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tRNA Nuclear Export in *Saccharomyces cerevisiae*: In Situ Hybridization Analysis

Srimonti Sarkar and Anita K. Hopper*

Department of Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

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To understand the factors specifically affecting tRNA nuclear export, we adapted in situ hybridization procedures to locate endogenous levels of individual tRNA families in wild-type and mutant yeast cells. Our studies of tRNAs encoded by genes lacking introns show that nucleoporin Nup116p affects both poly(A) RNA and tRNA export, whereas Nup159p affects only poly(A) RNA export. Los1p is similar to exportin-t, which facilitates vertebrate tRNA export. A *los1* deletion mutation affects tRNA but not poly(A) RNA export. The data support the notion that Los1p and exportin-t are functional homologues. Because *LOS1* is nonessential, tRNA export in vertebrate and yeast cells likely involves factors in addition to exportin-t. Mutation of *RNA1*, which encodes RanGAP, causes nuclear accumulation of tRNAs and poly(A) RNA. Many yeast mutants, including those with the *rna1-1* mutation, affect both pre-tRNA splicing and RNA export. Our studies of the location of intron-containing pre-tRNAs in the *rna1-1* mutant rule out the possibility that this results from tRNA export occurring before splicing. Our results also argue against inappropriate subnuclear compartmentalization causing defects in pre-tRNA splicing. Rather, the data support "feedback" of nucleus/cytosol exchange to the pre-tRNA splicing machinery.

INTRODUCTION

Nucleus/cytosol exchange of macromolecules is a complicated process requiring participation of "shared" gene products affecting exchange of many types of macromolecular cargo, as well as participation of "specific" gene products affecting a subset of the types of cargo. Generally, karyophilic proteins are imported into the nuclear interior, and newly synthesized RNAs are exported out to the cytosol, but some macromolecules pass in both directions. Entry and exit proceed through the same nuclear pores (Dworetzky and Feldherr, 1988). Nuclear pores are large supramolecular complexes comprising ~50–100 separate proteins, called nucleoporins, that span the nuclear inner and outer membranes, creating an aqueous channel (for review see Fabre and Hurt, 1997; Ohno *et al.*, 1998). Small molecules can diffuse through the pore, but most macromolecules are transported by an energy-requiring, signal-mediated process.

* Corresponding author. E-mail address: ahopper@psu.edu.

Nucleus/cytosol exchange seems to require a specific category of exchange components, called importin-β proteins, which are instrumental in docking of the cargo to the nuclear pore. The prototype of this family, importin-β, was identified as a cytoplasmic receptor for the nuclear localization signal containing karyophilic proteins (for review see Izaurralde and Adam, 1998; Ohno *et al.*, 1998). Subsequent members of this family of receptors have been demonstrated to have different substrate specificity (Rout *et al.*, 1997; Schlenstedt *et al.*, 1997). RNA export, like protein import, requires the participation of receptors or exportins that are members of the importin-β family. Export of particular types of RNAs, i.e., tRNA, small nuclear RNA (snRNA), rRNA, and mRNA, is competed by an excess of that RNA type. However, the excess does not inhibit the export of other RNA types, indicating existence of limiting quantities of species-specific transit factors (Jarmolowski *et al.*, 1994). Recent data on the roles of particular RNA binding proteins such as cap binding proteins, necessary for snRNA export, and hnRNP proteins, important for mRNA export, support

the concept of RNA species-specific export pathways (Izaurralde and Adam, 1998). Some of the RNA binding proteins participating in the export process possess leucine-rich nuclear export sequences recognized by importin-β family member Crm1p/Xpo1p. Crm1p/Xpo1p has been shown to function as an exportin for the leucine-rich nuclear export sequence containing nucleus-localized proteins and to affect mRNA export to the cytosol (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Kudo *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997; Ohno *et al.*, 1998). Recently, another importin-β-like protein, exportin-t, has been proposed to serve as a tRNA-specific receptor for tRNA export in *Xenopus* and human cells by binding tRNA directly (Arts *et al.*, 1998; Kutay *et al.*, 1998).

Nucleus/cytosol exchange also requires participation of a small GTPase, Ran, and at least four proteins that regulate its GTP- or GDP-bound states. Although the role(s) of the Ran cycle in nucleus/cytosol exchange is not completely understood, several lines of evidence support the model that RanGDP/GTP exchange functions to release imported cargo from import receptors, and conversely, RanGTP hydrolysis functions to release exported cargo from export receptors (Izaurralde and Adam, 1998; Ohno *et al.*, 1998). Although a functional Ran cycle is required for translocation of most RNAs (Izaurralde *et al.*, 1997), export of the yeast heat shock mRNAs is apparently independent of the Ran cycle (Saavedra *et al.*, 1996).

Our studies focus on tRNA biogenesis including those gene products necessary for pre-tRNA processing and export of the mature tRNAs to the cytosol. Yeast pre-tRNAs differ from their mature counterparts by possession of extra sequences located at the 5′ and 3′ extremities and, for ~25% of tRNA families, by the presence of intron sequences located one nucleotide 3′ to the anticodon. Pre-tRNAs also lack numerous nucleoside modifications that are present on the mature tRNAs, posttranscriptionally added CCA nucleotides located at the 3′ end and sometimes a G located at the 5′ terminus (for review see Hopper and Martin, 1992). Although there appears to be a preferred order of processing steps, genetic studies and molecular analyses show that most of the steps are not in an obligatory order (Hopper *et al.*, 1982; Martin and Hopper, 1982; O'Connor and Peebles, 1991; Hopper and Martin, 1992). Some order to the eukaryotic processing pathway may be imposed by the subcellular distribution of the processing activities, because particular processing activities such as m^2_2G tRNA methyltransferase and splicing tRNA endonuclease appear to be located at the surface of the inner nuclear membrane (Peebles *et al.*, 1983; Clark and Abelson, 1987; Rose *et al.*, 1995), whereas other activities appear to be nucleolar (Bertrand *et al.*, 1998; Hunter *et al.*, 1998). Given what appears to be a nonobligatory order of processing steps, it is curious that those mutations that act indirectly in tRNA processing all affect the same step: excision of introns from the pre-tRNA.

Many of the genes that indirectly affect pre-tRNA splicing are known to play a role in nucleus/cytosol exchange, such as Rna1p and Prp20p (Amberg *et al.*, 1992; Forrester *et al.*, 1992; Kadowaki *et al.*, 1993), and/or to be nucleoporins, such as Nsp1p, Nup49p, Nup116p, Nup133p, and Nup145p (Sharma *et al*, 1996). Why the first step of pre-tRNA splicing is often affected by gene products that act indirectly in pre-tRNA processing and how intervening sequence removal is coupled to nucleus/cytosol exchange are intriguing questions. In an effort to learn about gene products important for tRNA export and the coupling of nuclear export and pre-tRNA splicing, we successfully adapted in situ hybridization to locate endogenous levels of particular tRNAs in wild-type and mutant yeast cells. Here we describe the roles of particular yeast proteins in export of tRNAs to the cytosol.

MATERIALS AND METHODS

Strains and Media

The following yeast strains were used. EE1b-35 (*MAT***a** *RNA1 rnh1::URA3 ura3-52 ade1 tyr1 his7 his4* Gal$^-$) and EE1b-6 (*MAT***a** *rna1-1 rnh1::URA3 ura3-52 ade1 tyr1 his7 his4* Gal$^-$) were described in Traglia *et al.* (1989). Strains W303 α (*MAT*α *ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 NUP116*) and SWY27 (*MAT*α *ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 nup116*Δ*::HIS*) were obtained from S. Wente (Wente *et al.*, 1993), and strains FY86 (*MAT*α *RAT7 his3*Δ*200 ura3-52 leu2*Δ*1*) and LGY101 (*MAT*α *rat7-1 his3*Δ*200 ura3-52 leu2*Δ*1*) were obtained from C.N. Cole (Gorsch *et al.*, 1995). X2316-3C (*MAT*α *LOS1 SUP4 ade2-1 can1-100 lys1-1 his5-2 trp5-48 ura3-1*) and IIIdIc-ΔV (*los1-*Δ*V SUP4 ade2-1 can1-100 ura3-1*) were described by Hurt *et al.* (1987). YEPD medium was used to grow yeast cells.

Oligonucleotide Probes

Probe 02 contains 50 residues of deoxythymidine. The sequences for probes 03, 04, and 05 are 5′-CGTTGCTTTTAAAGGCCTGTTTGAAAGGTCTTTGGCACAGAAACTTCGGAAACCGAATGTTGCTAT-3′, 5′-GTGGGGATTGAACCCACGACGGTCGCGTTATAAGCACGAAGCTCTAACCACTGAGCTACA-3′, and 5′-GCGGGATCGAACCGCTGATCCCCGCGTTATTAGCACGGTGCCTTAACCAACTGGGCCAAG-3′, respectively. All oligonucleotides used as probes for Northern analysis and in the subsequent fluorescence in situ hybridization analyses were synthesized by the Pennsylvania State University College of Medicine Macromolecular Core Facility.

Preparation of RNA and Northern Analysis

RNA was isolated by phenol extraction from log phase yeast cells as described by Hopper *et al.* (1980). Approximately 15 μg of each RNA sample were used for Northern analysis, which was done according to Wang *et al.* (1988).

