any pre-tRNA was observed in strains mutant for Nup1p, Nup85p, or Nup100p. Other RNA processing reactions tested, pre-rRNA processing, pre-mRNA splicing, and small nucleolar and small nuclear RNA synthesis, were not clearly affected for any nucleoporin mutant. These data provide evidence for a coupling between pre-tRNA splicing and nuclear-cytoplasmic transport. Mutation of *NUP49*, *NUP116*, or *NUP145* has previously been shown to lead to nuclear poly(A)$^+$ RNA accumulation, indicating that these nucleoporins play roles in the transport of more than one class of RNA.

In all eukaryotic cells, nuclear-cytoplasmic transport across the nuclear envelope occurs through a large transmembrane structure termed the nuclear pore complex (NPC). The NPC supports bidirectional transport of small molecules by passive diffusion, while larger proteins, RNAs and ribonucleoprotein complexes are actively transported. To date no pore specialization has been detected, and all pores are presumed to be competent both for protein import and the export of all classes of RNA.

A number of NPC components have been identified and cloned from yeasts and higher eukaryotes. Monoclonal antibodies against mammalian nucleoporins cross-react with yeast nucleoporins (2), arguing for the evolutionary conservation of nuclear pore components from yeasts to humans. Using an immunochemical approach, Nup1p (9), Nup2p (26), Nup49p, Nup100p, and Nup116p (50) were identified. Antibodies raised against a yeast insoluble nuclear fraction led to the identification of Nsp1p (17), which is 30% identical (50% homologous) to the vertebrate nucleoporin p62 (6). Other nucleoporins have been isolated by using an elegant synthetic lethal screen. The basis of this technique is the identification of pairs of mutations, neither of which is lethal on its own but which are lethal in combination (13, 25). The detection of synthetic-lethal interactions provides prima facie evidence that the components functionally interact, either by directly binding to each other or because their roles in some way overlap. A temperature-sensitive *nsp1* mutant is synthetically lethal with mutant alleles of *NUP49*, *NUP116*, *NUP85*, and *NUP145* (11, 42a, 53), while mutation of *NUP133* is synthetically lethal with a mutation in *NUP49* (10).

The mechanism of protein import across the NPC has been extensively studied, but despite a number of recent studies, much less is known about the mechanism of export of RNA from the nucleus. RNA export shows several similarities to protein import. It is an energy-dependent process which is sensitive to low temperatures and ATP depletion, is saturable, and can be inhibited by the lectin wheat germ agglutinin (for recent reviews, see references 12 and 19). tRNA export has been shown to be via a carrier-mediated translocation process which can be saturated with competitor tRNA and is sensitive to mutations in highly conserved regions of the tRNA molecule (8, 20, 44, 54). Dargemont and Kühn (8) reported that in competition experiments, excess tRNA inhibits mRNA export when injected in *Xenopus* oocytes, indicating that tRNA and mRNA may use common pathways. In contrast, Jarmolowski et al. (20) concluded that distinct factors are limiting for the export of different classes of RNA; microinjection of tRNA, U small nuclear RNAs, or mRNA into *Xenopus* oocytes competitively inhibited their own export at concentrations which do not affect the transport of heterologous RNAs. Given that all RNAs must pass through the same NPCs, it is likely that even if the initial binding factors are distinct, all RNAs will interact with common components at later steps in transport.

All tRNAs are synthesized as 5′- and 3′-extended precursors (pre-tRNAs). In addition, some pre-tRNAs are interrupted by intervening sequences. Of the 360 tRNA genes in *Saccharomyces cerevisiae*, approximately 40 to 45 encode intron-containing pre-tRNAs (14). These genes encode 10 of the 46 isoacceptor tRNA families (34) and contain introns of variable length (14 to 60 nucleotides), which are always present 1 nucleotide 3′ to the anticodon (see reference 52 for a listing of intron sizes in different pre-tRNA species). Processing of the ends of the tRNA generally precedes splicing, producing pre-tRNAs which are unspliced but have the mature ends. This order is not obligatory, however, and pre-tRNAs which are spliced but immature at their ends can also be readily detected in wild-type cells (32). Genetic and biochemical data suggest that there is a coupling between pre-tRNA splicing and transport (reviewed in reference 52). The 18S, 5.8S, and 25S rRNAs are also synthesized as large pre-rRNAs, which undergo a complex series of processing reactions before the rRNAs are transported to the cytoplasm. Interestingly, the processing reaction which converts the 20S pre-rRNA to the mature 18S rRNA is reported to occur in the cytoplasm (47). This reaction should therefore serve as an indicator that nuclear-cytoplasmic transport has occurred.

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TABLE 1. Yeast strains used

Strain	Genotype
RS453	*MAT***a**/α *ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 CAN1-100/CAN1-100*
Wild type	*MAT*α *ade2 leu2 ura3 trp1 his3 CAN1-100*
nup85-Δ	*MAT***a** *ade2 leu2 ura3 his3 trp1 CAN1-100 nup85*::*HIS3*
nup133-Δ	*MAT***a** *ade2 his3 trp1 leu2 ura3 CAN1-100 nup133*::*HIS3*
nup1-Δ	*MAT***a** *ura3-52 ade2-1 trp1-1 leu2-3 his3-11 CAN1-100 nup1*::*HIS3*
nsp1^{ts}*L→S*	*MAT***a** *ade2 leu2 ura3 trp1 his3 CAN1-100 HIS3*::*nsp1*^{ts} *L→S*
nsp1^{ts}*-S5*	*MAT***a** *ade1 his3 leu2 lys1 URA3*::*nsp1*^{ts}*-S5*
los1-Δ	*MAT*α *ade2 leu2 ura3 his3 trp1 CAN1-100 los1*::*HIS3*
los2-Δ	*MAT***a** *ade2 leu2 ura3 his3 trp1 CAN1-100 los2*::*HIS3*
nup100-Δ	*MAT***a** *ade2 leu2 ura3 his3 trp1 CAN1-100 nup100*::*LEU2*
nup116-Δ	*MAT*α *ade2 leu2 ura3 his3 trp1 CAN1-100 nup116*::*URA3*
NUP49^{+}	*MAT*α *ade2 ade3 his3 leu2 lys2 ura3 nup49*::*TRP1* (pCH1122-ADE3-URA3-NUP49)
nup49-313	*MAT*α *ade2 ade3 his3 leu2 lys2 ura3 nup49*::*TRP1* (pUN90-NUP49-313)
nup49-316	*MAT*α *ade2 ade3 his3 leu2 lys2 ura3 nup49*::*TRP1* (pUN90-nup49-316)
GAL10::Prot	*MAT***a** *ade2 leu2 his3 trp1 ura3 nup145*::*HIS3*
A-*NUP145*	*CAN1-100* (pUN100-LEU2-GAL10::ProtA-NUP145)
pop1-1	*MAT***a** *ade2 arg4*-Δ *leu2-3,112 ura3-52 trp1-289 GAL*-U4:: *TRP pop1-1*

