The nuclear tRNA aminoacylation-dependent pathway may be the principal route used to export tRNA from the nucleus in *Saccharomyces cerevisiae*

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Nuclear tRNA export in *Saccharomyces cerevisiae* has been proposed to involve three pathways, designated Los1p-dependent, Los1p-independent nuclear aminoacylation-dependent, and Los1p- and nuclear aminoacylation-independent. Here, a comprehensive biochemical analysis was performed to identify tRNAs exported by the aminoacylation-dependent and -independent pathways of *S. cerevisiae*. Interestingly, the major tRNA species of at least 19 families were found in the aminoacylated form in the nucleus. tRNAs known to be exported by the export receptor Los1p were also aminoacylated in the nucleus of both wild-type and mutant Los1p strains. FISH (fluorescence *in situ* hybridization) analyses showed that $tRNA^{Tyr}$ co-localizes with the U18 small nucleolar RNA in the nucleolus of a tyrosyl-tRNA synthetase mutant strain defective in nuclear $tRNA^{Tyr}$ export because of a block in nuclear $tRNA^{Tyr}$ aminoacylation. $tRNA^{Tyr}$ was also found in the nucleolus of a *utp8* mutant strain defective in nuclear tRNA export but not nuclear tRNA aminoacylation. These results strongly suggest that the nuclear aminoacylation-dependent pathway is principally responsible for tRNA export in *S. cerevisiae* and that Los1p is an export receptor of this pathway. It is also likely that in mammalian cells tRNAs are mainly exported from the nucleus by the nuclear aminoacylation-dependent pathway. In addition, the data are consistent with the idea that nuclear aminoacylation is used as a quality control mechanism for ensuring nuclear export of only mature and functional tRNAs, and that this quality assurance step occurs in the nucleolus.

Key words: Los1p, nucleolus, nuclear tRNA export, nuclear tRNA aminoacylation, protein translation, Utp8p.

INTRODUCTION

In eukaryotic cells, tRNA genes are transcribed by RNA polymerase III to produce pre-tRNAs. The pre-tRNA transcripts undergo trimming at the 5′ and 3′ ends, base modification, addition of CCA to their 3′ ends and, in a small percentage of the transcripts, removal of an intron. This multi-step maturation process occurs in the nucleolus, nucleoplasm and nuclear envelope in an order that is undefined (for a review see [1]). Like mRNA, tRNA maturation is an essential prerequisite for nuclear tRNA export [2–9]. However, this is now being questioned, since a recent report has provided evidence that two subunits of the endonuclease responsible for cleaving the exon–intron junction of intron-containing pre-tRNAs are located on the surface of the mitochondria in *Saccharomyces cerevisiae* [10]. Studies first conducted in *Xenopus laevis* and later in *S. cerevisiae* led to the suggestion that, in addition to tRNA maturation, aminoacylation of tRNAs in the nucleus also plays a role in nuclear tRNA export [2–6,11,12]. However, unlike tRNA maturation, nuclear aminoacylation does not appear to be absolutely required for tRNA export in both *X. laevis* and *S. cerevisiae* [2,12]. While the role of nuclear tRNA aminoacylation is not understood, it has been proposed to provide a mechanism for selecting fully matured tRNAs for export to the cytoplasm [4].

Utp8p is a nucleolar protein that plays a critical role in nuclear tRNA export in *S. cerevisiae* [13]. Depletion of Utp8p resulted in a block in nuclear export of tRNAs derived from both intronless and intron-containing pre-tRNAs, but did not affect tRNA maturation or nuclear aminoacylation. Overexpression of Utp8p also alleviated nuclear retention of $tRNA^{Tyr}$ in a temperature-sensitive TyrRS (tyrosyl-tRNA synthetase) mutant strain expressing a nuclear-import defective TyrRS mutant protein that is catalytically active. Utp8p binds tRNAs directly, but is not a nuclear tRNA export receptor, since it does not shuttle between the nucleus and cytoplasm. These findings suggest that Utp8p is required for nuclear export of both aminoacylated and non-aminoacylated tRNAs and that it acts at a step in between tRNA maturation/aminoacylation and translocation of the tRNA out of the nucleus [13]. Utp8p was proposed to function as an intranuclear factor that picks up tRNAs in the nucleolus and delivers them to the tRNA export receptors [13].

Translocation of tRNAs across the nuclear pore complex located in the nuclear envelope is facilitated by exportin-t and possibly by exportin-5 in mammalian cells, and by Los1p, the orthologue of exportin-t in *S. cerevisiae* [14–18]. These proteins are members of the β-karyopherin family of nucleocytoplasmic transport factors that bind the tRNA cargo directly in a RanGTP-dependent manner *in vitro*. Exportin-5 has been shown to facilitate nuclear export of the eukaryotic elongation factor (eEF)-1A using aminoacyl-tRNA as an adaptor. Since exportin-5 stimulates tRNA export in cells depleted of exportin-t and interacts with aminoacyl-tRNA, this suggests that exportin-5 may also function as an export receptor for aminoacylated tRNAs [17,18]. However, it is not known whether Los1p and exportin-t facilitate nuclear export of aminoacylated or non-aminoacylated tRNAs. Cca1p [ATP(CTP):nucleotidyltransferase], an essential enzyme that prepares tRNAs for aminoacylation in the nucleus, cytoplasm and mitochondrion by adding the nucleotides C, C and A to the 3′ ends of tRNAs, is also involved in nuclear export of some tRNAs in *S. cerevisiae* [19]. Cca1p is thought to function as a tRNA export receptor or an adaptor in a Los1p- and nuclear aminoacylation-independent pathway that permits export of tRNAs obtained from intronless pre-tRNAs [19]. The finding that overexpression of Cca1p did not restore export of $tRNA^{Tyr}$, a tRNA derived from

Abbreviations used: Cca1p, ATP(CTP):nucleotidyltransferase; eEF, eukaryotic elongation factor; TyrRS, tyrosyl-tRNA synthetase; U18 snoRNA, U18 small nucleolar RNA; FISH, fluorescence *in situ* hybridization.