Fluorescence In Situ Hybridization

This procedure has been adapted from the previously published procedure of Kadowaki *et al.* (1992). Strains were grown at 23°C to log phase in YEPD and were either maintained at 23°C or shifted to 37°C for the indicated periods of time. Cells were prefixed in the culture by the addition of 0.1 volume of 37% formaldehyde. After 15

min, 5 ml cells were harvested by centrifugation and resuspended in 6 ml 4% paraformaldehyde, 0.1 M KPO_4 (pH 6.5), and 5 mM $MgCl_2$. After 3 h, cells were washed twice with solution B (1.2 M sorbitol and 0.1 M KPO_4, pH 6.5) and resuspended in 2.8 ml solution B containing 0.05% β-mercaptoethanol and 50 μl 2 mg/ml freshly prepared 100T Zymolyase (ICN Biochemicals, Cosa Mesa, CA). Spheroplasting was conducted at 37°C for 20 min. Spheroplasts were washed three times in solution B and resuspended in 0.3 ml solution B. Cells were adhered to the wells of Teflon-faced slides (Cel-Line/Erie Scientific, Portsmouth, NH) that had been pretreated with a 0.1% (wt/vol) poly-L-lysine-containing solution (Sigma Chemical, St. Louis, MO). Nonadhered cells were removed by aspiration. Cells were treated with 70, 90, and 100% ethanol successively for a duration of 5 min each. The slides were placed in a dessicator for 5 min. Cells were then incubated in prehybridization buffer containing 10% dextran sulfate, 0.2% BSA, 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M Na-citrate), 125 μg *Escherichia coli* tRNA/ml, and 500 μg denatured sonicated salmon sperm DNA/ml for 2 h at 37°C in a humid chamber. Hybridization buffer had the same composition with 450 pg/ml digoxigenin-labeled probes. All probes were labeled at their 3′ end using terminal transferase (Life Technologies, Gaithersburg, MD) and digoxigenin-11-UTP (Boehringer Mannheim, Indianapolis, IN) according to the previously described procedure of Amberg *et al.* (1992). Both the prehybridization and hybridization buffers contained RNasin (Promega, Madison, WI) at a concentration of 1 U/μl. Hybridization was carried out at 37°C overnight. Cells were washed three times with 2× SSC at 45°C for tRNA probes and at 37°C for the oligo$(dT)_{50}$ probe. Cells were then washed three times for 10 min with 1× SSC at room temperature. Cells were briefly washed with 4× SSC containing 1% Triton X-100 and then blocked for 2 h using 1% BSA containing 4× SSC. Fluoresceinated anti-digoxigenin Fab fragment (Boehringer Mannheim) was diluted according to the manufacturer's recommendation in solution containing 1% BSA and 4× SSC, and the cells were incubated with the diluted antibody for 2 h. Cells were washed twice with 4× SSC followed by two more washes with 4× SSC containing 1% Triton X-100, each wash lasting for 10 min. After two more rapid washes with 4× SSC, cell nuclei were counterstained with 0.1 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). After two rapid washes with water, the slides were mounted under 90% glycerol and 1× PBS containing 1 mg/ml *p*-phenylenediamine and stored at −20°C.

Fluorescence In Situ Hybridization and Indirect Immunofluorescence

Fluorescence in situ hybridization was carried out as described above, but after incubation with fluoresceinated anti-digoxigenin Fab fragment all subsequent washes were of only 5 min duration each. Cells were incubated with 1:20,000 dilution of mouse monoclonal antibody 32D6 (anti-Nsp1p) (Hunter *et al.*, 1998) for 2 h followed by five rapid washes with 1× SSC and then incubated for 1 h with 1:400 dilution of CY3-conjugated goat-anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were washed five times with 1× SSC and stained with DAPI as described above.

Microscopic Imaging

Fluorescence images were obtained by using a Nikon Microphot-FX microscope (Nikon Instrument Group, Melville, NY) equipped with a SenSys charged-coupled device camera (Photometrics, Tucson, AZ). Image processing was done with QED software (Pittsburgh, PA), NIH Image (http://rsb.info.nih.gov/nih-image), and Adobe Photoshop (Adobe Systems, Mountain View, CA).

RESULTS

In Situ Hybridization Analysis of the Subcellular Distributions of Endogenous Levels of tRNAs in Wild-Type Cells

In situ hybridization is a powerful method to study gene products important for the export of RNAs from their nuclear site of synthesis to their final cytoplasmic destination. Using oligo(dT) to detect total poly(A)-containing mRNA populations, numerous yeast mutants altered in mRNA transport have been identified (Amberg *et al,* 1992; Kadowaki *et al*., 1992). It has also been possible to study the location of particular mRNAs via in situ hybridization. Usually single mRNA species are detected by overexpression of the gene in question and by using multiple probes complementary to the mRNA species (Saavedra *et al*., 1996; Long *et al*., 1997); however, there is one report of successful use of in situ hybridization to locate endogenous levels of a mRNA (Takizawa *et al*., 1997). Because tRNAs have no sequences such as poly(A) in common with each other, it is not possible to study the cellular distribution of the total tRNA population. However, tRNAs are rather abundant molecules. Only $\sim 2.5 \times 10^4$ of the 1.2×10^7 nucleotide yeast genome is devoted to tRNA genes (for review see Hani and Feldmann, 1998), yet tRNAs constitute ~20% of the total RNA population. One reason for tRNA abundance is long half-life. Given 42 tRNA species (Hani and Feldmann, 1998), an individual species should constitute ~0.5% of the total cellular RNA, within an order of magnitude of the sum of the total mRNA population (~1–5% of total RNA). Therefore, we anticipated that in situ hybridization would be sufficiently sensitive to locate a single species of mature tRNA within yeast cells. In contrast, tRNA precursors (pre-tRNAs) have shorter half-lives than their mature counterparts, and the ability to detect individual pre-tRNAs by in situ techniques could be problematic.

To determine whether it is possible to use in situ hybridization to locate individual tRNAs within yeast, we chose to study $tRNA^{Ile}$ and designed oligonucleotide probes to detect particular $tRNA^{Ile}$ species (Figure 1). Probe 05 contains 60 nucleotides and is complementary to $tRNA^{Ile}_{AAU}$, which is encoded by 13 identical genes that do not contain introns (Hani and Feldmann, 1998). The specificity of the probe was determined by RNA blot analysis. Using this assay, a single RNA species that migrates at the expected molecular weight for mature $tRNA^{Ile}_{AAU}$ was detected (Figure 2). $tRNA^{Ile}_{UAU}$ is encoded by two identical genes that contain 60-nucleotide-long introns, the longest of the yeast tRNA introns (Hopper and Martin, 1992). To detect intron-containing pre-$tRNA^{Ile}_{UAU}$, we used a 66-nucleotide probe complementary to the entire intron plus 3 nucleotides 5′ and 3′ to the intron (Figure 1, Probe 03). To detect the entire $tRNA^{Ile}_{UAU}$ population, we used an oligonucleotide containing 60

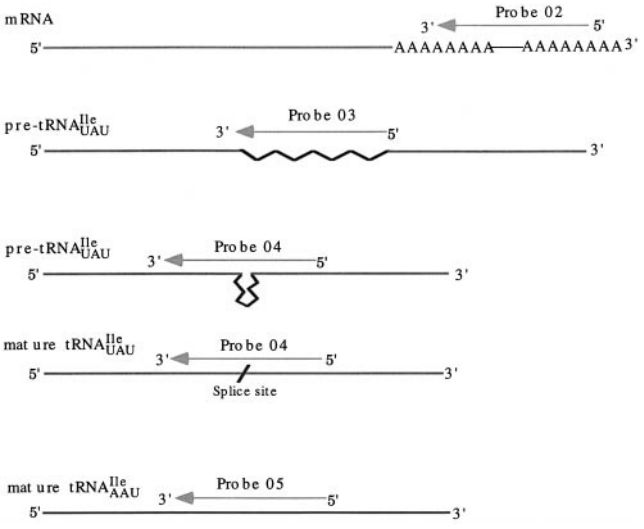

Figure 1. Diagram of the regions of RNAs complementary to oligonucleotide probes. The straight line depicts mature RNA regions; the wavy line represents the IVS sequence, and the interrupted line marks the position from where an intron has been removed. Lines with arrows indicate the complementary regions of each probe.