The effects of mutations in nucleoporins, and other proteins, on nuclear-cytoplasmic transport of poly(A)$^{+}$ RNA has been studied by in situ hybridization using oligo(dT) probes (1, 23). This approach is less readily used to study the localization of the stable cytoplasmic RNAs, tRNA and rRNA. The long lifetimes of these RNAs mean that even in strains inhibited for transport, a strong residual cytoplasmic signal is expected. We therefore screened all of the then available nucleoporin mutants for their effects on pre-tRNA splicing and pre-rRNA processing as indicators of defects in tRNA and rRNA transport. We report that a subset of nucleoporin mutations do indeed impair pre-tRNA splicing.

MATERIALS AND METHODS

Strains and media. The genotypes of the strains used are shown in Table 1. The *los1*-Δ, *los2*-Δ (42a), *nsp1-L→S* (53), *nup1*-Δ (39), *nup85*-Δ (42a), *nup100*-Δ (11), *nup116*-Δ (11), *nup133*-Δ (10), and *GAL*::*nup145* (11) strains, as well as the wild-type control, are haploid progeny derived from strain RS453 and are therefore expected to be isogenic. Exceptions are the *nup49* (10), *nsp1-S5* (39), and *pop1-1* strains (27). For *NUP49*, an isogenic strain carrying the wild-type *NUP49* gene on a plasmid (strain *NUP49*$^{+}$ in Table 1) (10) was also analyzed for pre-tRNA processing defects and is shown as the control strain in Fig. 5A. The *nsp1-S5* strain shows little alteration in pre-tRNA processing, and the RNA from the *pop1-1* strain was used solely as an aid to the characterization of the pre-tRNA species.

Cells were grown in YPD or YPGal medium or plates containing 1% yeast extract, 2% Bacto Peptone, 2% glucose, or 2% galactose or selective medium or plates complemented with the appropriate nutrients (SD or SGal [2% glucose or galactose, 0.7% yeast nitrogen base plus nutrients]) (41). For Nup145p depletion, cells growing exponentially in galactose minimal medium at 30°C were harvested by centrifugation, washed, and resuspended in glucose minimal medium. Other strains were pregrown at 25°C to early log phase and then transferred to 37°C. During growth, cells were diluted with prewarmed medium and constantly maintained in early exponential phase.

RNA extraction and Northern (RNA) hybridization. Exponentially growing cells (approximately 5×10^8 cells) were quickly chilled by addition of crushed ice to the culture medium. Cells were harvested by centrifugation at 4°C, washed with ice-cold water, centrifuged again, and frozen at −80°C. Frozen cell pellets were resuspended in 0.5 ml of 4 M guanidine thiocyanate (GTC) prepared as described previously (28) and 0.5 ml of phenol. Cells were lysed by vortexing at 4°C for 5 min with 1 ml of glass beads (0.45- to 0.5-mm diameter). A further 7.5 ml of GTC and 7.5 ml of phenol were added, and the samples were incubated at 65°C for 5 min. Eight milliliters of chloroform and 4 ml of 100 mM sodium acetate were added, and the aqueous phase was recovered after centrifugation. Following two further extractions with phenol-chloroform-isoamyl alcohol, RNA was recovered by ethanol precipitation. For analysis of low-molecular-weight species, RNA was separated by migration for 1,600 V · h on a 20-cm 10% polyacrylamide gel containing 8.3 M urea and 1× Tris-borate-EDTA (TBE) (28) and electroblotted to a Hybond N^{+} membrane (Amersham International, Amersham, United Kingdom) with 0.5× TBE as the transfer buffer. For the analysis of high-molecular-weight species, RNA was separated on 1.2% agarose gels containing formaldehyde as described previously (45) and transferred to a Hybond N^{+} membrane (Amersham) with 10× (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) as the transfer buffer. Northern hybridization was performed in 6× SSPE–0.5% sodium dodecyl sulfate–5× Denhardt's solution (28) at 37°C; filters were washed in 6× SSPE at 8°C below the calculated melting temperature. For Northern hybridization, 4 μg of total RNA was used for each sample. Oligonucleotide hybridization probes used are listed in Table 2.

Metabolic labeling. Metabolic labeling of RNA was performed as described previously (46). Cultures of the *GAL*::*nup145* strain growing exponentially in galactose minimal medium were harvested, washed, and resuspended in prewarmed glucose minimal medium. Cultures of the *NUP49* and *nup49-313* strains growing exponentially in glucose minimal medium at 23°C were transferred to 37°C. The cells were subsequently maintained in an early exponential phase by dilution with prewarmed medium. Metabolic labeling was carried out 8 h after transfer. Four milliliters of cells (optical density at 600 nm of ~0.3) was labeled for 1 min with 133 μCi of prewarmed [^{3}H]uracil. After this time, prewarmed, unlabeled uracil was added to a final concentration of 5 mM. Samples of 0.8 ml were taken 0, 1.5, 4, and 9 min after addition of unlabeled uracil. Cells were rapidly harvested by centrifugation for 8 s in an Eppendorf centrifuge, the supernatant was discarded, and the cell pellet was frozen by transferring the tube to a dry ice-ethanol bath. Harvesting takes 1 min, which is added to the chase times. RNA was extracted from the frozen pellets as described above except that all volumes were reduced 10-fold. The entire RNA recovered was separated by migration for 1,600 V · h on a 20-cm 10% polyacrylamide gel containing 8.3 M urea and 1× TBE. RNA was electroblotted to a GeneScreen Plus membrane (Dupont, Boston, Mass.) with 0.5× TBE as the transfer buffer. Following transfer, the membrane was sprayed with En^{3}Hance (Dupont) and exposed to film at −80°C.