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intron-containing pre-tRNA, in the temperature-sensitive TyrRS mutant strain expressing the nuclear import-defective TyrRS mutant protein also suggests that another unidentified Los1p- and nuclear aminoacylation-independent pathway facilitates nuclear export of tRNAs derived from intron-containing pre-tRNAs [13].

The studies reported to date suggest that nuclear tRNA export in *S. cerevisiae* is facilitated by three pathways designated Los1p-dependent, Los1p-independent nuclear aminoacylation-dependent, and Los1p- and nuclear aminoacylation-independent. However, these studies have not established whether all three pathways are used concurrently, or the identity of the receptors responsible for exporting aminoacylated tRNAs, or the tRNAs transported by each pathway. In this report we present data suggesting that the nuclear aminoacylation-dependent pathway may be the principal pathway utilized by *S. cerevisiae* to export tRNAs from the nucleus to the cytoplasm, and that Los1p is an export receptor of this pathway. It is, therefore, possible that exportin-t, the orthologue of Los1p, can also facilitate nuclear export of aminoacylated tRNAs in mammalian cells. The results also suggest that nuclear tRNA aminoacylation occurs in the nucleolus. Moreover, the data are consistent with the proposal that nuclear tRNA aminoacylation is used mainly as a quality control mechanism for ensuring nuclear export of properly folded matured tRNAs. The nuclear aminoacylation-independent pathway most likely serves an auxiliary role, which may be used when the efficiency of nuclear aminoacylation is affected or the demand for protein synthesis has increased significantly.

MATERIALS AND METHODS

Strains and plasmids

The plasmids pRSTYS1-nls1-myc and pRSTYS1-myc, and ts2 (*MATa*, *ade2-101*, *his3Δ200*, *tyr1*, *ura3-52*, *tys1-1*) were provided by Dr A. K. Hopper (Department of Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, Hershey, PA, U.S.A.) [12]. The *los1* strain was obtained from Dr E. Hurt (University of Heidelberg, Heidelberg, Germany) [8] and W303 (*MATa*, *ade2-1*, *his3-11*, *trp1-1*, *leu2-3*, *112*) was obtained from the American Type Culture Collection. BYU8 (a derivative of BY4743, *MATa*, *utp8::KAN^R*, pCEN-URA-GAL1-UTP8) was described previously [13].

Analysis of the state of aminoacylation of nuclear tRNA

The W303 and *los1* strains were grown in rich medium at 30 °C to a D_{600} of 0.8 and washed with 40 ml of 0.5 % 2-mercaptoethanol and resuspended in 40 ml of SB [1.2 M sorbitol, 10 mM EDTA (pH 8.0), 10 mM potassium phosphate (pH 7.5) and 0.1 % 2-mercaptoethanol] [13,20]. The cells were converted to spheroplasts by incubating the suspension with 5 mg of Zymolyase 100T at 30 °C with gentle agitation. The incubation mixture was centrifuged at 2500 ***g*** for 1 min. All subsequent steps were performed at 4 °C. The cells were washed with 15 ml of AMC (300 mM sodium acetate, pH 5.0, 5 mM magnesium acetate and 0.5 M sucrose) and resuspended in 25 ml of AMS (300 mM sodium acetate, pH 5.0, 5 mM magnesium acetate and 0.25 M sucrose) with 0.1 % Nonidet P-40. The cells were lysed by homogenization with a Dounce homogenizer, using 25 strokes with a loose pestle followed by 10 strokes with a tight pestle [21]. The unlysed cells were removed by centrifugation at 2500 ***g*** for 10 min. The supernatant was applied to an 8 ml AMC cushion and centrifuged at 8000 ***g*** for 10 min. The resulting nuclear (pellet) and post-nuclear (supernatant) fractions were separated, and the nuclear pellet was washed once with AMS and resuspended in 100 μl of AMS. An equal volume of phenol was added to the nuclear and post-nuclear fractions, and the mixture was vortex-mixed every 2 min for 30 s. After 10 cycles, the mixture was centrifuged and the aqueous phase was extracted with an equal volume of phenol/chloroform, followed by final extraction with an equal volume of chloroform. To the aqueous phase was added 3 vol. of 95 % ethanol. The mixture was incubated at -20 °C overnight and centrifuged at 5000 ***g*** for 30 min. The RNA precipitate was resuspended in an appropriate volume of 20 mM sodium acetate, pH 5.0. Total RNA from the nuclear and post-nuclear fractions was separated by electrophoresis on a 6.5 % polyacrylamide gel containing 8 M urea at 4 °C using 100 mM sodium acetate buffer, pH 5.0, and transferred on to Nytran Plus membranes. Deacylated tRNA marker was prepared by incubating nuclear and cytoplasmic RNA in 50 mM Tris/HCl, pH 9.0, at 37 °C for 1 h. Hybridization was performed at 37 °C overnight using 5′-end ^{32}P-labelled oligonucleotide [$(1–2) \times 10^6$ c.p.m./ml] specific for the mature tRNA [13,21].