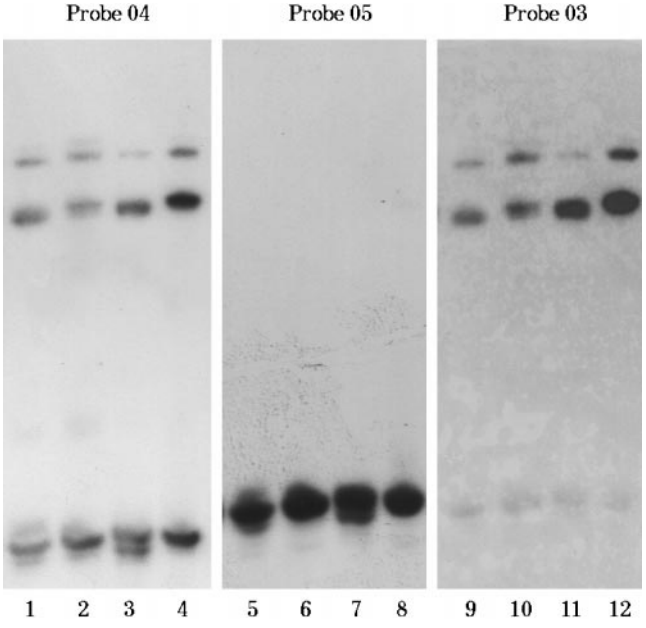

Figure 2. Northern blot detection of pre-tRNA$^{Ile}_{UAU}$, mature tRNA$^{Ile}_{UAU}$, and mature tRNA$^{Ile}_{AAU}$. tRNAs were isolated from EE1b-35 (lanes 1, 2, 5, 6, 9, and 10) and EE1b-6 (lanes 3, 4, 7, 8, 11, and 12) cells grown either at 23°C (lanes 1, 3, 5, 7, 9, and 11) or shifted to 37°C for 1 h (lanes 2, 4, 6, 8, 10, and 12). Lanes 1–4 were incubated with probe 04, lanes 5–8 were incubated with probe 05, and lanes 9–11 were incubated first with probe 05 and then stripped and reprobed with probe 03. In lanes 1–4, the top band corresponds to 5′ and 3′ extended intron-containing pre-tRNA species, the next band corresponds to end-matured intron-containing pre-tRNA, and the bottom band corresponds to mature tRNA$^{Ile}_{UAU}$.

nucleotides complementary to a region of the mature tRNA sequence that spans the anticodon loop (Figure 1, Probe 04). By Northern analysis probe 04 detected three RNAs migrating at the expected molecular weights for mature tRNA$^{Ile}_{UAU}$, end-matured intron-containing pre-tRNA, and 5′ and 3′ end-extended intron-containing pre-tRNA species. Probe 03, as expected, detected only the two intron-containing pre-tRNAs. Therefore, the probes detect only the anticipated tRNA species.

In situ hybridization studies using probes 03, 04, and 05 were conducted to locate the tRNAs within wild-type yeast strain EE1b-35 (Figure 3; see MATERIALS AND METHODS). The positions of nuclei were assessed by costaining cells with the DNA-specific dye DAPI (Figure 3) or by combining in situ hybridization with immunofluorescence techniques and using an antibody specific for nuclear pores (see below). Control cells treated identically to experimental cells except for the absence of probe had little or no fluorescent signal (Figure 3, A and B). Using probe 05, which is specific for mature tRNA$^{Ile}_{AAU}$, wild-type cells grown at 23°C had signal throughout the cells, with some accumulation in nuclei (Figure 3, C and D). When the same cells were incubated for 1 or 3 h at 37°C, the signal was primarily cytoplasmic, and the nucleus appeared to be rather depleted for tRNA$^{Ile}_{AAU}$ (see below). Controls in which 1000-fold excess of unlabeled probe 05 was included during hybridization resulted in loss of the fluorescent signal (Figure 3, E and F), whereas including 1000-fold excess of probes 03 or 04 during hybridization did not affect the intensity or location of the probe 05 signal (our unpublished results). The results show that the hybridization signal detected is specific for tRNA$^{Ile}_{AAU}$ and indicate, as expected, that mature tRNAs are located primarily in the cytosol. Detection of a nuclear pool at 23°C but not at 37°C probably reflects a lower rate of nuclear export of tRNA$^{Ile}_{AAU}$ at the lower temperature growth conditions.

In situ hybridization using probe 04 complementary to the two forms of precursor and the mature tRNA$^{Ile}_{UAU}$ gave a cytosolic signal along with evidence of nuclear staining when the cells were grown at 23°C (Figure 3, G and H) or when incubated at 37°C for 1 or 3 h (our unpublished results). This signal was completely competed by the addition of 1000-fold excess of unlabeled probe 04 in the hybridization mix but not by heterologous probe 05 (our unpublished results), again documenting specificity. Because probe 04 detects pre-tRNA$^{Ile}_{UAU}$ as well as mature tRNA$^{Ile}_{UAU}$, whereas probe 05 detects only mature tRNA$_{AAU}$, the nuclear signal detected for wild-type cells incubated at 37°C using probe 04 but not 05 could be indicative of a nuclear pool of pre-tRNA$^{Ile}_{UAU}$ at both low and high temperatures. To test this, we used

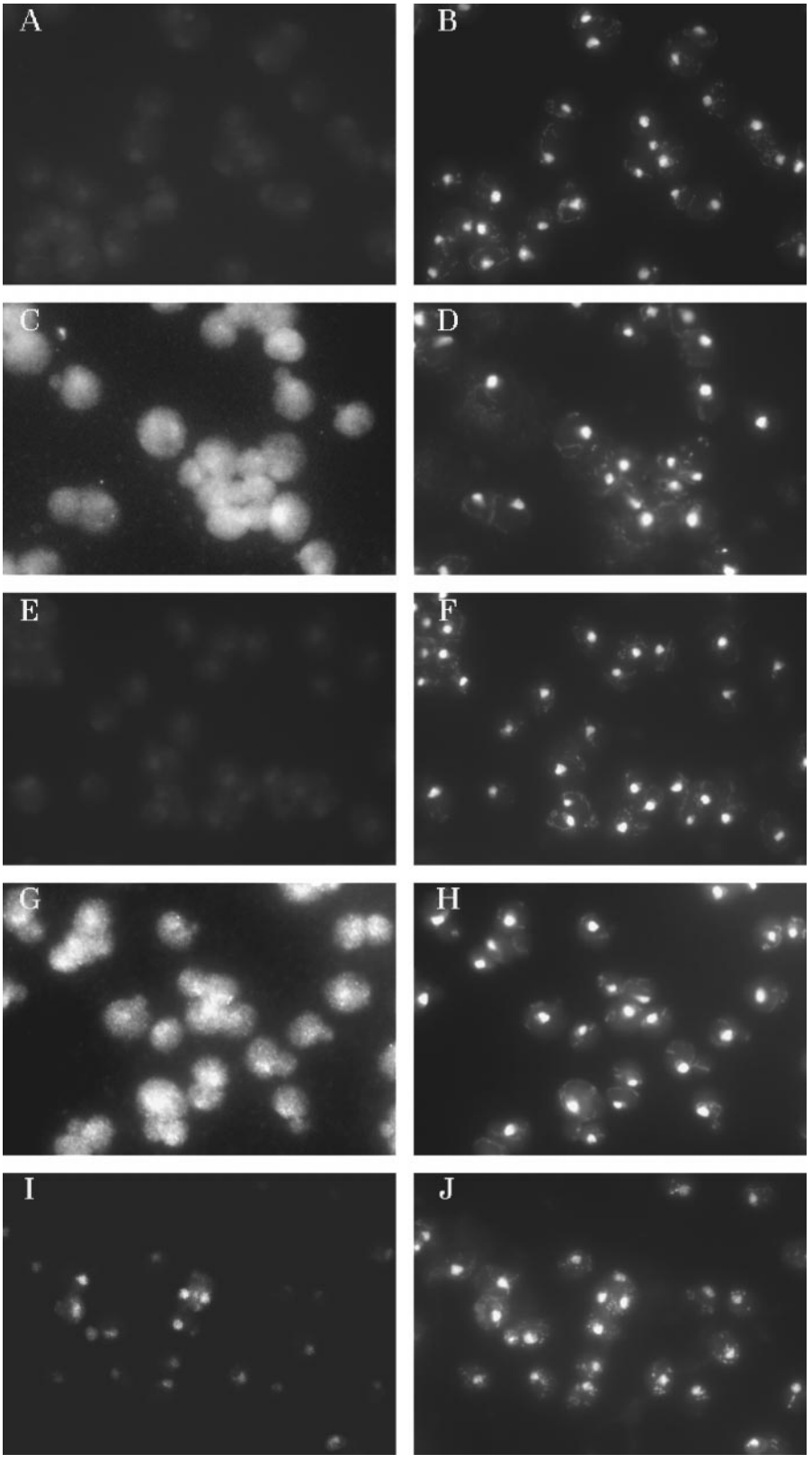

Figure 3. Detection of endogenous levels of single species of tRNA is possible by fluorescence in situ hybridization. EE1b-35 cells were grown at 23°C and then subjected to fluorescence in situ hybridization. (A) In situ hybridization done in the absence of digoxigenin-labeled probe. (C) In situ hybridization detection of mature $tRNA^{Ile}_{AAU}$ using digoxigenin-labeled probe 05. (E) In situ hybridization detection of mature $tRNA^{Ile}_{AAU}$ using digoxigenin-labeled probe 05 in the presence of 1000-fold excess of unlabeled probe 05. (G) In situ hybridization detection of mature $tRNA^{Ile}_{UAU}$ using digoxigenin-labeled probe 04. (I) In situ hybridization detection of pre-$tRNA^{Ile}_{UAU}$ using digoxigenin-labeled probe 03. (B, D, F, H, and J) DAPI staining of cells shown in A, C, E, G, and I, respectively.

probe 03, which detects only pre-tRNA$^{Ile}_{UAU}$ as documented by Northern analysis for in situ hybridization. These studies revealed only a somewhat uniformly distributed nuclear signal whether the cells were grown at 23°C (Figure 3, I and J) or incubated at 37°C for 1 or 3 h (see below).