RESULTS

The nucleoporins analyzed in this study include both essential and nonessential species. For the nonessential nucleoporins, *NUP1*, *NUP85*, *NUP100*, *NUP116* and *NUP133* gene disruption constructs were analyzed. Of these, deletion of *NUP116* (11, 51) or *NUP133* (10), or insertional disruption of *NUP85* (42a), gives rise to a temperature-sensitive (*ts*) growth phenotype. The original deletion of *NUP1* was nonconditionally lethal (9), but in the strain used here, the *nup1*-Δ mutation confers a slow-growth phenotype at all temperatures and is *ts* lethal (39). Deletion of *NUP100* (11, 50) is not associated with a growth defect. *NSP1*, *NUP49*, and *NUP145* are essential for viability. Two *ts*-lethal alleles of *NSP1*, *nsp1*^{ts}*-S5* (31) and

TABLE 2. Oligonucleotides used

Name	DNA sequence (5′→3′)
α$tRNA^{Trp}_{CCA}$ mature	AAC CTG CAA CCC TTC GA
α$tRNA^{Pro}_{UGG}$ mature	ACC CAG GGC CTC TC
α$tRNA^{Ala}_{UGC}$ mature	CTA CCA ACT GCG CCA TG
α$tRNA^{Gly}_{GCC}$ mature	TAC CAC TAA ACC ACT TGC
α$tRNA^{Leu}_{CAA}$ mature	GCA TCT TAC GAT ACC TG
α$tRNA^{Phe}_{GAA}$-int	TTG ACC GAA GTA TTT C
α$tRNA^{Ile}_{UAU}$-int	AGG CCT GTT TGA AAG GTC
α$tRNA^{Trp}_{CCA}$-int	CTT ATT CCG TGG AAT TTC C
α$tRNA^{Lys}_{UUU}$-int	CCT TGC TTA AGC AAA TGC
α$tRNA^{Pro}_{UGG}$-int	TGT CTT CCT GTT TAA TCA
α$tRNA^{Ser}_{CGA}$-int	AGC GAA CTT TTT TAT TCC A
α$tRNA^{Ser}_{GCU}$-int	ATT GCT TTT CTG AGG A
α$tRNA^{Leu}_{UAG}$-int	AAT TTA GAG GTT AAA TCC A
α$tRNA^{Tyr}_{G\Psi A}$-int	AAG ATT TCG TAG TGA TAA
α20S-2	AGC TCT CAT GCT CTT GCC
αrna2.1	GGC CAG CAA TTT CAA GTT A
α18S	CAT GGC TTA ATC TTT GAG AC
α25S+40	CTC CGC TTA TTG ATA TGC
α5S	CTA CTC GGT CAG GCT C

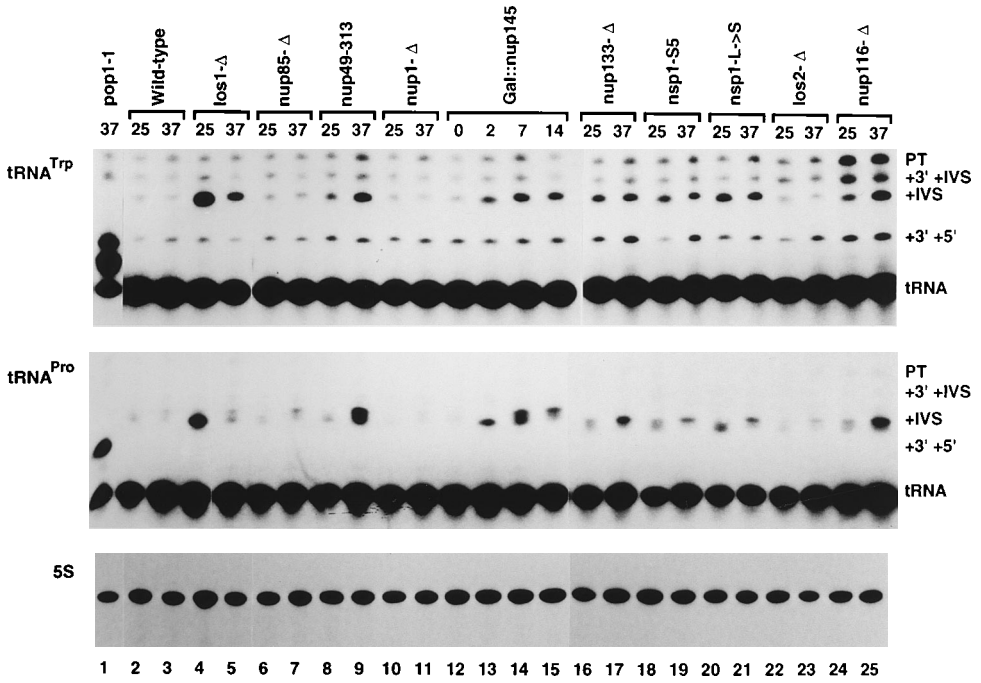

FIG. 1. Pre-tRNA splicing in a panel of nucleoporin mutant strains. Lane 1, *pop1-1* strain following growth at 37°C for 6 h; lane 2, wild-type strain grown at 25°C; lane 3, wild-type strain grown at 37°C; lane 11, *GAL*::*nup145* strain grown on galactose medium; lanes 12 to 15, *GAL*::*nup145* strain grown on glucose medium for 2, 7, and 14 h, respectively. Other lanes show the mutant strains indicated following growth at 25°C or after 8 h at 37°C. RNA was extracted and separated by polyacrylamide gel electrophoresis. Duplicate filters were hybridized with probes specific for mature $tRNA^{Trp}$ (upper panel) or $tRNA^{Pro}$ (center panel). The positions of the pre-tRNA primary transcripts (PT), the 5′ processed pre-tRNAs (+3′ +IVS [intervening sequence]), the 5′ and 3′ processed but nonspliced pre-tRNAs (+IVS), the spliced but 5′ and 3′ nonprocessed pre-tRNAs (+3′ +5′), and the mature tRNAs (tRNA) are shown. As a control for RNA recovery, the level of 5S rRNA is also shown (lower panel).