FISH (fluorescence *in situ* hybridization) analysis of the cellular location of tRNAs

The *tys1^{ts}* (ts2) strain carrying the pRSTYS1-myc or pRSTYS1-nls1-myc plasmid was grown to mid-logarithmic phase at 25 °C in CS medium [11,13] lacking uracil and containing 2 % glucose, and then incubated at 37 °C for 3 h. *utp8* (BYU8) carrying pCEN-URA-GAL1-UTP8 was grown in CS medium containing 2 % raffinose and 200 μg/ml G418 to mid-exponential phase at 30 °C, and then in CS medium containing 2 % raffinose and 2 % glucose or 2 % galactose for 6 h. Hybridization was carried out at 37 °C for 12 h in hybridization buffer [4 × SSC, 50 % formamide, 10 % dextran sulphate, 125 μg/ml *Escherichia coli* 5 S rRNA, 500 μg/ml salmon sperm DNA, 0.5 units/μl RNasin (Promega) and 1 × Denhardt's solution] [11,13] containing 0.5 pmol/μl 5′-end fluorescein-labelled U18 snoRNA (U18 small nucleolar RNA)-specific oligonucleotide (5′-GGTCTTACTTCCCATC-3′) and 0.5 pmol/μl 5′-end Cy3-labelled oligonucleotide specific for mature tRNA^{Tyr} (5′-GCGAGTCGAACGCCCGATCTCAAGATTTACAGTCTTGCGCCTTAAACCAACTTGGCTACC-3′). The slides were viewed under a 60 × objective lens of a Nikon Eclipse 6600 microscope. The images were recorded using a Coolsnapfx monochrome charge-coupled-device digital camera (Roper Scientific) and processed using Metamorph (Universal Imaging).

RESULTS

The major tRNA species from 19 families are present mainly in the aminoacylated form in the nucleus of *S. cerevisiae*

tRNA^{Tyr} and tRNA^{Gly} are the only tRNAs that have been shown definitively to be exported by the nuclear aminoacylation-dependent pathway in *S. cerevisiae* [12,13]. Consequently, a biochemical approach capable of separating the aminoacylated and non-aminoacylated forms of tRNA was utilized to identify tRNAs exported by the nuclear aminoacylation-dependent and -independent pathways of *S. cerevisiae*. To preserve the ester bond linking the amino acid to the tRNA during preparation of nuclei, and extraction of total nuclear tRNA, acidic conditions were used [13,21]. We and others have shown previously that nuclei isolated by the sucrose-density centrifugation fractionation procedure used in this study are essentially free of cytosolic contaminants [20,21]. The portion of cellular tRNAs in the nuclear fraction obtained from *S. cerevisiae* is approx. 1–5 % (results not shown).

Table 1 tRNA species analysed

tRNA species	tDNA anti-codon	Major or minor species	Intron
Ala1	AGC	Major	−
Arg2	ACG	Major	−
Asn	GTT	Major	−
Asp	GTC	Major	−
Cys	GCA	Major	−
Gln1	TTG	Major	−
Glu3	TTC	Major	−
Gly1	GCC	Major	−
His2	GTG	Major	−
Ile2	AAT	Major	−
Ile1	TAT	Minor	+
Leu3	CAA	Major	+
Lys2	TTT	Major	+
Met3	CAT	Major	−
Phe	GAA	Major	+
Pro1	TGG	Major	+
Ser2	AGA	Major	−
Thr1a	AGT	Major	−
Trp	CCA	Major	+
Tyr	GTA	Major	+
Val1a	AAC	Major	−

To assess the aminoacylation status of each nuclear tRNA species (Table 1) at steady state, total nuclear RNA was isolated from exponentially growing *S. cerevisiae* cells (Figure 1A, lanes 1) and separated by PAGE under acidic conditions to prevent deacylation of the tRNAs; this electrophoretic system has been used extensively to monitor the aminoacylation status of tRNAs from a number of organisms, including yeast [4,6,13,23,24]. Each tRNA family, except the Asn, Asp, Cys, Phe, Trp and Tyr families, consists of one major and several minor species. For our analysis we focused on the major tRNA species of each family (Table 1). The aminoacylated and non-aminoacylated forms of the major tRNA species from the 20 families were detected by Northern blot analysis using an oligonucleotide that is complementary to nucleotides of the anticodon stem and loop of tRNAs derived from intronless pre-tRNAs, and with an oligonucleotide that is complementary to nucleotides of the anticodon stem loop and the variable loop of tRNAs made from intron-containing pre-tRNAs. Total cytosolic RNA treated with base to cleave the ester bond between the amino acid and the tRNA served as the source of deacylated tRNA markers (Figure 1A, lanes 2). We have shown previously that nuclear aminoacyl-tRNA^{Tyr}, a tRNA derived from intron-containing pre-tRNA and -tRNA^{Gly} obtained from intronless precursor, co-migrated with their corresponding cytosolic aminoacylated form during electrophoresis under acidic conditions [13]. In addition, nuclear and cytosolic aminoacylated tRNA^{Tyr} or tRNA^{Gly} deacylated by base treatment has the same electrophoretic mobility and they migrated faster than their aminoacylated form [13].

Figure 1(A) shows that 19 of the 20 major tRNA species in the nucleus are present primarily in the aminoacylated form. The nuclear tRNAs are aminoacylated, since they co-migrate with their corresponding cytosolic aminoacylated form (see Figure 1B for analysis of tRNA^{Trp}, compare lanes 1 and 2 with lane 3); deacylation by treatment with base also caused the nuclear tRNAs to co-migrate with their cytosolic deacylated form (see Figure 1B for analysis of tRNA^{Trp}, compare lanes 4 and 5). This finding indicates that the difference in mobility between the nuclear tRNAs and their cytosolic deacylated forms is not due lack of modification or processing of the tRNAs. A small amount of the deacylated form was observed for the majority of the nuclear tRNAs. This is most likely due to the steady-state rate of tRNA aminoacylation being slower than that of tRNA synthesis and maturation.

In contrast to the other tRNAs, nuclear tRNA^{Cys} appears to be deacylated, since it co-migrates with the deacylated tRNA^{Cys} marker (Figure 1A2, bottom right-hand panel). To verify that nuclear tRNA^{Cys} is deacylated, the mobility of nuclear and cytosolic tRNA^{Cys} was compared. Both nuclear (Figure 1C, lane 1) and cytosolic (Figure 1C, lane 2) tRNA^{Cys} have similar mobility and co-migrate with deacylated tRNA^{Cys} (Figure 1C, lane 3). This result, combined with other reports showing that the aminoacylated and non-aminoacylated forms of tRNA^{Cys} are resolved by the electrophoretic system employed, suggests that tRNA^{Cys} is present in the deacylated form in both compartments [25]. Since the major tRNA species from 19 families are found in the aminoacylated form, it is plausible that tRNA^{Cys} is also aminoacylated in the nucleus. Deacylation of tRNA^{Cys} could have occurred during extraction of the RNA from nuclei by acid-catalysed intramolecular cleavage of the ester linkage by the side-chain thiol group. Thus it is conceivable that the major tRNA species from all 20 families are exported from the nucleus in the aminoacylated form.