Absence of signal in the absence of probe, competition of signal for each probe when an excess of unlabeled probe was included during hybridization, and lack of competition when the same molar excess of either heterologous unlabeled probe was used, provide strong evidence that in situ hybridization can be used to detect endogenous levels of specific tRNAs in yeast. Moreover, the nuclear location of pre-tRNA and primarily cytosolic location of mature tRNAs substantiate the efficacy of the in situ hybridization procedure and further indicate the usefulness of this method for studies of subcellular distributions of tRNA species and/or pre-tRNA processing.

The Effects of Nucleoporins, Los1p, and RanGAP on Nuclear Export of tRNAs Encoded by Genes Lacking Introns

Nucleoporins. Our goal is to identify genes important for the export of tRNAs to the cytosol. As described above, tRNA export is coupled to pre-tRNA splicing. To study export of tRNAs independent of pre-tRNA splicing, we chose to assess the location of tRNA$^{Ile}_{AAU}$, which is encoded by genes lacking introns. In wild-type yeast cells tRNA$^{Ile}_{AAU}$ is located primarily in the cytosol (Figure 3, C and D). Because tRNAs are very stable molecules, cells will have high levels of cytosolic tRNA$^{Ile}_{AAU}$ whether or not they are blocked in tRNA nuclear export when incubated at nonpermissive conditions. Therefore, it was not clear whether it would be possible to detect by in situ hybridization increased nuclear pools over high cytosolic signals in cells with tRNA export defects. To determine this, we chose to compare the location of tRNA$^{Ile}_{AAU}$ in the *nup116*Δ mutant to its location in the parent strain. *NUP116* encodes a nucleoporin, and a deletion of this gene causes a temperature-sensitive growth defect resulting from aberrant, sealed nucleopores and subsequent defects in nucleus/cytosol exchange (Wente and Blobel, 1993). Because of the aberrant nucleopores, we anticipated that export of tRNA would also be defective and that this strain would be useful to test whether in situ hybridization could be used to study tRNA nuclear retention.

Parent W303 strain and the *nup116*Δ strain were grown at a permissive temperature and incubated at 37°C for 1 h, and the locations of RNAs were determined by in situ hybridization (Figure 4). To be certain that the cells showed the appropriate defect in nucleus/cytosol exchange under these conditions and in the in situ procedures we use, we assessed the location of poly(A)-containing RNA in these cells using a 50-nucleotide oligo(d)T probe. Appropriate controls to confirm the specificity of the oligo(dT)$_{50}$ probe were conducted (our unpublished results). For the parent grown at 23°C (our unpublished results) or incubated for 1 h at 37°C (Figure 4, A and B), poly(A)-containing RNA was distributed throughout the cells. For *nup116*Δ cells grown at the permissive temperature, the signal was indistinguishable from the parent strain (our unpublished results). In contrast, for *nup116*Δ cells incubated for 1 h at 37°C (Figure 4, C and D), poly(A)-containing RNA was predominately nuclear. Thus, *nup116*Δ cells display the previously reported defect in mRNA nuclear export (Wente and Blobel, 1993). Mature tRNA$^{Ile}_{AAU}$ was distributed throughout parental cells when grown at 23°C (our unpublished results). When the parent strain was incubated for 1 h at 37°C (Figure 4, E and F), the tRNA$^{Ile}_{AAU}$ nuclear signal was less prominent than at 23°C (our unpublished results). The signal for the *nup116*Δ mutants grown at 23°C was indistinguishable from its parent (our unpublished results). However, upon incubation of the *nup116*Δ cells for 1 h (Figure 4, G and H) at 37°C, prominent nuclear accumulation of tRNA$^{Ile}_{AAU}$ resulted. Even though there is considerable tRNA$^{Ile}_{AAU}$ in the cytosol of *nup116*Δ cells incubated at 37°C, within 1 h there is clear nuclear accumulation of tRNA$^{Ile}_{AAU}$ above this background. We conclude that it is possible to detect RNA export defects by in situ hybridization even for a stable molecule such as tRNA and that Nup116p is important for the movement of tRNA from the nucleus to the cytosol.

Mutations of certain nucleoporins appear to affect nucleus/cytosol exchange in a single direction only. *RAT7* encodes an essential nucleoporin, Nup159p, which contains GLFG and XFXFG repeats found in numerous other nucleoporins (Gorsch *et al.*, 1995). The *rat7-1* mutation causes temperature-sensitive growth defects at 37°C and defects in mRNA nuclear export, as assessed by in situ hybridization using oligo(dT) probes, but does not appear to affect nuclear import of at least some particular protein cargoes (Gorsch *et al.*, 1995). To address the possibility that Nup159p could have substrate specificity for exported cargo, we assessed the locations of poly(A) RNA and tRNA$^{Ile}_{AAU}$ in *rat7-1* cells and the parent to this mutant, strain FY86. As anticipated, *rat7-1* cells accumulate substantially more poly(A) RNA in the nucleus when incubated for 1 h at 37°C than do the parental cells (Figure 5, A–D). As for the parent strain of *nup116*Δ, tRNA$^{Ile}_{AAU}$ was distributed throughout the FY86 cells when they were grown at 23°C, and there was a less prominent nuclear signal when these cells were incubated for 1 h at 37°C (Figure 5, E and F). However, in contrast to the results obtained for *nup116*Δ, tRNA$^{Ile}_{AAU}$ distribution in *rat7-1* cells was indistinguishable from the isogenic FY86 parent cells. No tRNA$^{Ile}_{AAU}$ nuclear accumulation was

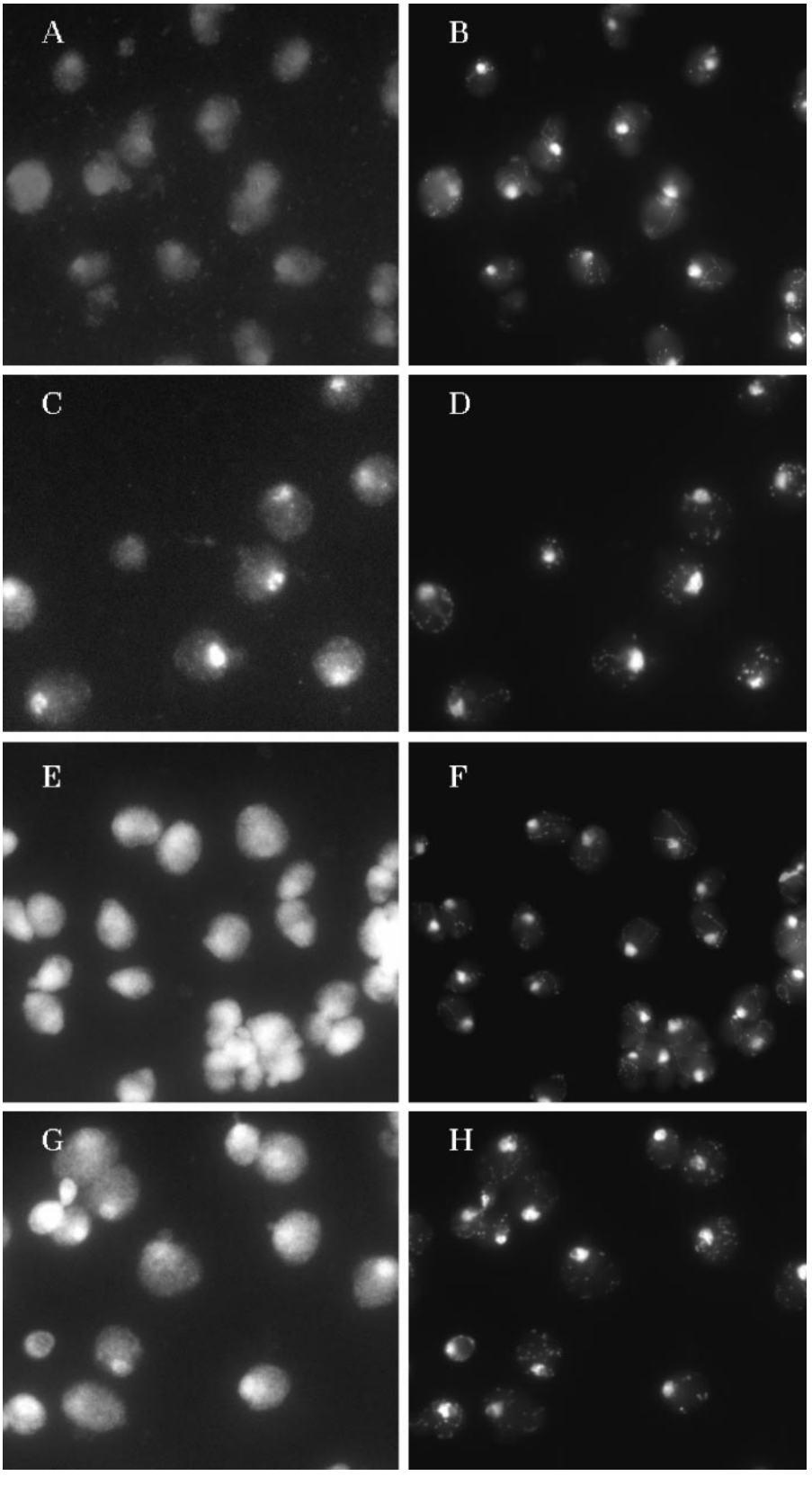

Figure 4. Identification of tRNA transport mutants is possible using fluorescence in situ hybridization. Parent W303 and *nup116*Δ strain SWY27 were grown at 23°C, and log phase cells were shifted to 37°C for 1 h. (A) In situ hybridization detection of Poly(A) RNA with digoxigenin-labeled probe 02 in W303 cells. (C) Detection of Poly(A) RNA with digoxigenin-labeled probe 02 in SWY27 cells. (E) Detection of mature $tRNA^{Ile}_{AAU}$ with digoxigenin-labeled probe 05 in W303 cells. (G) Mature $tRNA^{Ile}_{AAU}$ detection with digoxigenin-labeled probe 05 in SWY27 cells. (B, D, F, and H) DAPI staining of cells shown in A, C, E, and G, respectively.

