Nucleotides of the tRNA D-stem that play an important role in nuclear-tRNA export in *Saccharomyces cerevisiae*

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Nuclear export of tRNA in *Saccharomyces cerevisiae* involves Los1p and Arc1p. Los1p facilitates tRNA translocation across the nuclear pore complex whereas Arc1p plays a role in delivering some species of tRNA exiting the nucleus to their cognate aminoacyl-tRNA synthetases. Here, we show that mutations of C11 and G24 of the D-stem of the yeast tyrosine amber-suppressor tRNA have different effects on nuclear export of the tRNA. Changing G24 had no effect on export of the tRNA to the cytoplasm. In contrast, mutating C11 resulted in nuclear retention of the tRNA. Nuclear retention of the tRNA mutants was not due to lack of processing, since only the mature forms of the tRNA mutants were found. The fact that mutations of G24 did not affect export of the tRNA also indicates that the effect of mutating C11 is not due to gross alteration of the tertiary structure resulting from disruption of the C11/G24 base pair.

Expression of Los1p and the mammalian tRNA export receptor exportin-t rescued nuclear export of the tRNA with changes at position 11. The export-defective mutations of the tRNA mutants were suppressed by introducing the complementary nucleotides at position 24. Taken together, these findings suggest that C11 is important for binding of the tRNA to the export receptor, and that this binding is influenced by the conformation of the base. Finally, the export-defective tRNA mutants described can be used as reporters to identify eukaryotic proteins involved in the nuclear-tRNA export process, and characterize the molecular interactions between known receptors and the tRNA substrate.

Key words: export-defective tRNA mutants, exportin-t, Los1p, tRNA export receptors.

INTRODUCTION

Macromolecular movement between the nucleus and the cytoplasm of eukaryotes occurs through the nuclear pore complex (NPC), and is a protein-mediated energy-dependent process (for review, see [1–4]). The factors that facilitate nuclear transport of proteins and nucleic acids are called import/export receptors, and many have been identified in both mammalian and yeast cells [1–4]. These receptors are frequently members of the β -karyopherin family of nuclear import/export receptors. β -Karyopherins contain an N-terminal Ran-binding domain (RBD) motif and their function is regulated by RanGTPase, a small Ras-like protein [1–5].

Like other classes of RNA, only mature tRNAs are exported from the nucleus [6–12]. In eukaryotic cells, pre-tRNA transcripts are synthesized by RNA polymerase III [13]. 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 [14,15]. Maturation of the 5' end is performed by endonuclease RNase P whereas 3'-end trimming is accomplished by an unknown endonuclease [14]. There are some reports suggesting that these reactions occur in the nucleoplasm and nucleolus [16]. Removal of the intron is an atypical process that is not spliceosome-dependent. tRNA splicing in *Saccharomyces cerevisiae* has been shown to involve excision of the intron by an endonuclease, ligation of the two parts of the tRNA by a tRNA ligase and removal of a 2'-phosphate contained in the ligated tRNA by an

NAD-dependent phosphotransferase [15]. These splicing reactions most probably occur at the nuclear envelope since the endonuclease is a membrane protein, and the ligase and phosphotransferase were found to be associated with the inner nuclear membrane [15]. Upon completion of the maturation process, the tRNAs are transported to the cytoplasm, and delivered to their correct cytoplasmic locations. Evidence reported strongly suggests that tRNA maturation is a key checkpoint required for nuclear-tRNA export [6–12].

tRNA translocation of the NPC in *S. cerevisiae* and mammalian cells is facilitated by Los1p and exportin-t, respectively [12,17–19]. Both proteins contain an N-terminal RBD motif and bind tRNA directly in a RanGTP-dependent manner *in vitro* [5,17–19]. In contrast to exportin-t, it is not known whether Los1p accompanies the tRNA through the NPC, since there is no evidence that it shuttles between the nucleus and the cytoplasm. Genetic evidence suggests that in addition to Los1p, an unidentified receptor is involved in nuclear-tRNA export in *S. cerevisiae* [12].