Nuclear aminoacylation of tRNAs known to be exported by Los1p is not affected by loss of Los1p function

tRNA^{Met}, derived from intronless pre-tRNA, and the minor tRNA^{Ile} species and tRNA^{Leu}, obtained from intron-containing pre-tRNAs (Table 1), are thought to be exported to the cytoplasm by the Los1p-dependent pathway, based on the finding that these tRNAs are retained in the nucleus of a *los1* mutant strain [11,19]. Since tRNA^{Met} and tRNA^{Leu} obtained from nuclei of wild-type *S. cerevisiae* are found in the aminoacylated form, this suggests that Los1p facilitates nuclear export of aminoacylated tRNAs. To investigate whether aminoacylation of these tRNAs is affected by loss of Los1p function, we assessed the aminoacylation status of nuclear tRNA^{Met}, tRNA^{Leu} and the minor tRNA^{Ile} species in a *los1* mutant strain (Figure 2). This analysis showed that the nuclear tRNAs (lane 1) are also present mainly in the aminoacylated form. A small amount of nuclear tRNA^{Met}, tRNA^{Leu} and tRNA^{Ile} are not aminoacylated (compare lane 1 with lane 2), but not in appreciably higher proportion than the wild-type strain (compare tRNA^{Met} and tRNA^{Leu} in lane 1 of Figures 1 and 2). These results combined with those presented in Figure 1 are consistent with Los1p being a receptor of the aminoacylation-dependent nuclear tRNA-export pathway.

Mature tRNA^{Tyr} is retained in the nucleolus of a TyrRS mutant strain defective in nuclear export of the RNA

FISH has detected nuclear retention of mature tRNA^{Tyr} in a mutant strain (*tys1^{ts}*) harbouring a chromosomal temperature-sensitive *tys1* gene and expressing a catalytically active TyrRS mutant protein (TYS1-nls1) that is defective in nuclear import [12,13]. Retention of tRNA^{Tyr} in the nucleus of the mutant strain is in part due to lack of nuclear aminoacylation of the RNA, since the synthetase is not efficiently imported into the nucleus [12]. Therefore, to investigate the possibility that tRNA aminoacylation occurs in the nucleolus, FISH was used to assess whether mature tRNA^{Tyr} retained in the nucleus co-localizes with U18 snoRNA in the *tys1^{ts}* TYS-nls1 strain (Figure 3). This strategy was used to show that pre-tRNA maturation occurs in the nucleolus of *S. cerevisiae* [26]. The cellular location of mature tRNA^{Tyr} was detected with a Cy3-labelled-oligonucleotide, which is specific

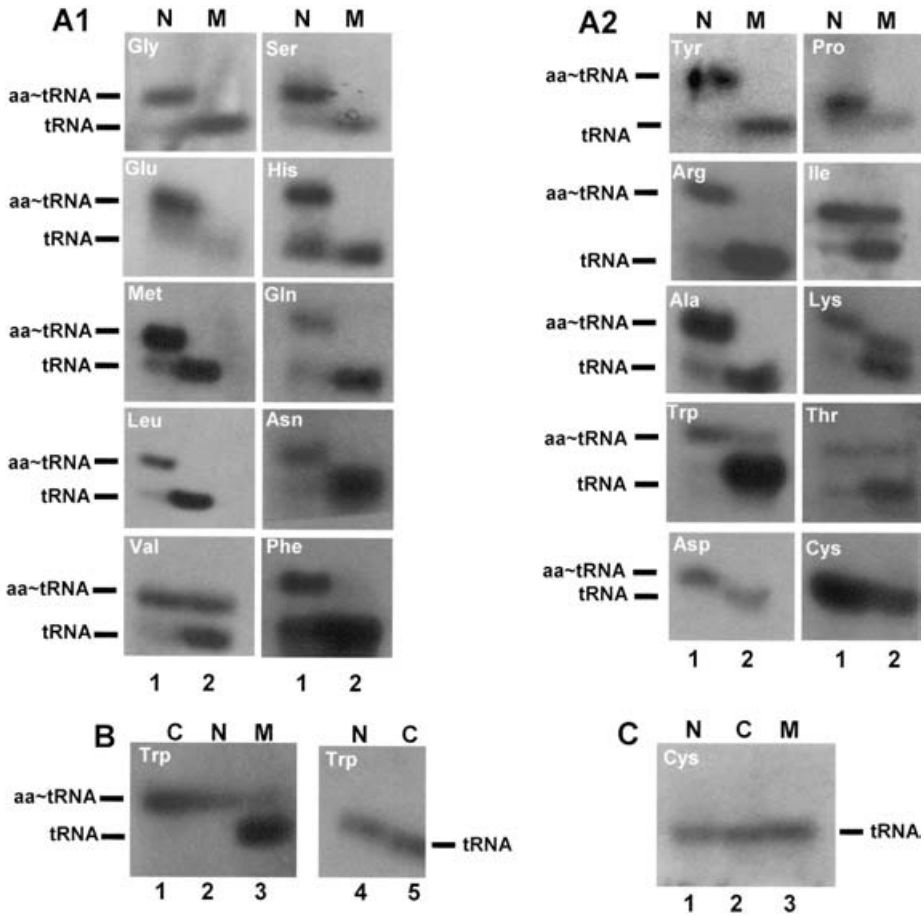

Figure 1 Analysis of the aminoacylation status of all major nuclear tRNAs obtained from wild-type *S. cerevisiae*