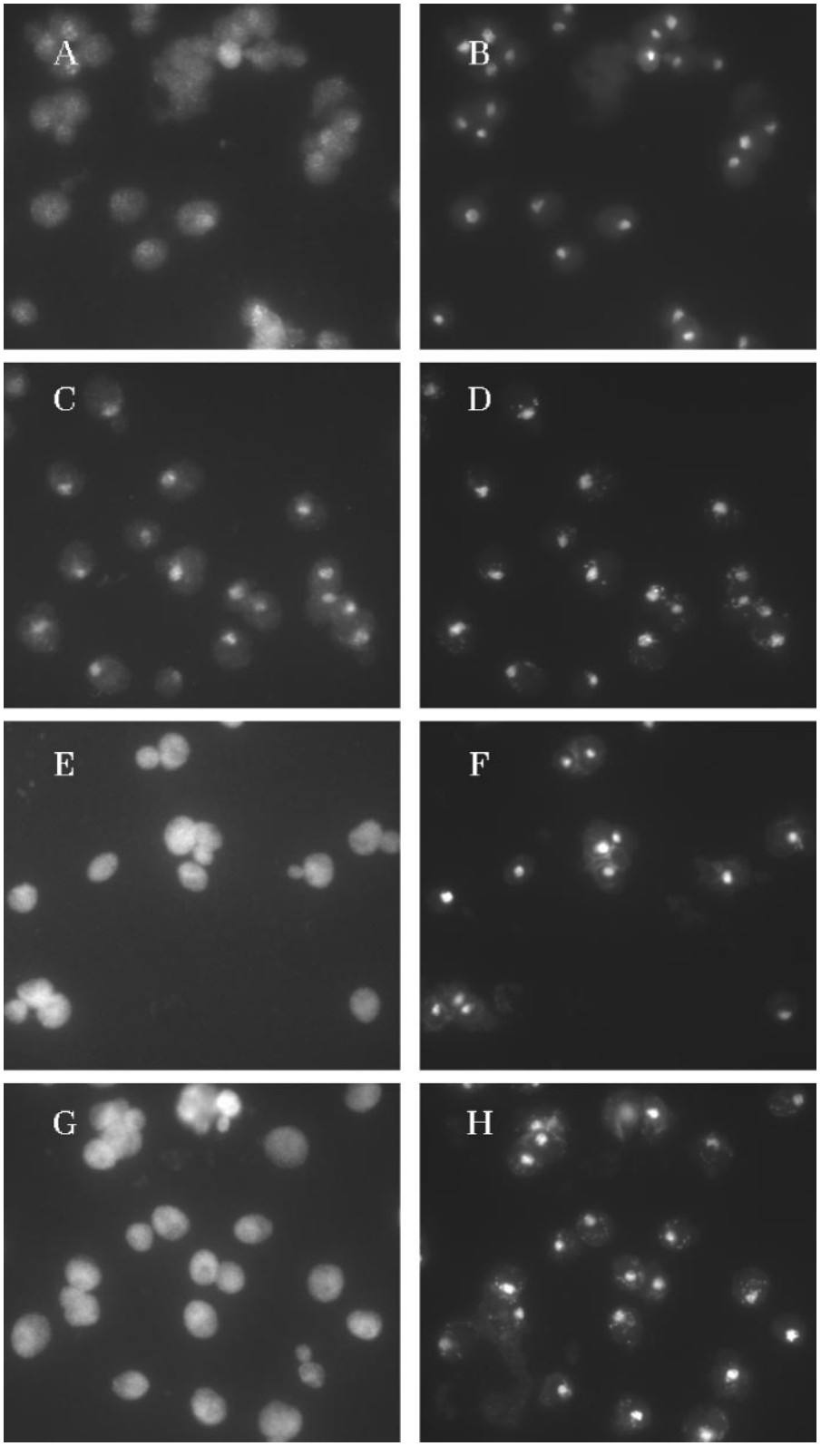

Figure 5. Nucleoporins that are required for Poly(A) RNA export may not be required for mature tRNA export out of the nucleus. Parent FY86 and *rat7-1* mutant strain LGY101 were grown at 23°C, and log phase cells were shifted to 37°C for 1 h. In situ hybridization was performed using the following probes and cells: (A) poly(A) RNA probe 02, FY86 cells; (C) poly(A) RNA probe 02, LGY101 cells; (E) mature $tRNA^{Ile}_{AAU}$ probe 05, FY86 cells; (G) mature $tRNA^{Ile}_{AAU}$ probe 05, LGY101 cells. (B, D, F, and H) DAPI staining of cells shown in A, C, E, and G, respectively.