Arc1p is another protein involved in the nuclear-export process in *S. cerevisiae* [20]. The protein was shown to be cytoplasmic and to interact with methionyl- and glutamyl-tRNA synthetases [20]. This interaction increased the catalytic efficiency of methionyl-tRNA synthetase by increasing its tRNA affinity. Mutational studies indicated that an N-terminal segment consisting of the first 132 residues of Arc1p is required for interaction with the synthetases, whereas the rest of the protein is involved in tRNA binding [20,21]. Based on these findings, it was proposed

Abbreviations used: NPC, nuclear pore complex; RBD, Ran-binding domain; tRNA^{Tyr_{am}}, tyrosine amber-suppressor tRNA; CSDD, complete synthetic dextrose-dropout; snoRNA, small-nucleolar RNA.

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that Arc1p is responsible for delivering tRNA^{Met} and tRNA^{Glu} from the nucleus to their cognate aminoacyl-tRNA synthetases [20,21]. However, the function of Arc1p is not essential, since the viability of *arc1* mutants were not affected [20].

Mutational analyses have identified signals in mRNA, small-nuclear RNA and rRNA that are important for their nuclear export in *Xenopus laevis* [22–24]. Similar analyses have been conducted to identify export signals in mammalian tRNAs. Zasloff and coworkers [25] have shown that nuclear export of the human initiator tRNA in *X. laevis* oocytes was severely impaired when nucleotides 19, 22 and 24 of the D-stem and nucleotides 52, 53, 56 and 57 of the T-stem and loop were mutated individually, suggesting that these nucleotides are nuclear-export-recognition signals. Recent studies *in vitro* showed that exportin-t had reduced affinity for tRNA^{Lys} when nucleotides 50, 52, 56 and 57 of the T-stem and loop were mutated individually [7]. The findings imply that these nucleotides may interact directly with exportin-t. Recent RNA protection studies also indicate that nucleotides of the acceptor stem and the D-stem may participate in tRNA binding to exportin-t [6].

Nucleotides in *S. cerevisiae* tRNAs that are important for their nuclear export have not been defined. In this work we used an RNA nucleocytoplasmic distribution approach to identify nucleotides in an *S. cerevisiae* tyrosine amber-suppressor tRNA (tRNA^{Tyr_{am}}) that are required for tRNA export. Of the nucleotides investigated only mutations of C11 of the D-stem completely blocked export of the tRNA to the cytoplasm in *S. cerevisiae*. This effect of the mutations to C11 is not due simply to a structural change, since mutations of G24, which forms a base pair with C11, did not affect nuclear export of the tRNA. The fact that overproduction of Los1p and exportin-t rescued nuclear export of the tRNA with changes at nucleotide 11 suggests that C11 is involved in binding of the tRNA to the nuclear-export receptor. This binding is not strictly dependent on the nature of the base; the essential determinant appears to be the conformation of the base of nucleotide 11, which is dictated by pairing of the bases between nucleotides 11 and 24.

MATERIALS AND METHODS

Strains, media and plasmids

The *S. cerevisiae* HEY301-129 (a *met8-1*, *trp1-1*, *his4-580*, *leu2-3,112*, *ura3-1*, *ade1*, *can1-100*) was described previously [26,27]. The *met8*, *trp1* and *his4* alleles contain amber mutations. Transformants of HEY301-129 were grown in complete synthetic dextrose-dropout (CSDD) medium lacking the component required for selection of the plasmid. The plasmid pYX242 carrying a *LEU* selection marker was obtained from Novagen. The 2 μ *URA3* yEPlac195 vector was described previously [28]. The *Escherichia coli* XL1-blue and DH5 α strains were purchased from Stratagene and New England Biolabs, respectively. The synthetic yeast tRNA^{Tyr_{am}} gene in the pGFIBI plasmid was obtained from Dr. U. L. RajBhandary (Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, U.S.A.). The Human Marathon-Ready cDNA was purchased from Clontech.

Site-directed mutagenesis of the tRNA^{Tyr_{am}} gene

A RNA polymerase III transcription-termination sequence of seven T bases was introduced at the 3' end of the tRNA^{Tyr_{am}} gene by PCR using the pGFtRNA^{Tyr_{am}} vector as the template. The amplified product was digested with *EcoRI* and *SphI* and cloned into the same sites in yEPlac195. Site-specific mutations were introduced into the tRNA gene on yEPlac195 using *Pfu* DNA

polymerase and the Stratagene QuikChange[™] mutagenesis protocol.