(**A**) Analysis of all 20 tRNAs. An aliquot of total nuclear RNA (5–10 μg; N, lanes 1) and total cytosolic RNA (5–10 μg) treated with base to deacylate the tRNAs (M, lanes 2) were separated by electrophoresis on 6.5 % polyacrylamide gels containing 8 M urea at 4 °C and transferred to Nytran membranes. Each blot was probed with a mature tRNA-specific oligonucleotide to detect the aminoacylated (aa ~ tRNA) and non-aminoacylated (tRNA) forms of the tRNA. (**B**) Electrophoretic mobility of nuclear and cytosolic $tRNA^{Trp}$. Total RNA from cytosol (C, lane 1), and nuclei (N, lane 2), deacylated cytosolic RNA (M, lane 3), and deacylated nuclear (N, lane 4) and cytosolic RNA (C, lane 5) was separated by electrophoresis on a 6.5 % polyacrylamide gel containing 8 M urea at 4 °C and transferred to Nytran membrane. The blot was probed with an oligonucleotide specific for mature $tRNA^{Trp}$. (**C**) Analysis of $tRNA^{Cys}$. Total RNA from nuclei (N, lane 1), cytosol (C, lane 2) and total cytosolic RNA treated with base (M, lane 3) was analysed as described above. The tRNAs derived from intron-containing pre-tRNAs are $tRNA^{Tyr}$, $tRNA^{Leu}$, $tRNA^{Lys}$, $tRNA^{Phe}$, $tRNA^{Pro}$ and $tRNA^{Trp}$.

for the mature RNA (Figure 3, upper left panels) whereas that of U18 snoRNA was monitored with a fluorescein-labelled probe (Figure 3, upper right panels). As reported previously, $tRNA^{Tyr}$ was detected mainly in cytoplasm of the *tys1*^{ts} strain expressing the wild-type TyrRS enzyme provided *in trans* whereas the RNA is concentrated in the nucleus of the *tys1*^{ts} strain expressing the mutant TyrRS (compare the top left panels of Figures 3A and 3B) [12,13]. Overlay analysis of the $tRNA^{Tyr}$ and U18 snoRNA signals obtained from the *tys1*^{ts} TYS1 strain indicated that the two RNAs are not in the same compartment, even in those cells showing some amount of $tRNA^{Tyr}$ in the nucleus. In contrast, co-localization of the two RNAs was observed in the *tys1*^{ts} TYS1-nls1 strain. This finding suggests that non-aminoacylated $tRNA^{Tyr}$ accumulates in the nucleolus of the *tys1*^{ts} TYS1-nls1 strain.

Aminoacylated $tRNA^{Tyr}$ is retained in the nucleolus of an *utp8* mutant strain defective in nuclear export

We have shown previously that Utp8p, a nucleolar protein, plays an essential role in nuclear tRNA export in *S. cerevisiae*. Depletion of Utp8p in *utp8* harbouring a centromeric vector with the *UTP8* gene under the control of the *GAL1* promoter blocked nuclear export of $tRNA^{Tyr}$ [13]. This defect, however, was not due to a lack of nuclear aminoacylation or maturation of the tRNA [13]. Thus co-localization analysis using FISH was performed to ascertain whether aminoacylated $tRNA^{Tyr}$ is retained in the nucleolus of *utp8*. As shown before, nuclear export of $tRNA^{Tyr}$ was not affected in *utp8* expressing Utp8p, and the RNA does not co-localize with the U18 snoRNA (Figure 4, left-hand panels). $tRNA^{Tyr}$ accumulates in the nucleus of *utp8* depleted of Utp8p and the RNA co-localizes with U18 snoRNA (Figure 4, right-hand panels). This finding suggests that depletion of Utp8p causes nucleolar retention of aminoacyl-tRNAs.

DISCUSSION

Nuclear tRNA export in *S. cerevisiae* is thought to involve three pathways designated Los1p-dependent, Los1p-independent nuclear aminoacylation-dependent and Los1p- and nuclear aminoacylation-independent. Of these, the Los1p- and nuclear aminoacylation-independent pathway has been proposed to employ two potential mechanisms for facilitating tRNA export from the nucleus. The first involves Cca1p for export of only

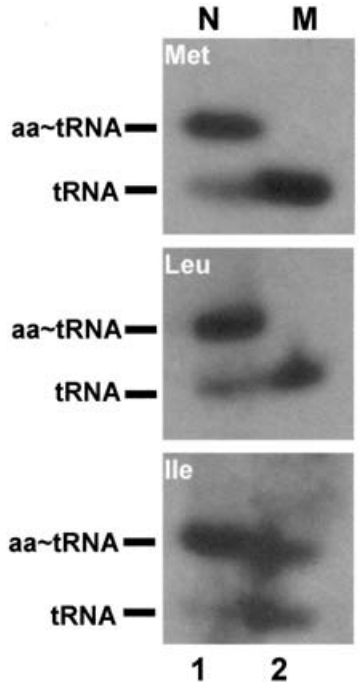

Figure 2 Analysis of the aminoacylation status of $tRNA^{Met}$, $tRNA^{Leu}$ and $tRNA^{Ile}$ in the nucleus of a *los1* mutant strain

An aliquot of total nuclear RNA (5–10 μg; N, lane 1) and total cytoplasmic RNA (5–10 μg) treated with base to deacylate the tRNAs (M, lane 2) was separated and analysed as described in the legend of Figure 1.