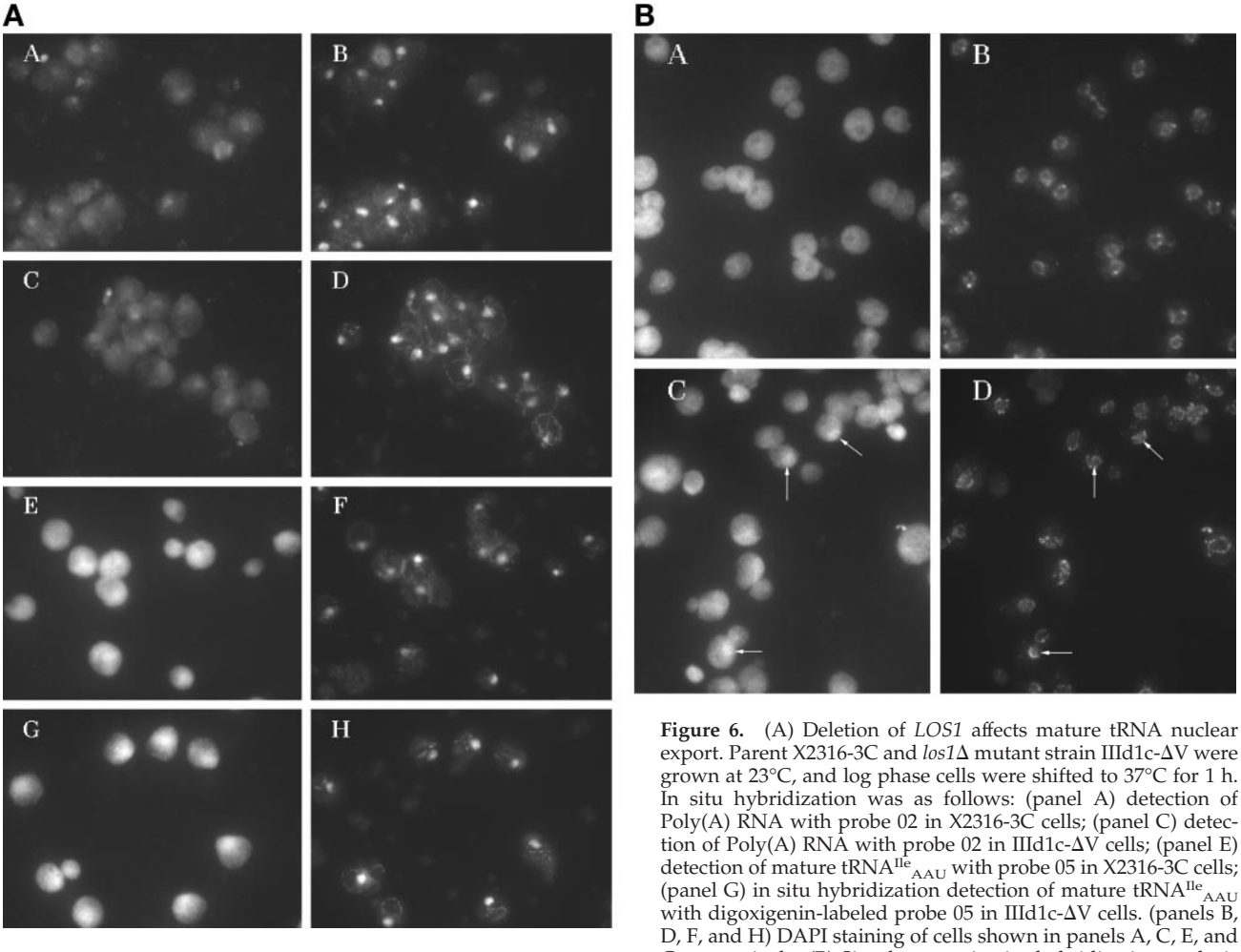

Figure 6. (A) Deletion of *LOS1* affects mature tRNA nuclear export. Parent X2316-3C and *los1*Δ mutant strain IIId1c-ΔV were grown at 23°C, and log phase cells were shifted to 37°C for 1 h. In situ hybridization was as follows: (panel A) detection of Poly(A) RNA with probe 02 in X2316-3C cells; (panel C) detection of Poly(A) RNA with probe 02 in IIId1c-ΔV cells; (panel E) detection of mature $tRNA^{Ile}_{AAU}$ with probe 05 in X2316-3C cells; (panel G) in situ hybridization detection of mature $tRNA^{Ile}_{AAU}$ with digoxigenin-labeled probe 05 in IIId1c-ΔV cells. (panels B, D, F, and H) DAPI staining of cells shown in panels A, C, E, and G, respectively. (B) Simultaneous in situ hybridization analysis of $tRNA^{Ile}_{AAU}$ and immunofluorescent location of Nsp1. Parent X2316-3C and *los1*Δ mutant strain IIId1c-ΔV were grown at 23°C and log phase cells were shifted to 37°C for 1 h. (Panel A) Detection of mature $tRNA^{Ile}_{AAU}$ with probe 05 in X2316-3C cells. (Panel B) Indirect immunofluorescence detection of nucleoporin Nsp1p with 32D6 antibody in X2316-3C cells. (Panel C) Detection of mature $tRNA^{Ile}_{AAU}$ with probe 05 in IIId1c-ΔV cells. (Panel D) Indirect immunofluorescence detection of nucleoporin Nsp1p with 32D6 antibody in IIId1c-ΔV cells. Arrows point to cells displaying nuclear accumulations.

evident when the *rat7-1* cells were incubated for 1 h at 37°C (Figure 5, G and H). We conclude that not all nucleoporins that are important for the distribution of mRNA to the cytosol are important for the distribution of tRNA to the cytosol.

Los1p. Yeast Los1p bears similarity to the importin-β family of proteins (Görlich *et al.*, 1997), specifically to vertebrate exportin-t, which has been shown to facilitate nuclear tRNA export (Arts *et al.*, 1998; Kutay *et al.*, 1998). *LOS1* is an unessential yeast gene. Mutations of the *LOS1* gene cause accumulation of intron-containing pre-tRNAs (Hopper *et al.*, 1980, Simos *et al.*, 1996) but do not appear to affect production of rRNA or most mRNAs (Hopper *et al.*, 1980; Shen *et al.*, 1996). If yeast Los1p is the functional homologue of exportin-t, then one might expect that in addition to the defects in pre-tRNA splicing, *los1* mutants might show defects in the distribution of tRNA to the cytosol. To study the effects of Los1p on nuclear export independent of the affects on pre-tRNA splicing, we used in situ hybridization to locate $tRNA^{Ile}_{AAU}$ that is encoded by intronless genes.

The locations of poly(A) RNA and $tRNA^{Ile}_{AAU}$ (Figure 6A) were determined for wild-type strain X2316-3C and the related strain IIId1c-ΔV, which possesses a deletion allele, *los1ΔV* (Hurt *et al.*, 1987). As expected, poly(A)-containing RNA and $tRNA^{Ile}_{AAU}$ were distributed throughout the X2316-3C cells when they were grown at 23°C (our unpublished results). When X2316-3C cells were incubated for 1 h (Figure 6) or 3 h at 37°C (our unpublished results), $tRNA^{Ile}_{AAU}$ was substantially depleted from nuclei (Figure 6, A,

panels E and F, and B). In contrast, *los1ΔV* mutant cells showed significant $tRNA^{Ile}_{AAU}$ nuclear accumulation when the cells were incubated for 1 h (Figure 6, A, panels G and H, and B) or 3 h at 37°C (our unpublished results). The accumulation of nucleus-located RNA appeared to be tRNA specific, because the same cells showed no accumulation of nucleus-located poly(A) RNA (Figure 6A, panels C and D). Thus, *los1* mutations affect export of tRNA but not mRNA at the nonpermissive temperature. Although it is difficult to obtain quantitative information regarding the amounts of nuclear signal to cytosolic signal by these methods, the *nup116Δ* strain appears to accumulate $tRNA^{Ile}_{AAU}$ in the nucleus more rapidly and to a higher level than do *los1ΔV* cells.

The $tRNA^{Ile}_{AAU}$ nuclear signal in *nup116Δ* (Figure 4) or *los1*ΔV (Figure 6A) strains appeared more diffuse and to extend beyond the DAPI signal. To confirm that accumulated $tRNA^{Ile}_{AAU}$ was within the confines of the nuclear border, we probed for $tRNA^{Ile}_{AAU}$ and simultaneously stained the nuclear membrane using monoclonal antibody 32D6, which is specific for nucleoporin Nsp1p (Hunter *et al*., 1998; see MATERIALS AND METHODS). Despite the diffuse signal, the vast majority of the accumulated $tRNA^{Ile}_{AAU}$ is indeed located within the nuclear interior in *nup116Δ* (our unpublished results) and *los1ΔV* mutant cells (Figure 6B, panels A–D).

RanGAP. Alteration of components of the RanGTPase cycle causes nuclear accumulation of mRNA (Amberg *et al*., 1992; Forrester *et al*., 1992; Kadowaki *et al*., 1993; Schlenstedt *et al*., 1995) and defects in nuclear protein import (Corbett *et al*., 1995). The yeast *RNA1* gene encodes the RanGTPase-activating protein RanGAP, which is necessary for GTP hydrolysis of RanGTP to RanGDP (Becker *et al*., 1995; Bischoff and Ponstingl, 1995; Corbett *et al*., 1995). If alteration of Ran components also affects tRNA export, then one might expect mutations in *RNA1* to cause nuclear accumulation of mature tRNAs. We determined the location of mature $tRNA^{Ile}_{AAU}$ encoded by intronless genes in *rna1-1* cells. As for the studies of *nup116Δ* and *rat7-1* mutants, we compared the cellular distributions of poly(A)-containing RNA and $tRNA^{Ile}_{AAU}$ in EE1b-6 *rna1-1* mutant cells with the distributions in EE1b-35, the isogenic wild-type strain. Poly(A)-containing RNA distributions were, as anticipated, largely cytoplasmic in the wild-type strain and nuclear in the *rna1-1* mutant strain when the cells were incubated at 37°C for 1 h (our unpublished results) or 3 h (Figure 7, A–D). Also as anticipated, $tRNA^{Ile}_{AAU}$ was predominantly cytosolic when EE1b-35 cells were incubated at 37°C for 1 h (our unpublished results) or 3 h (Figure 7, E–F). Interestingly, *rna1-1* mutant cells showed nuclear accumulation of $tRNA^{Ile}_{AAU}$ when exposed for 1 h (our unpublished results) or 3 h (Figure 7, G–H) at the nonpermissive temperature. As for the studies of *los1ΔV*, it appears that the *nup116Δ* cells accumulate $tRNA^{Ile}_{AAU}$ in the nucleus more rapidly and to a higher level than *rna1-1* cells. Our results are in agreement with the studies of Izaurralde *et al.* (1997), who demonstrated a role for a functional RanGTPase cycle for the export of all tested RNAs.

Defects in RanGAP Cause Nuclear Accumulation of Intron-Containing Pre-tRNA

As pre-tRNA splicing precedes export of mature tRNA from the nucleus, it is remarkable that mutations of genes involved in nucleus/cytosol exchange—nuclear pore structural components and the RanGTPase pathway—affect pre-tRNA intron removal (Hopper *et al*., 1978; Kadowaki *et al*., 1993; Sharma *et al*., 1996). At least four different scenarios, or combinations thereof, could account for this conundrum. First, there could be "feedback" of information from the exchange process to the splicing endonuclease machinery thereby indirectly causing intron-containing species to accumulate within the nucleus. Second, pre-tRNAs could fail to be delivered from their site of synthesis to the nuclear membrane where the tRNA splicing machinery is located (Peebles *et al*., 1983; Clark and Abelson, 1987). Third, alteration of the nuclear pores and/or the Ran pathway could lead to structural changes in the nuclear membrane which, in turn, could alter the topology of the nuclear membrane-located tRNA splicing endonuclease or cause leakage of nuclear components. Previous studies have implicated a role for the RanGTPase cycle in nuclear membrane integrity in mammalian and *Schizosaccharomyces pombe* cells (for review see Sazer, 1996). Fourth, alteration of nuclear transport components could affect the regulation of the ordered path of pre-tRNA splicing preceding nuclear export causing export of intervening sequence (IVS)-containing RNAs. There is precedence for alterations in the nucleus/cytosol exchange machinery affecting the ordered steps of RNA processing and export. For example, when the HIV Rev gene is expressed in yeast, unspliced RRE-containing mRNAs are detected in the cytosol and the levels of these cytoplasmic pre-mRNAs are modulated by overexpression or disruption of *RIP1/NUP42*, a gene encoding a yeast nucleoporin (Stutz *et al*., 1995). Consequences of either the third or fourth scenarios could generate cytoplasmic pools of intron-containing pre-tRNAs that could not be spliced because they would be physically separated from the tRNA splicing endonuclease located at the inner surface of the nuclear membrane.