Isolation of total RNA

HEY301-129 transformants harbouring the yEPlac195 vector carrying the mutant tRNA genes were grown overnight at 30 °C in CSDD medium lacking uracil. An aliquot of the culture was diluted 50-fold into 100 ml of CSDD medium lacking uracil and grown at 30 °C to an A_{600} of 0.6. The cells were pelleted by centrifugation at 2000 *g* for 15 min at 4 °C, and total RNA was prepared as described in [29]. To the cell pellet was added 2 ml of a solution containing 0.5 M NaCl, 0.2 M Tris/HCl (pH 7.4), 10 mM EDTA, 2 g of glass beads (425–600 nm) and 2 ml of phenol/chloroform (1:1). The RNA was released from the cells by vigorous vortexing for 1 min. Phase separation was achieved by centrifugation at 3000 *g* for 10 min. The aqueous phase was removed and the remaining phenol layer was re-extracted with 2 ml of the same buffer. The pooled aqueous layers were then extracted with 4 ml of chloroform. The RNA in the aqueous phase was precipitated with 0.4 ml of 3 M lithium acetate and 10 ml of 95 % ethanol at –20 °C for 12 h. The RNA pellet was dissolved in an appropriate volume of TE (10 mM Tris/HCl, pH 8.0/1 mM EDTA), pH 8.0.

Distribution of tRNA^{Tyr_{am}} mutants between the nucleus and cytoplasm

Nuclear and post-nuclear fractions were prepared by sucrose density centrifugation [29]. HEY301-129 transformants harbouring the yEPLAC195 carrying the mutant tRNA^{Tyr_{am}} genes were grown in 500 ml of CSDD medium lacking uracil to an A_{600} of 0.6 and pelleted by centrifugation. The cells were washed with 40 ml of 0.5 % β -mercaptoethanol and resuspended in 40 ml of SB [1.2 M sorbitol, 10 mM EDTA (pH 8.0), 10 mM KPO₄ (pH 7.5) and 0.1 % β -mercaptoethanol]. 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. The cells were washed with 15 ml of HMC [25 mM Hepes (pH 7.6), 5 mM magnesium acetate and 0.5 M sucrose] and resuspended in 25 ml of HMS [25 mM Hepes (pH 7.6), 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. The unlysed cells were removed by centrifugation at 2500 *g* for 10 min. The supernatant was applied to an 8-ml HMC cushion and centrifuged at 8000 *g* for 10 min. The resulting nuclear (pellet) and post-nuclear (supernatant) fractions were removed and an equal volume of sterile water was added. An equal volume of hot (65 °C) phenol was added and the mixture was incubated at 65 °C for 20 min with vortexing every 2 min for 30 s. The mixture was centrifuged and the aqueous phase was extracted with an equal volume of phenol:chloroform, followed by a final extraction with an equal volume of chloroform. To the aqueous phase was added 1/10 volume of 4 M sodium acetate, EDTA to a final concentration of 20 mM and 3 volumes 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 TE, pH 8.0.

Northern-blot analysis

The RNAs were separated on a 10 % polyacrylamide gel containing 8 M urea, and transferred electrophoretically on to Nytran Plus membrane (0.2 μ m; Schleicher and Schuell) at 20 V

Figure 1 Strategy for identification of $\text{tRNA}^{\text{Tyr}}_{\text{am}}$ mutants defective in nuclear export in *S. cerevisiae*

NE, nuclear envelope; U18, U18 small-nucleolar RNA; Cyto., cytoplasm; Nuc., nucleus. Suppression refers to translation of amber premature termination codons in reporter genes in *S. cerevisiae*.