tRNAs derived from intronless pre-tRNAs, and the second is the participation of an unidentified protein for export of tRNAs made from intron-containing pre-tRNAs [13,19]. As a first step to understanding the relative contribution of each pathway to overall nuclear tRNA export, a biochemical approach was used to ascertain the aminoacylation status of the major tRNA species of the 20 families in the nucleus of *S. cerevisiae* (Table 1) [13]. Interestingly, tRNAs from 19 families were found mainly in the aminoacylated form in the nucleus (Figure 1). This finding suggests that it is likely that most species of each tRNA are aminoacylated in the nucleus, and implies that the nuclear aminoacylation-dependent pathway is the primary pathway used to export tRNAs from the nucleus to the cytoplasm in *S. cerevisiae*. This conclusion is in accordance with the notion that nuclear aminoacylation serves as a quality control mechanism necessary for ensuring nuclear export of fully matured and functional tRNAs [4], and with reports suggesting that nuclear export of tRNAs by the aminoacylation-independent pathway is rather inefficient [11,12]. Nevertheless, we cannot rule out the possibility that some minor tRNA species are exported from the nucleus by the aminoacylation-independent pathway. Furthermore, the data are consistent with Los1p being a component of the aminoacylation-dependent export pathway. Based on the results of the present study we also suggest that it is possible that the nuclear aminoacylation-dependent pathway is mainly responsible for exporting tRNAs from the nucleus in mammalian cells.

The export receptor that facilitates nuclear export of aminoacylated tRNAs in *S. cerevisiae* is not known. In mammalian cells, exportin-5 may export aminoacylated tRNAs, since it can bind aminoacylated tRNAs *in vitro* and stimulate tRNA export in cells depleted of exportin-t [17,18]. Presently, it is not known whether exportin-t is involved in the aminoacylation-dependent pathway. Los1p has been shown to facilitate nuclear export of $tRNA^{Leu}$, $tRNA^{Met}$ and the minor species of $tRNA^{Ile}$, and the finding that overexpression of methionyl-tRNA synthetase or Cca1p restored export of $tRNA^{Met}$ in a *los1* mutant strain suggest that Los1p exports these tRNAs in the non-aminoacylated form [11,19]. We have found that $tRNA^{Leu}$, $tRNA^{Met}$ and $tRNA^{Ile}$ are in the aminoacylated form in the nucleus of the *los1* strain (Figure 2). Since $tRNA^{Met}$ and $tRNA^{Leu}$ are also aminoacylated in the nucleus of the wild-type strain (Figure 1), this excludes the possibility that in *los1* the tRNAs are aminoacylated because they have been rerouted to the aminoacylation-dependent export pathway.

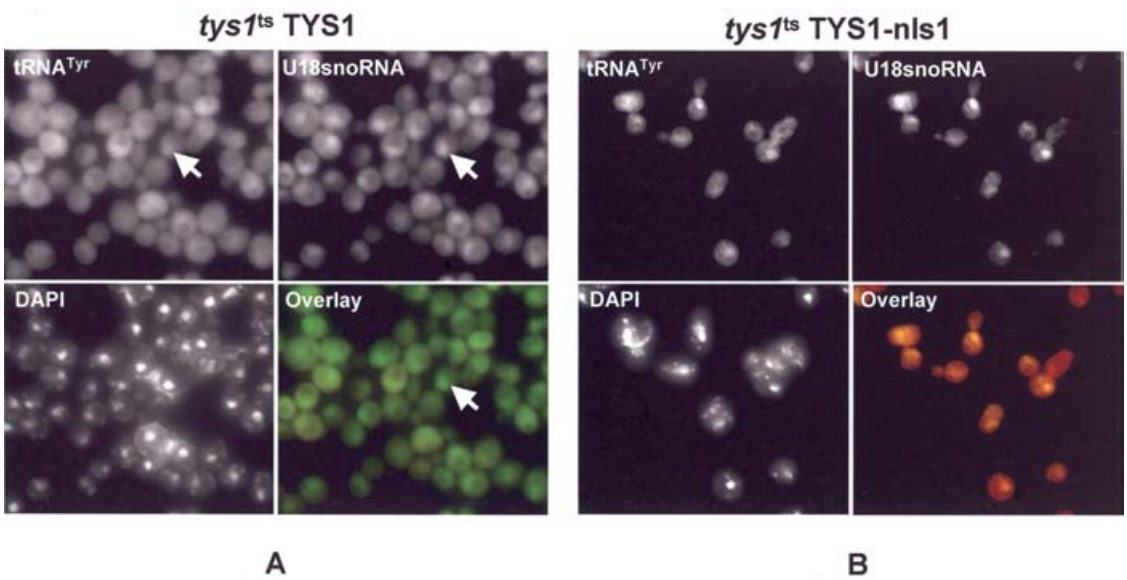

Figure 3 FISH analysis of co-localization of $tRNA^{Tyr}$ and U18 snoRNA in a TyrRS mutant strain

The *tys1*^{ts} TYS1 and *tys1*^{ts} TYS1-nls1 strains were grown in selection medium at 25 °C to mid-logarithmic phase and shifted to 37 °C for 3 h. The cellular location of $tRNA^{Tyr}$ and U18 snoRNA was detected by FISH. Co-localization of the two RNAs was determined by overlay analysis of the two images. DAPI, 4,6-diamidino-2-phenylindole.