nsp1^{ts}*-L→S* (53), and two *ts*-lethal alleles of *NUP49*, *nup49-313* and *nup49-316* (10), were tested. Pre-tRNA and pre-rRNA processing was assessed in these strains, and in the deletion mutants, following growth at 23°C and 8 h after transfer to 37°C. For *NUP145*, a construct in which the *NUP145* promoter is replaced by the *GAL10* promoter (*GAL*::*nup145*) was used (11). Growth of this strain on glucose medium represses the *GAL* promoter, leading to depletion of Nup145p. Pre-tRNA and other RNA processing reactions were assessed during growth on galactose medium and at times after transfer to glucose medium.

As a positive control for pre-tRNA accumulation, a *los1*-Δ strain (42a) was analyzed (Fig. 1 and 2, lanes 4 and 5). Mutations in *LOS1* have previously been reported to result in the accumulation of unspliced pre-tRNAs (16). RNA extracted from a *pop1-1* strain following growth at 37°C was also analyzed (Fig. 1 and 2, lanes 1). Pop1p is a component of RNase P (27), which cleaves the 5′ end of pre-tRNAs. In *pop1-1* strains, pre-tRNAs which are spliced but 5′ and 3′ unprocessed accumulate, while the unspliced pre-tRNAs are specifically underaccumulated. The absence of the unspliced pre-tRNA species from the *pop1-1* strain confirms their identification.

Ten families of intron-containing pre-tRNAs have been identified in *S. cerevisiae*; probes specific for the mature $tRNA^{Pro}_{UGG}$, $tRNA^{Trp}_{CCA}$, and $tRNA^{Leu}_{CAA}$ ($tRNA^{Leu}_{3}$) sequences were used, as were probes to the introns of $tRNA^{Phe}_{GAA}$, $tRNA^{Ile}_{UAU}$, $tRNA^{Trp}_{CCA}$, $tRNA^{Lys}_{UUU}$, $tRNA^{Pro}_{UGG}$, $tRNA^{Ser}_{CGA}$, $tRNA^{Ser}_{GCU}$, $tRNA^{Leu}_{UGA}$, and $tRNA^{Tyr}_{G\Psi A}$. Figure 1 shows hybridization with probes specific for mature $tRNA^{Pro}_{UGG}$ and $tRNA^{Trp}_{CCA}$. Among the nucleoporin mutants, strongest accumulation of pre-tRNA was observed in strains carrying a deletion of *LOS1* (Fig. 1, lanes 4 and 5), in strains carrying the *ts nup49-313* mutation at the nonpermissive temperature (37°C) (Fig. 1, lane 9), and in strains carrying the *GAL*::*nup145* mutation after transfer to glucose medium (Fig. 1, lanes 13 to 15). A slightly weaker effect was seen in strains deleted of *NUP116* (Fig. 1, lanes 24 and 25) following transfer to the nonpermissive temperature (37°C). For several tRNA species, accumulation of the primary transcript was seen in the *nup116*-Δ strain but not in other mutants. The significance of this finding is not clear. Strains carrying other mutations, *nup133*-Δ (Fig. 1, lanes 16 and 17) or either *nsp1* allele (Fig. 1, lanes 18 to 21), also showed some accumulation of pre-tRNA. In contrast, no pre-tRNA accumulation was observed for strains carrying *nup85*-Δ (Fig. 1, lanes 7 and 8), *nup1*-Δ (Fig. 1, lanes 10 and 11), or *nup100*-Δ (data not shown). The *los2*-Δ strain (Fig. 1, lanes 22 and 23) has a deletion of a nonessential gene identified on the basis of genetic interaction with *LOS1* (42a); no accumulation of pre-tRNA was seen in this strain. In all strains, some shift in the mobility of pre-$tRNA^{Pro}$ is seen at 37°C; we assume that this is due to incomplete 3′ processing of the pre-tRNA, but this possibility has not been further investigated. As a control for recovery, the mutants were analyzed for the level of 5S rRNA (Fig. 1). No significant differences were observed, indicating that the variations in the levels of pre-tRNAs are not due to differences in RNA extraction.

Duplicate filters carrying RNA extracted from all of the nucleoporin mutants were probed for all 10 families of intron-containing pre-tRNAs. For simplicity and ease of comprehension, we show the remaining pre-tRNAs only for the *nup116*-Δ, *nup49-313*, and *GAL*::*nup145* strains, together with the wild-type, *los1*-Δ, and *pop1-1* control strains, in Fig. 2. Accumulation of all 10 families of intron-containing pre-tRNAs was observed. The degree of accumulation is, however, not the