Temperature-sensitive *rna1-1* mutants are defective in nucleus/cytosol exchange at the nonpermissive temperature (Amberg *et al*., 1992; Corbett *et al*., 1995), and they accumulate intron-containing pre-tRNAs (Hopper *et al*., 1978; Knapp *et al*., 1978). As assessed by Northern analysis, using probe 03 complementary to the entire pre-$tRNA^{Ile}_{UAU}$ intron, there was no difference in the amount of intron containing pre-

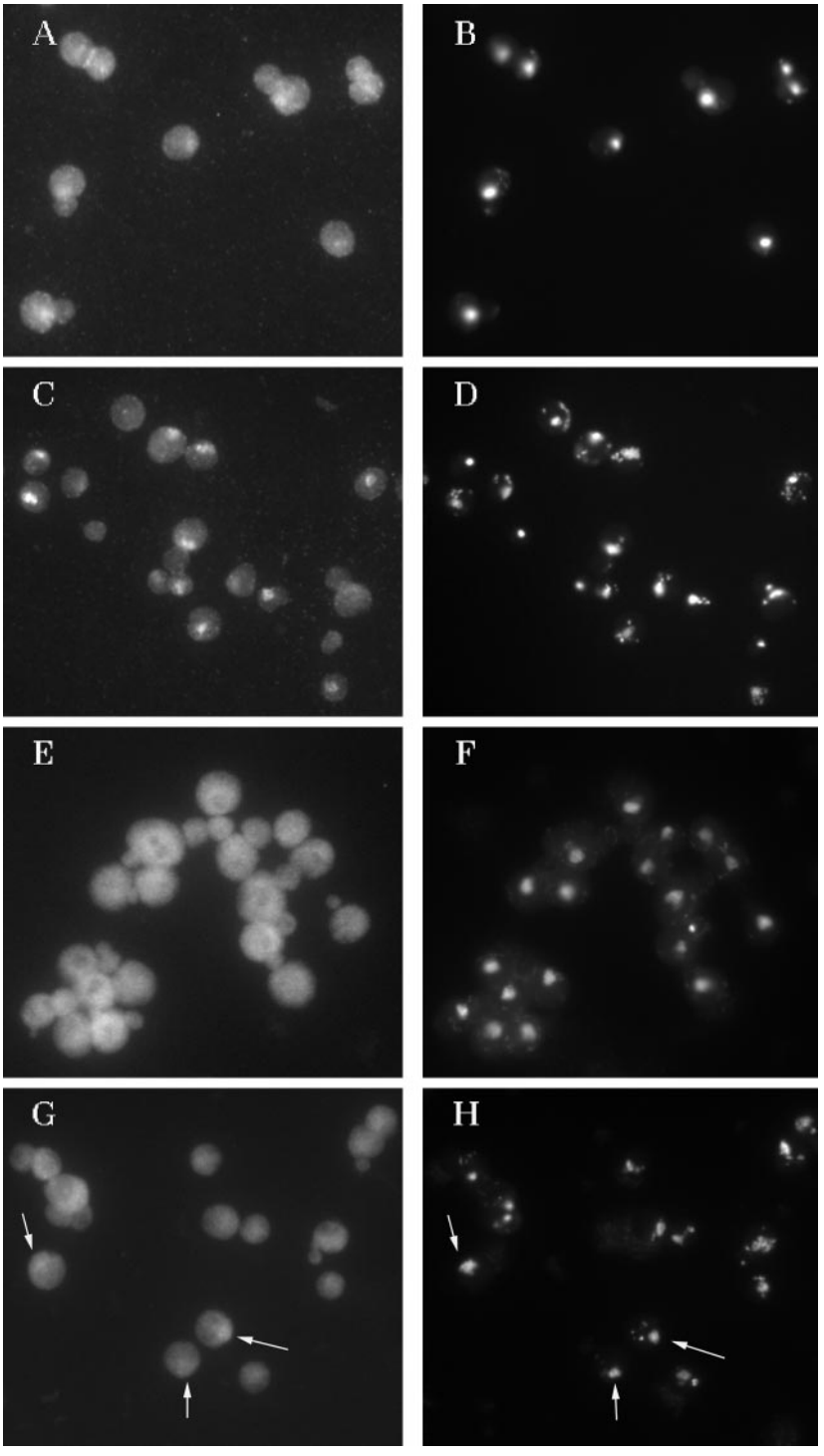

Figure 7. Mutation in *RNA1* causes accumulation of mature tRNA in the nucleus. The same cultures of parent EE1b-35 and *rna1-1* mutant strain EE1b-6 that were grown at 23°C and used for Figure 3 were shifted to 37°C for 3 h. (A) Detection of Poly(A) RNA with probe 02 in EE1b-35 cells. (C) Detection of Poly(A) RNA with probe 02 in EE1b-6 cells. (E) Detection of mature $tRNA^{Ile}_{AAU}$ with probe 05 in EE1b-35 cells. (G) Detection of mature $tRNA^{Ile}_{AAU}$ with probe 05 in EE1b-6 cells. (B, D, F, and H) DAPI staining of cells shown in A, C, E, and G, respectively. Arrows point to cells displaying nuclear accumulations.

$tRNA^{Ile}_{UAU}$ when the wild-type, EE1b-35 or *rna1-1* mutant, EE1b-6, cells were grown at the permissive temperature (our unpublished results). However, after an exposure to the elevated temperature of 37°C for 1 h, *rna1-1* cells had an increased level pre-$tRNA^{Ile}_{UAU}$ compared with the isogenic wild-type cells (Figure 2, compare lane 4 with lanes 1–3). To test whether the increased levels of pre-$tRNA^{Ile}_{UAU}$ in the *rna1-1* cells were due to precocious movement of pre-tRNAs to the cytosol in cells with an altered RanGTPase pathway,

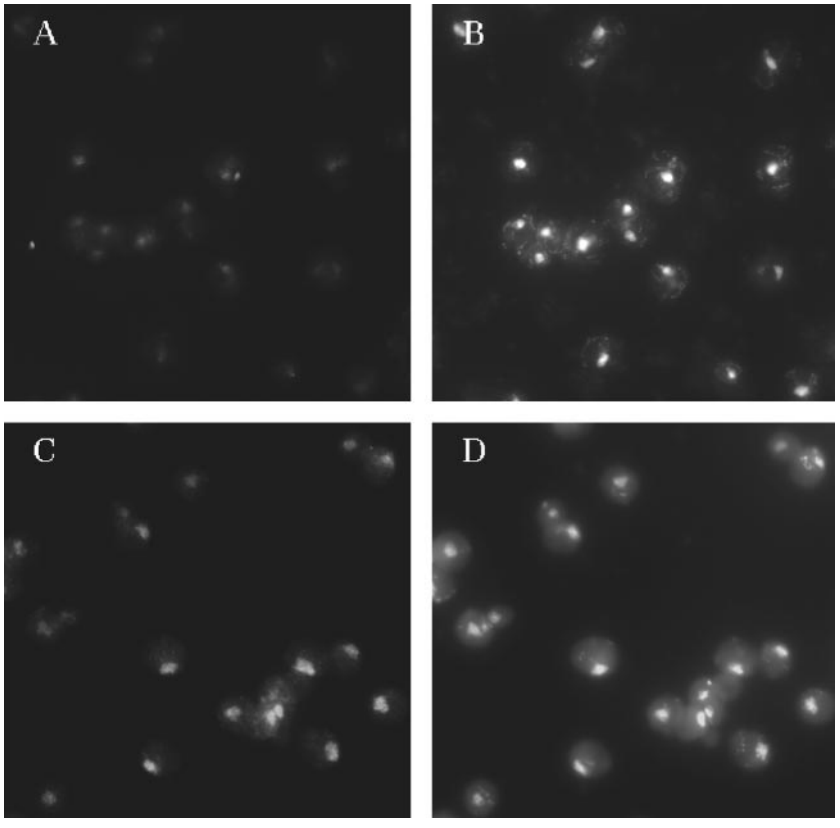

Figure 8. Accumulation of pre-tRNAs in *rna1-1* cells is not due to precocious movement of pre-tRNAs out of the nucleus. Parent EE1b-35 and *rna1-1* mutant strain EE1b-6 were grown at 23°C, and log phase cells were shifted to 37°C for 1 h. (A) Detection of pre-tRNA$^{Ile}_{UAU}$ with probe 03 in EE1b-35 cells. (C) Detection of pre-tRNA$^{Ile}_{UAU}$ with probe 03 in EE1b-6 cells. (B and D) DAPI staining of cells shown in A and C, respectively.

we used in situ hybridization to locate intron-containing pre-tRNAs in wild-type cells and *rna1-1* cells incubated at the nonpermissive temperature. In agreement with the Northern analysis, the in situ hybridization signal using probe 03 was substantially more intense in *rna1-1* cells when they were incubated at the nonpermissive temperature in comparison with the control cells (Figure 8, compare A with C). This intron-specific signal is restricted to the nucleus in both parental and *rna1-1* mutant cells, and the signal is somewhat uniformly distributed throughout the nucleus in both. Thus, the data indicate that pre-tRNA accumulation in *rna1-1* cells does not result from precocious pre-tRNA nuclear export or accumulation in an inappropriate nuclear subcompartment. The nuclear location of intron-containing pre-tRNAs supports the notion that the tRNA splicing pathway is tightly coupled to nucleus/cytosol exchange (Hopper *et al*., 1978; Kadowaki *et al*., 1993; Sharma *et al*., 1996).