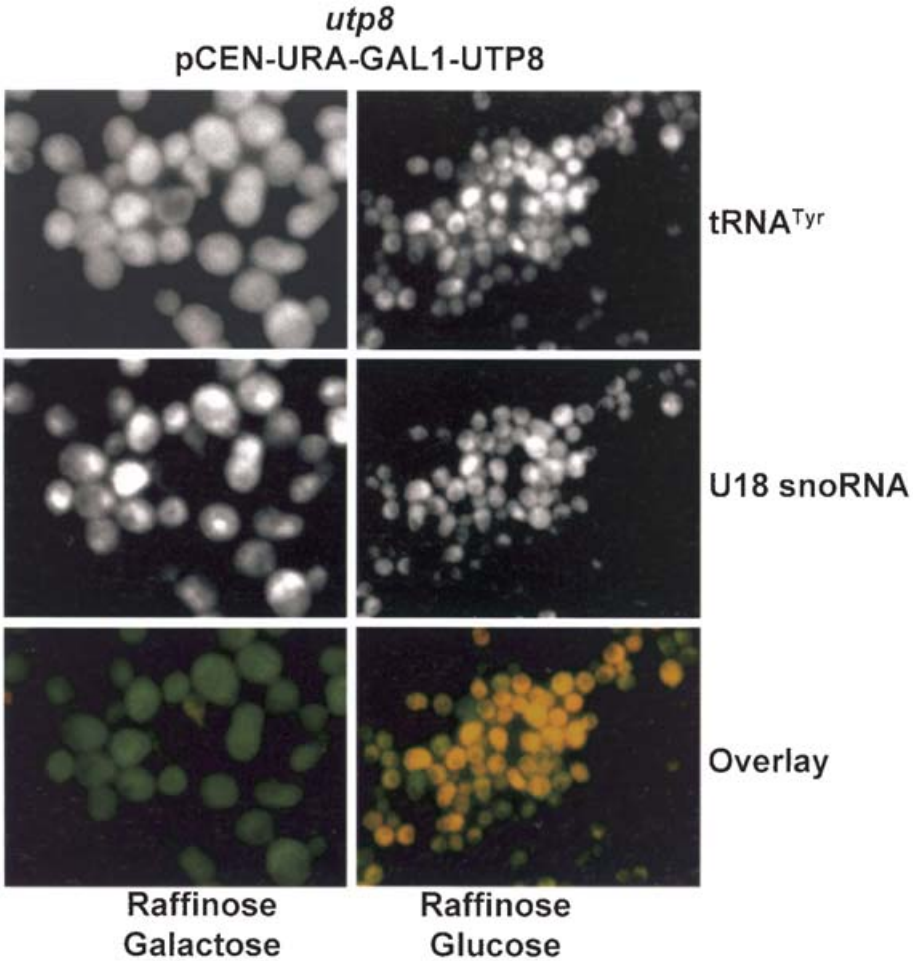

Figure 4 FISH analysis of co-localization of aminoacylated tRNA^{Tyr} and U18 snoRNA in *utp8* depleted of Ut8p

The *utp8* strain was grown in minimal medium containing 2 % raffinose and 2 % galactose or 2 % glucose for 6 h at 30 °C and the cellular location of tRNA^{Tyr} and U18 snoRNA was detected by FISH. Co-localization of the two RNAs was determined by overlay analysis of the two images.

Thus the most likely explanation of the results is that Los1p exports aminoacylated tRNAs and is an export receptor of the nuclear aminoacylation-dependent export pathway. However, our results do not exclude the possibility that Los1p can also export non-aminoacylated tRNA when the efficiency of the nuclear aminoacylation step is impaired. These results, combined with genetic and cell biological studies that have shown that loss of Los1p function is not lethal and that tRNA^{Gly} and tRNA^{Glu} are not substrates for Los1p, suggest that an additional receptor is utilized by the nuclear aminoacylation-dependent pathway to transport tRNA to the cytoplasm in *S. cerevisiae* [11,27]. This receptor is not Msn5p, the yeast homologue of exportin-5, since nuclear retention of tRNAs was not observed in a *msn5* strain [19]. Since Los1p appears to be a receptor of the nuclear aminoacylation-dependent pathway, this suggests that its orthologue exportin-t may also be competent for nuclear export of aminoacylated tRNAs in mammalian cells.

Co-localization studies using FISH detected mature tRNA^{Ile} and tRNA^{Leu} in the nucleolus of *S. cerevisiae* defective in nuclear tRNA export due to loss of Los1p function or inhibition of tRNA^{Leu} and tRNA^{Ile} aminoacylation by amino acid starvation and inhibition of isoleucyl-tRNA synthetase activity, respectively, but not by inhibition of protein synthesis [11]. However, the significance of this finding was not understood. We also showed by FISH analysis that mature tRNA^{Tyr} co-localizes with the U18 snoRNA in the nucleolus of the *tys1^{ts}*TYS1-nls1 strain that is defective in nuclear aminoacylation of tRNA^{Tyr} (Figure 3). tRNA^{Tyr} was also retained in the nucleolus of the *utp8* mutant strain, which is defective in nuclear tRNA export but not nuclear aminoacylation (Figure 4) [13]. Furthermore, we found tRNA^{Leu} and tRNA^{Ile} primarily in their aminoacylated forms in the nucleus of *los1* (Figure 2). These results taken altogether strongly suggest that nuclear aminoacylation is occurring in the nucleolus of *S. cerevisiae*. By comparison, one may predict that this step in mammalian cells occurs in the nucleolus. This possibility is consistent with the finding of methionyl-tRNA synthetase in the nucleolus of mammalian cells [28]. Why the nuclear aminoacylation step should take place in the nucleolus is unclear. A likely possibility is that the final tRNA maturation step, which is not presently known, occurs in this compartment. The data also suggest that nuclear tRNA export is initiated from the nucleolus. By analogy to ribosome biogenesis and export (for review see [29]), it is possible that the aminoacylated tRNAs are transported from the nucleolus to the tRNA export receptors by a component that links the tRNA maturation and proofreading apparatus with the tRNA export machinery. Such a step was proposed to involve