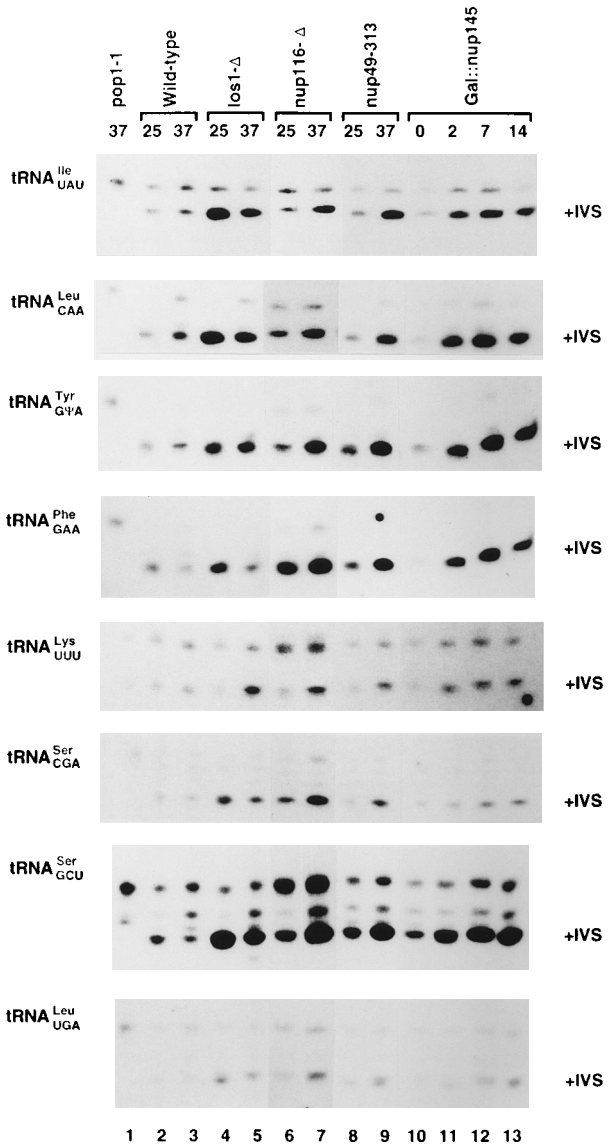

FIG. 2. Pre-tRNA splicing in *nup49*, *nup116*, and *nup145* mutant strains. Lane 1, *pop1-1* strain following growth at 37°C for 6 h; lane 2, wild-type strain grown at 25°C; lane 3, wild-type strain grown at 37°C; lane 4, *los1*-Δ strain grown at 25°C; lane 5, *los1*-Δ strain grown at 37°C; lane 6, *nup116*-Δ strain grown at 25°C; lane 7, *nup116*-Δ strain grown at 37°C; lane 8, *nup49-313* strain grown at 25°C; lane 9, *nup49-313* strain grown at 37°C; lane 10, *GAL*::*nup145* strain grown on galactose medium; lanes 11 to 13, *GAL*::*nup145* strain grown on glucose medium for 2, 7, and 14 h, respectively. RNA was extracted and separated by polyacrylamide gel electrophoresis. Duplicate filters were hybridized with probes specific for the introns of tRNAs indicated. The positions of the 3′ and 5′ processed but unspliced pre-tRNAs (+IVS [intervening sequence]) are indicated. The lanes shown are selected regions of the same filters as used for Fig. 1.

same for all pre-tRNAs. The original *los1-1* allele was selected to be *ts* defective in the splicing of pre-tRNA^{Tyr} and is reported to be generally *ts* for pre-tRNA accumulation (16). However, complete deletion of the *LOS1* gene resulted in stronger accumulation of pre-tRNA at 25 than at 37°C for most pre-tRNAs, although pre-tRNA^{Tyr} and pre-tRNA^{Lys} accumulated to higher levels at 37°C. Other nucleoporin mutants, strains carrying both *nsp1* alleles and *nup133*-Δ strains, showed low levels of accumulation for all species of pre-tRNA; the *nsp1^{ts}-L→S* mutant showed stronger pre-tRNA accumulation than the *nsp1^{ts}-S5* strain. In contrast, no accumulation of any pre-tRNA is observed for the *nup1*-Δ, *nup85*-Δ, and *nup100*-Δ strains (data not shown). In addition, the mutants were analyzed with probes specific for the nonspliced tRNAs, tRNA$^{Ala}_{UGC}$ and tRNA$^{Gly}_{GCC}$; no differences were observed with probes specific for nonspliced pre-tRNAs (data not shown).

A number of other RNA species were also analyzed. The small nuclear RNAs U4 and U6 probably associate in the cytoplasm and therefore require nuclear export for their association, while the small nucleolar RNAs snR190 and U14 are excised from larger transcripts by posttranscriptional processing (3, 35a). No clear differences in the steady-state levels of these RNAs were detected. The splicing of the pre-mRNAs for actin and ribosomal protein S10 was also examined for all nucleoporin mutants; no accumulation of the pre-mRNAs was observed (data not shown).

In addition, the synthesis and processing of pre-rRNA and rRNA species were analyzed for all nucleoporin mutants (shown for the *nup49-313*, *nup116*-Δ, and *GAL*::*nup145* strains in Fig. 3). No clear differences in the levels of mature 5.8S, 18S, or 25S rRNA were observed. No strong effects on pre-rRNA processing or accumulation of mature rRNA were observed for any mutant. Mild accumulation of the 35S pre-rRNA primary transcript was observed in the strains shown in Fig. 3 and in both the *nsp1^{ts}-S5* and *nsp1^{ts}-L→S* mutants (data not shown) but not in other strains. In the Northern blot shown in Fig. 3, some reduction in mature 18S rRNA is apparent in the *nup116*-Δ strain; this was not observed on ethidium staining of five other gels.

To further characterize the pre-tRNA splicing defect of the *nup49-313* and *GAL*::*nup145* mutations, pulse-chase labeling was performed (shown for *nup49-313* in Fig. 4). Cells were labeled with [^{3}H]uracil for 1 min and then chased with a large excess of unlabeled uracil for 1, 2.5, 5, or 10 min. In the *nup49-313* strain, some increase in the level of RNA species with the gel mobility expected for unprocessed pre-tRNAs can be seen. As only approximately 10% of pre-tRNAs are spliced, a larger effect is not to be expected. A similar but less marked effect was seen for the *GAL*::*nup145* strain following a transfer to glucose medium (data not shown).