DISCUSSION

Three lines of evidence document successful adaptation of in situ hybridization to assess intracellular locations of endogenous levels of individual tRNA species: 1) competition studies showing specificity of the signals for particular tRNA probes used, 2) location of mature tRNAs in the cytosol and IVS-containing pre-tRNAs in the nucleus, and (3) nuclear accumulation of mature tRNA in yeast cells with sealed nuclear pores. Detection of endogenous levels of individual species of IVS-containing tRNA processing intermediates is due, in part, to pre-tRNA splicing being a slow step in the biogenesis pathway. Our ability to detect nuclear accumulation of mature tRNAs above the cytosolic pool has allowed analyses of particular yeast mutants for defects in tRNA export. We intend to extend this approach to characterize roles of other known nucleoporins in tRNA export. In principle, we should be able to adapt this type of analysis to screen among collections of yeast temperature-sensitive mutants to uncover novel essential genes important to the tRNA export pathway.

Studies of yeast mutants with lesions in genes encoding nucleoporins have demonstrated that many of the nucleoporins are important for both nuclear import and export. However, other nucleoporins have been found to affect transit in a single direction (Fabre and Hurt, 1997). Yeast *RAT7* encoding nucleoporin Nup159p has been reported to affect only outward-bound nuclear traffic (Gorsch *et al*., 1995). Here we show that even though *rat7-1* cells are defective in poly(A) RNA export, they appear not to be defective

in nuclear export of mature $tRNA^{Ile}_{AAU}$. Thus, Rat7p/Nup159p appears to have a species-specific role in RNA export. Yeast *NUP42/RIP1* encoding nucleoporin Nup42p provides another example of an RNA species-specific nucleoporin. *NUP42/RIP1* is an unessential gene and *rip1* mutants show no defect in RNA nuclear export when the location of poly(A) mRNA is analyzed using oligo(d)T probes for in situ hybridization. However, the mutant cells are unable to export heat shock mRNAs (Saavedra *et al*., 1997). These two examples, the role of Nup159p in poly(A) export but not in tRNA export, and the role of Nup42p in heat shock mRNA export but not in general poly(A) export, indicate that the roles of other nucleoporins in the exchange processes need to be evaluated for multiple types of RNA cargo.

Pre-tRNA splicing is highly coupled to nuclear export. Perhaps the most compelling evidence for this is accumulation of IVS-containing pre-tRNAs in yeast strains with mutations in any of several genes encoding nucleoporins (Nsp1p, Nup49p, Nup116p, Nup133p, and Nup145p; Sharma *et al*., 1996). We originally identified the *RNA1* and *LOS1* genes in searches for yeast mutants defective in pre-tRNA processing, and we and others showed that *rna1-1* and *los1* mutants accumulate intron-containing end-matured pre-tRNAs (Hopper *et al*., 1978, 1980; Knapp *et al*., 1978; Simos *et al*., 1996). In this study we demonstrate that the *rna1-1* and *los1*Δ*V* mutations cause nuclear accumulation of tRNAs encoded by genes lacking introns. Thus, *RNA1* and *LOS1* functions are also important for tRNA export independent of the effects on pre-tRNA splicing.

Los1p is located primarily in nuclei and it is a member of the importin-β family of proteins (Shen *et al*., 1993; Görlich *et al*., 1997). Recently, a human Ran binding protein, exportin-t, was shown to interact with tRNA and, when overexpressed, to facilitate export of tRNA from the nucleus to the cytosol in *Xenopus* oocytes and HeLa cells (Arts *et al*., 1998; Kutay *et al*., 1998). Human exportin-t is 19% identical to yeast Los1p (Kutay *et al*., 1998). Here we demonstrate that a disruption of the *LOS1* gene causes nuclear accumulation of mature tRNA. Thus, an excess of human exportin-t facilitates tRNA nuclear export and yeast *los1* deletion inhibits tRNA nuclear export. Hence our data is consistent with the model that Los1p and human exportin-t are functionally homologous because both affect tRNA export. Because the yeast genome contains a single *LOS1* gene and it is unessential (Hurt *et al*., 1987; Goffeau *et al*., 1997), Los1p cannot be absolutely required for tRNA export. Moreover, our results indicate that *los1* mutant cells accumulate nuclear tRNA more slowly and to a lesser extent than do *nup116* mutant cells, which have nucleopore structural defects. If Los1p is indeed the yeast exportin-t homologue, then there must be other factors, at least in yeast, that also play a role in tRNA export.

In our efforts to identify other proteins that may function like Los1p, we found the *SOL* family of genes as multicopy suppressors of *los1* mutations (Shen *et al*., 1996). However, the *SOL* genes do not appear to be involved in tRNA nuclear export (Sarkar, Stanford, and Hopper, unpublished results) or pre-tRNA splicing, because we do not see any accumulation of pre-tRNAs in the *sol* mutants by in situ hybridization (Sarkar, Stanford, and Hopper, unpublished results), and accumulation of intron-containing pre-tRNAs, observed in *los1* mutants, is not reversed by overexpressing the *SOL* genes (Shen *et al*., 1996). Using the strategy of synthetic lethality, Simos *et al.* (1996) uncovered three-way genetic interactions between *LOS1* and *PUS1*, which encodes tRNA pseudouridine synthase, and *NSP1. NSP1* encodes an essential member of the FXFG family of nucleoporins and *nsp1* mutants show defects in nuclear protein import but not nuclear export of poly(A) RNAs. A testable possibility is that *NSP1* affects tRNA nuclear export without affecting poly(A) export. It would also be valuable to determine the phenotypes of yeast strains that have lesions in *LOS1* in addition to lesions in genes encoding other members of the importin-β family.

Although the RanGTPase path has been shown to be required for nuclear exit of most RNAs, yeast heat shock mRNAs exit the nucleus by a Ran-independent path (Saavedra *et al*., 1996). The role of the RanGTPase pathway in tRNA nuclear export has been somewhat controversial. Studying cells with a mutant RanGDP/GTP exchange factor, Cheng *et al*. (1995) found that tRNA export was independent of the RanGTPase pathway. In contrast, injection of excess RanGAP into nuclei to deplete nuclei of RanGTP, led Izaurralde *et al*. (1997) to conclude that the RanGTPase pathway is necessary for tRNA nuclear export. However, even for the studies using excess nuclear RanGAP, tRNA export was affected to a lesser extent than were other RNAs such as snRNAs. Although not conclusive from our work here, it appears that *rna1-1* cells, like *los1*Δ*V* cells, accumulate nuclear tRNA slower and to lesser extent than do *nup116* mutant cells. Thus, in yeast as in higher eukaryotic cells, it may be that nuclear export of tRNAs is less dependent on the RanGTPase cycle than are other RNAs. Why the RanGTPase pathway has different effects on particular RNA substrates is an intriguing unresolved question.

How are pre-tRNA splicing and nuclear export coupled in *rna1-1* and *los1* mutants? Unless Los1p and Rna1p function at more than one step in the tRNA biogenesis pathway, the simplest explanation for their functions is that they play direct roles in tRNA nucleus/cytosol exchange and affect pre-tRNA splicing indirectly. Nuclear accumulation of intron-containing pre-tRNAs in *rna1-1* (Figure 8) and *los1*Δ*V* (our un-

published results) cells rule out one model wherein the IVS-containing pre-tRNAs accumulate because they are physically separated from the splicing machinery due to precocious movement to the cytosol. Our studies showing similar intranuclear distribution of IVS-containing pre-tRNAs in wild-type and mutant strains argue against yet another model wherein pre-tRNA accumulation in the mutant strains is caused by inappropriate subnuclear compartmentalization. A model (Sharma *et al.*, 1996) that could account for the phenotypes of *nup* mutants posits that the tRNA splicing machinery is located within nucleopore channels and is aberrant in cells with lesions of genes encoding nucleoporins. However, by this model it is more difficult to account for the coupling of nuclear export and pre-tRNA splicing evidenced by *los1* and *rna1* strains. It is possible that each alters nuclear pore structure, but there is no evidence to support this. Alternatively, it is possible that faulty export blocks upstream pre-tRNA splicing. It is not likely that splicing defects are caused by inappropriately high concentrations of mature tRNAs in nuclear export-deficient mutants because it has been demonstrated that high concentrations of mature tRNA failed to act as a competitor for endonuclease activity (Peebles *et al.*, 1979). An attractive and testable model for feedback inhibition is the possible shuttling of splicing endonuclease subunits between the nucleus and the cytosol (Trotta and Abelson, personal communication). This would result in pre-tRNA splicing dependence on continuous appropriate nucleus/cytosol exchange.

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