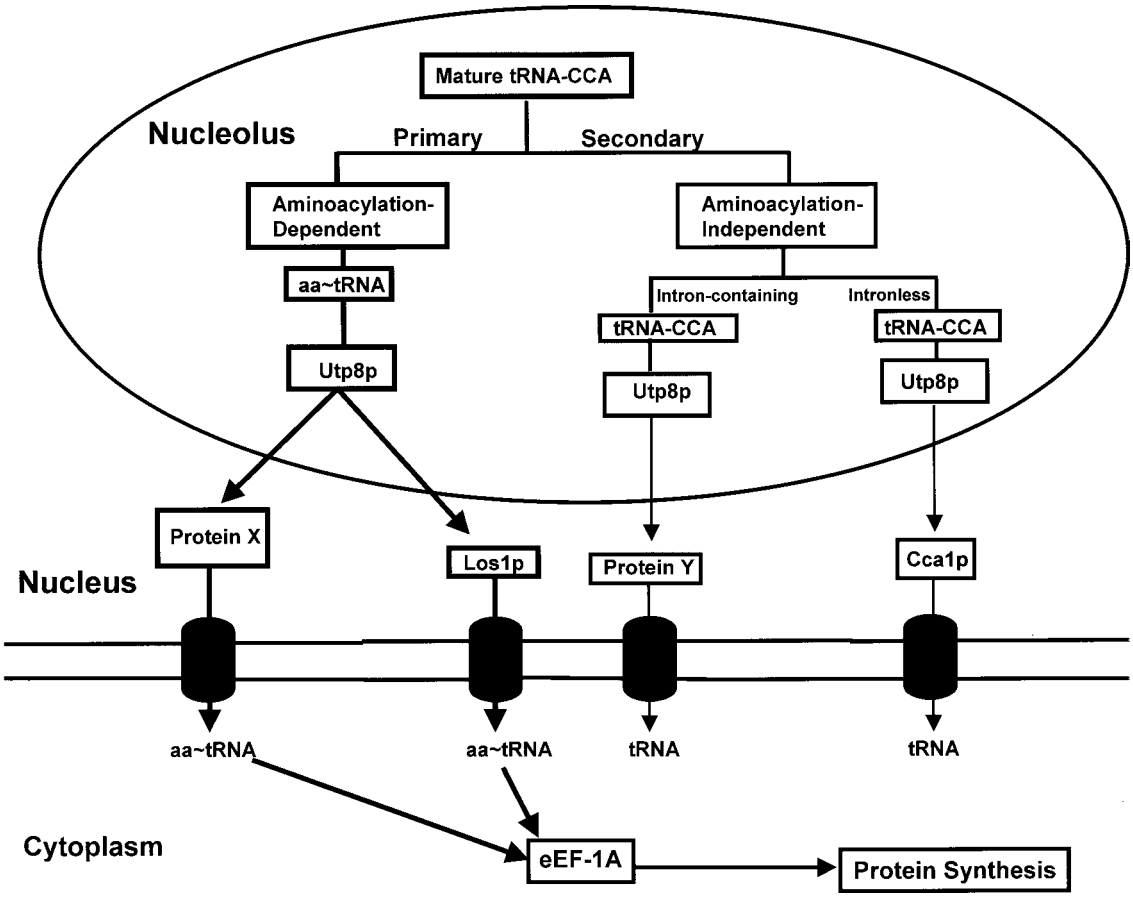

Figure 5 Model of nuclear tRNA export in *S. cerevisiae*

The model is based on results obtained in the present study as well as from those reported previously.

the nucleolar tRNA-binding protein Utp8p, which is absolutely required for nuclear export of all species of tRNA and acts at a step in between tRNA maturation/aminoacylation and translocation out of the nucleus [13]. This interpretation is consistent with the finding that $tRNA^{Tyr}$ is retained in the nucleolus of *utp8* depleted of Utp8p. However, further work will be required to definitively prove that Utp8p functions as an intranuclear factor that picks up tRNAs in the nucleolus and delivers them to the export receptors.

eEF-1A binds and delivers cytoplasmic aminoacyl-tRNAs to the ribosome. eEF-1A is encoded by two identical genes *TEF1* and *TEF2*, in *S. cerevisiae*. Inactivation of either gene causes yeast cells to grow slowly, whereas disruption of both genes is lethal [11]. Disruption of the *TEF2* gene has been shown to cause partial nuclear retention of mature tRNAs derived from both intronless and intron-containing pre-tRNAs [11]. In contrast, inhibition of protein synthesis did not result in a block in nuclear tRNA export [11]. These results led to the suggestion that eEF-1A is involved in nuclear tRNA export in *S. cerevisiae*. However, the function of the protein in nuclear tRNA export is not understood [11]. The finding that tRNAs are exported from the nucleus in the aminoacylated form suggests that they are used directly in protein synthesis upon entering the cytoplasm. It is possible that the role of eEF-1A in nuclear tRNA export is to pick up aminoacyl-tRNAs exiting the nucleus and deliver them directly to the ribosomes. This is consistent with studies showing that the mammalian eEF-1A does not function as a tRNA export receptor or as an adaptor in tRNA translocation across the nuclear pore complex [17,18]. Thus the nuclear accumulation of tRNA observed when the cellular level of eEF-1A was decreased by disruption of the *TEF2* gene could be accounted for by an accumulation of transport pools on the cytoplasmic side of the nuclear pore complex which in turn resulted in nuclear accumulation [11].

A model of nuclear tRNA export in *S. cerevisiae*, which is consistent with data obtained from this study and those reported previously, is shown in Figure 5. Following completion of the final tRNA maturation step, the tRNAs are subjected to a proofreading step to ensure that they are fully matured and properly folded. This quality assurance step occurs in the nucleolus and is mainly accomplished by aminoacylation of the tRNA. tRNAs that are aminoacylated are picked up by Utp8p in the nucleolus and delivered to the export receptors Los1p and/or Protein X, which translocates the tRNA across the nuclear pore complex. Upon entering the cytoplasm, the aminoacyl-tRNAs are picked up by eEF-1A and taken directly to the ribosomes. The nuclear aminoacylation-independent pathway most likely serves a back-up role, which may be used when the efficiency of nuclear aminoacylation is reduced or the demand for protein translation has increased dramatically. The non-aminoacylated mature tRNAs derived from intronless and intron-containing pre-tRNAs are delivered by Utp8p to Protein Y and Cca1p. However, it is possible that the receptors of the aminoacylation-dependent pathway could also facilitate nuclear export of non-aminoacylated tRNAs. After translocation across the nuclear pore complex, the tRNAs enter the cytoplasmic pool of deacylated tRNAs,

which are then aminoacylated by cytoplasmic aminoacyl-tRNA synthetases and utilized in protein synthesis.

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