The increase in pre-tRNA observed on Northern hybridization might have been due either to a delay in the processing of the spliced pre-tRNA population as a whole or to the synthesis of a low level of dead-end intermediates which accumulate over time. The detection of unspliced pre-tRNAs after short labeling times indicates that it is the splicing of the bulk of pre-tRNAs which is inhibited.

The time course for the *GAL*::*nup145* strain indicates that the accumulation of pre-tRNA occurs relatively quickly after a shift to nonpermissive conditions (Fig. 1 and 2). To determine whether this is also the case for *nup49-313* and *nup116*-Δ strains, the time course of pre-tRNA accumulation was analyzed following a transfer to 37°C. Clear accumulation of pre-tRNA^{Pro} was seen 1 h after the temperature shift (Fig. 5A, lower panel), while clear accumulation of pre-tRNA^{Trp} (Fig. 5A, upper panel), pre-tRNA^{Tyr}, and pre-tRNA^{Leu} (data not shown) was seen 3 h after the shift. In the *nup116*-Δ strain, accumulation of pre-tRNA^{Pro} (Fig. 5B, upper panel) and pre-tRNA^{Trp} (Fig. 5B, lower panel) is detected 3 h after the temperature shift. These relatively rapid effects make it less likely that the pre-tRNA splicing defect is a secondary effect of impaired protein import in the nucleoporin mutants.

The effects of two different alleles of *nup49* on protein import and nuclear poly(A)$^+$ RNA accumulation have been reported (10). The *nup49-313* allele has a stronger protein im-

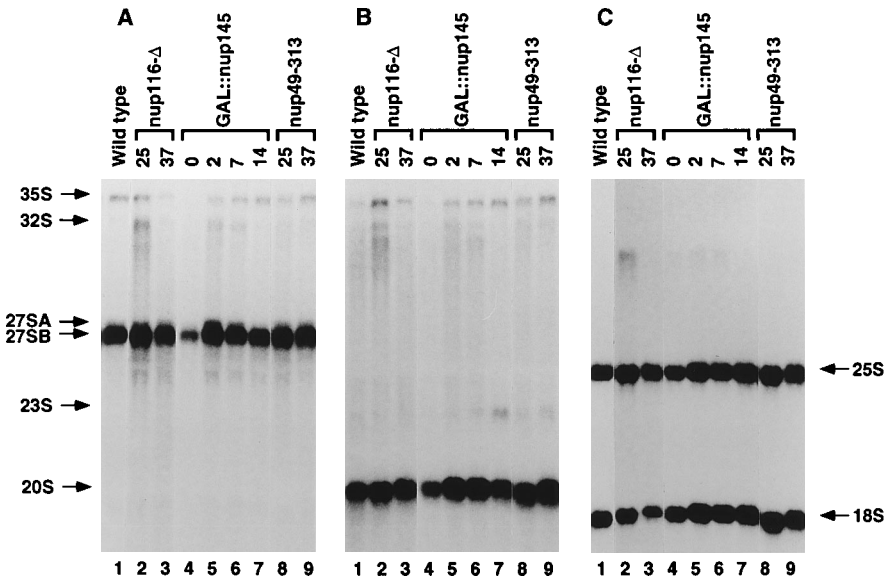

FIG. 3. Processing of pre-rRNA in nucleoporin mutants. Lane 1, wild-type strain grown at 30°C; lane 2, *nup116*-Δ strain grown at 25°C; lane 3, *nup116*-Δ strain grown at 37°C; lane 4, *GAL*::*nup145* strain grown on galactose medium; lanes 5 to 7, *GAL*::*nup145* strain grown on glucose medium for 2, 7, and 14 h, respectively; lane 8, *nup49-313* strain grown at 25°C; lane 9, *nup49-313* strain grown at 37°C. RNA was extracted, separated by electrophoresis on agarose-formaldehyde gels, and analyzed by Northern hybridization. (A) Hybridization with a probe specific for the 5′ region of internal transcribed spacer 1 (probe α20S-2); (B) hybridization with a probe specific for the 5′ region of internal transcribed spacer 2 (probe αrna2.1); (C) hybridization with probes specific for the mature 18S and 25S rRNAs. The positions of the major 35S, 32S, 27SA, 27SB, and 20S pre-rRNAs as well as the mature 25S and 18S rRNAs are indicated. Also indicated is the position of the 23S pre-rRNA, which accumulates in many pre-rRNA processing mutants, including those defective in ribosomal protein synthesis. RNA samples are the same as those used for Fig. 1 and 2.

port defect (10), while the *nup49-316* allele has a stronger effect on nuclear poly(A)$^+$ accumulation. As shown in Fig. 6, the *nup49-313* allele (Fig. 6, lane 6) also leads to a much stronger accumulation of pre-tRNA than does the *nup49-316* allele (Fig. 6, lane 8). Both alleles are fully *ts* for the pre-tRNA processing defect, with no pre-tRNA accumulation evident after growth at 25°C (Fig. 6, lanes 5 and 7). For $tRNA^{Pro}$, the differences in pre-tRNA accumulation are not clearly visible at an exposure which shows the level of the primary transcript (Fig. 6, middle panel), and a shorter exposure is therefore also shown (Fig. 6, lower panel). Thus, while Nup49p is required for both poly(A)$^+$ export and pre-tRNA splicing, these effects are partially separable by mutation.

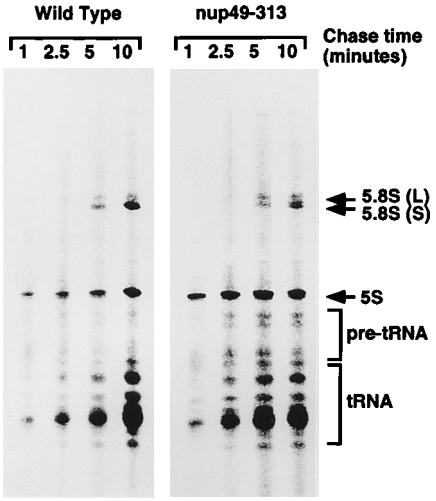

FIG. 4. Metabolic labeling of pre-tRNA in a *nup49-313* strain. The wild-type and *nup49-313* strains were analyzed 8 h after a transfer to 37°C. Strains were labeled for 1 min with [^{3}H]uracil and then chased with a large excess of unlabeled uracil for 1, 2.5, 5, and 10 min. RNA was recovered, separated by polyacrylamide gel electrophoresis, transferred to a GeneScreen Plus membrane, and visualized by fluorography. The positions of the 5.8S and 5S rRNAs, pre-tRNA, and the mature tRNAs are indicated.

DISCUSSION

We have tested the effects of a number of conditional mutations in nuclear pore complex components for their effects on the processing of pre-tRNA. Strains mutant for a subset of the nucleoporins tested, Nup49p, Nup145p, and Nup116p, show strong accumulation of unspliced pre-tRNAs, and for these the splicing defect is observed for all 10 families of intron-containing pre-tRNAs. Strains mutant for other nucleoporins, Nup133p and Nsp1p, show weaker accumulation of unspliced pre-tRNA. These defects are not simply due to general impairment of either growth or transport. Strains carrying an insertional disruption of the gene encoding Nup85p are *ts* for growth (42a), while the strain lacking Nup1p grows slowly at all temperatures and is *ts* lethal (39); however, neither of these strains shows pre-tRNA accumulation. In contrast, the strain lacking Los1p is not detectably impaired in growth but shows strong accumulation of pre-tRNA. The processing of pre-tRNA therefore joins protein import and nuclear poly(A)$^+$ accumulation as features for which future nucleoporin mutants can usefully be screened.

For the mutants showing strong accumulation of pre-tRNA, the *GAL*::*nup145*, *nup49-316*, and *nup116*-Δ mutants, the kinetics of the appearance of the accumulation of unspliced pre-tRNAs was assessed following a transfer to nonpermissive conditions. In the *GAL*::*nup145* mutant, the pre-tRNA splicing defect is detected 2 h after a transfer to glucose medium. In the *nup49-313* and *nup116*-Δ mutants, the defect can be detected 1 and 3 h, respectively, after the transfer to 37°C. The effects of these mutations on nuclear protein import are detected only at

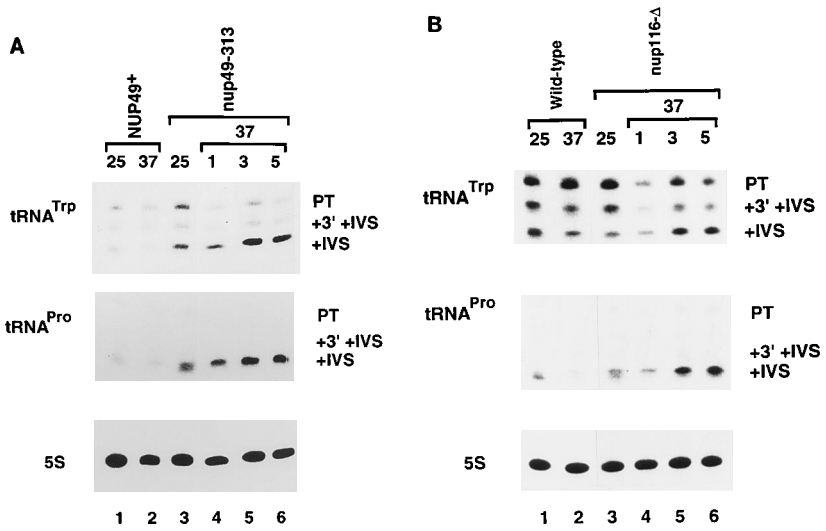

FIG. 5. (A) Time course of pre-tRNA splicing in a *nup49-313* strain. Lane 1, wild-type strain grown at 25°C; lane 2, wild-type strain grown at 37°C; lane 3, *nup49-313* strain grown at 25°C; lanes 4 to 6, *nup49-313* strain grown at 37°C for 1, 3, and 5 h, respectively. RNA was extracted and separated by polyacrylamide gel electrophoresis. The filter was hybridized successively with probes specific for the introns of $tRNA^{Trp}$ (upper panel) and $tRNA^{Pro}$ (center panel). As a control for RNA recovery, the level of 5S rRNA is also shown (lower panel). (B) Time course of pre-tRNA splicing in a *nup116*-Δ strain. Lane 1, wild-type strain grown at 25°C; lane 2, wild-type strain grown at 37°C; lane 3, *nup116*-Δ strain grown at 25°C; lanes 4 to 6, *nup116*-Δ strain grown at 37°C for 1, 3, and 5 h, respectively. RNA was extracted and separated by polyacrylamide gel electrophoresis. The filter was hybridized successively with probes specific for the introns of $tRNA^{Trp}$ (upper panel) and $tRNA^{Pro}$ (center panel). As a control for RNA recovery, the level of 5S rRNA is also shown (lower panel). Designations at the right of each panel are as in Fig. 1.

later time points (10, 11). This finding makes it unlikely that the accumulation of unspliced pre-tRNA is a secondary effect of the inhibition of protein import in the nucleoporin mutants. These mutants are probably delayed but not blocked in pre-tRNA splicing, since the accumulation of pre-tRNA is not accompanied by a reduction in the level of the mature tRNAs. Depletion of the mature tRNAs would, however, have to be by growth, and the simultaneous inhibition of tRNA synthesis and growth might not result in clear depletion.

In contrast to the effects on pre-tRNA processing, no strong effects on pre-rRNA processing or accumulation of mature rRNA were observed for any mutant. This result is unexpected, since pre-rRNA processing is very dependent on the continued synthesis and nuclear import of ribosomal proteins. Several of the mutants tested are reported to be defective in the nuclear import of reporter constructs. Import of these proteins would appear to be substantially more sensitive to NPC defects than is import of ribosomal proteins. It is striking that none of the nucleoporin mutants tested appears to result in a complete block in the process of protein import.

The nucleoporins in which mutations strongly inhibit pre-tRNA splicing have other features in common. *nup49*, *nup116*, and *nup145* mutants have all been reported to accumulate poly(A)$^+$ RNA in the nucleus, indicating a defect in nuclear export of mRNA (10, 11, 51). Nup49p, Nup116p, and Nup145p are all members of the GLFG family of nucleoporins (11, 50, 53). Moreover, Nup116p and Nup145p have also been reported to share a novel RNA-binding domain, the nucleoporin RNA-binding motif (11). It is possible that this domain is involved in binding both nuclear poly(A)$^+$ RNA and pre-tRNA.

Pre-tRNA splicing is likely to be a nuclear event. Nuclear injection experiments with *Xenopus* oocytes show that splicing occurs prior to transport to the cytoplasm (29), and the yeast splicing machinery is also located in the nucleus (7, 35). Circumstantial evidence supports a connection between pre-tRNA splicing and nuclear-cytoplasmic export. Rna1p is the GTPase-activating protein for the GTPase Ran1p (designated

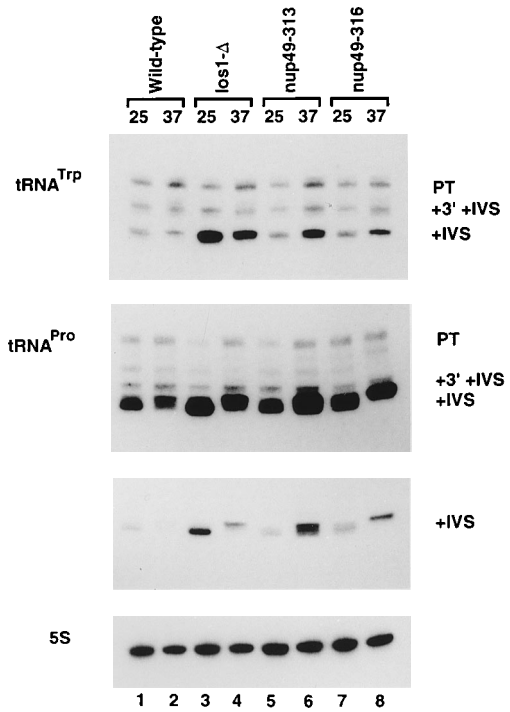

FIG. 6. Comparison of pre-tRNA splicing in *nup49-313* and *nup49-316* mutant strains. Lane 1, wild-type strain grown at 25°C; lane 2, wild-type strain grown at 37°C; lane 3, *los1*-Δ strain grown at 25°C; lane 4, *los1*-Δ strain grown at 37°C; lane 5, *nup49-313* strain grown at 25°C; lane 6, *nup49-313* strain grown at 37°C; lane 7, *nup49-316* strain grown at 25°C; lane 8, *nup49-316* strain grown at 37°C. RNA was extracted and separated by polyacrylamide gel electrophoresis. Duplicate filters were hybridized with probes specific for the introns of $tRNA^{Trp}$ (upper panel) and $tRNA^{Pro}$ (center panels). For $tRNA^{Pro}$, the differences in pre-tRNA accumulation are not clearly visible at an exposure which shows the level of the primary transcript, and a shorter exposure is therefore also shown. As a control for RNA recovery, the level of 5S rRNA is also shown (lower panel). Designations at the right are as in Fig. 1.

Gsp1p/Cnr1p and Gsp2p/Cnr2p in *S. cerevisiae*) (4, 5, 22), which is implicated in the nuclear-cytoplasmic transport of many components (for recent reviews, see references 12 and 19). Mutants in *RNA1* accumulate unspliced pre-tRNAs (15, 24) and are also defective in nuclear-cytoplasmic transport of poly(A)$^+$ RNA (1, 18, 42). Although the unspliced pre-tRNAs have not been demonstrated to accumulate in the nucleus, this finding suggests that the splicing defect is a consequence of the inhibition of nuclear-cytoplasmic transport. The yeast splicing endonuclease is an integral nuclear membrane protein (35). Immunogold labeling indicates that the tRNA ligase is localized both in the nucleoplasm close to the nuclear membrane and on the inner nuclear membrane and is enriched in regions close to NPCs (7). A model in which intron-containing pre-tRNAs are bound by the ligase in the nucleoplasm has been proposed (7, 32, 43; reviewed in reference 52). This complex then associates with the endonuclease in the nuclear membrane. Subsequently, the pre-tRNA–ligase–endonuclease complex may become associated with the NPC, possibly by diffusion within the nuclear membrane. Consistent with this model, the tRNA ligase can be cross-linked to intron sequences, even though the substrate for the ligation reaction is devoid of the intron (43). Splicing would then occur, followed by transport of the mature tRNA to the cytoplasm. The delay in pre-tRNA splicing observed in the nucleoporin mutants might be due either to inhibition of the interaction between the tRNA splicing complex and the NPC or to sequestration of the splicing components at the NPC following splicing. Interestingly, the number of splicing components (~400 ligase and ~100 endonuclease molecules per cell) (36, 37) is close to the estimated number of NPCs in yeast cells (21, 30), and thus sequestration is a real possibility. Alternatively, this observation may suggest that each NPC is associated with one pre-tRNA splicing complex.

A number of mutations which result in the accumulation of unspliced pre-tRNAs in vivo but are not defective in pre-tRNA splicing in vitro have been identified. These genes, *PTA1* (33), *TPD1* (48), *STP1* (49), and *LOS1* (16), presumably do not encode enzymatic components of the splicing endonuclease or ligase. The accumulation of unspliced pre-tRNAs reported for strains carrying these mutations appears similar to that observed in the nucleoporin mutants analyzed here. *TPD1* encodes a protein phosphatase (38), while Los1p behaves like a nuclear matrix protein (40) and has recently been shown to be localized at the NPC (42a). It is possible that other genes previously identified as defective in pre-tRNA splicing also encode nucleoporins.

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