for 180 min using 40 mM Tris/acetate/2 mM Na_2EDTA (pH 8.1) as the transfer buffer. The membrane was rinsed briefly with $6 \times \text{SSPE}$ ($1 \times \text{SSPE} = 0.18 \text{ M NaCl}/10 \text{ mM NaH}_2\text{PO}_4/1 \text{ mM Na}_2\text{EDTA}$) and then baked at 80°C for 1–2 h. The membrane was incubated at 37°C for 4 h in 20 ml of pre-hybridization solution consisting of $4 \times \text{SSPE}$, 250 $\mu\text{g}/\text{ml}$ sheared and denatured salmon sperm DNA, 0.1% SDS and $10 \times \text{Denhardt's}$ solution [$1 \times \text{Denhardt's}$ solution = 0.02% BSA/0.02% poly(vinylpyrrolidone) 40/0.02% Ficoll]. Hybridization was performed at 37°C overnight in 10 ml of pre-hybridization solution containing 5'-end [^{32}P]-labelled oligonucleotide ($1\text{--}2 \times 10^6$ c.p.m./ml). The membrane was washed twice for 30 min at room temperature and once for 30 min at 38°C with $1 \times \text{SSPE}$ and 0.1% SDS and subjected to autoradiography. The mutant $\text{tRNA}^{\text{Tyr}}_{\text{am}}$ was detected with an oligonucleotide complementary to nucleotides 29–47 of the $\text{tRNA}^{\text{Tyr}}_{\text{am}}$ (see Figure 2 below).

Co-expression of nuclear tRNA-export receptors and mutants of $\text{tRNA}^{\text{Tyr}}_{\text{am}}$ in *S. cerevisiae*

A DNA fragment containing the open reading frame of Los1p was obtained by PCR amplification of *S. cerevisiae* chromosomal DNA. A Los1p fragment lacking the codons for the first 150 amino acid residues ($\text{LOS1}\Delta\text{N150}$) was prepared by PCR amplification of chromosomal DNA. The PCR products were digested with *NcoI* and *BamHI* and ligated into the same sites of pYX242. An *EcoRI*–*XhoI* fragment containing the open reading frame of human exportin-t was obtained by PCR amplification of Human Marathon-Ready cDNA, and ligated into the same sites of

pYES2. An *NcoI*–*BamHI* fragment of the exportin-t open reading frame was obtained from the pYES2-exportin-t vector and ligated into the same sites of pYX242. In the pYX242 vector, the genes are under the control of the triose phosphate isomerase promoter and the transcription-termination sequence. The pYX242 recombinant plasmids were introduced into the HEY301-129 strain harbouring the yEPLAC195 vector carrying the various mutant tRNA genes using the lithium acetate method [30].

RESULTS

Strategy used to identify mutations affecting nuclear export of the yeast $\text{tRNA}^{\text{Tyr}}_{\text{am}}$

The yeast $\text{tRNA}^{\text{Tyr}}_{\text{am}}$ gene used in this study lacks the intron found in the natural gene. Expression of $\text{tRNA}^{\text{Tyr}}_{\text{am}}$ from this gene suppresses amber mutations in genes of enzymes involved in tryptophan and histidine biosynthesis in *S. cerevisiae* HEY301-129. This allows the strain to grow in the absence of one or all of the required amino acids. Thus the HEY301-129 strain will not grow under these conditions if a mutation affects nuclear export or delivery of the $\text{tRNA}^{\text{Tyr}}_{\text{am}}$ to its cognate aminoacyl-tRNA synthetase (Figure 1). In contrast, if the mutation does not affect export and utilization of the tRNA, the strain is able to grow in the absence of one or the other amino acid. To confirm that a particular tRNA mutant is defective specifically in nuclear export, RNA was extracted from nuclear and post-nuclear fractions, and Northern-blot analysis was used to detect the $\text{tRNA}^{\text{Tyr}}_{\text{am}}$.

Figure 2 Nucleotide sequence of the *S. cerevisiae* tRNA^{Tyr}_{am}

The mutated nucleotides are shown in boxes. Mutations in the main body of the tRNA are coupled to mutations in the anticodon sequence. The CUA anticodon sequence allows tRNA^{Tyr} to translate UAG premature termination codons. The tRNA with only the anticodon sequence mutation is designated the wild-type tRNA.

Table 1 Mutants of tRNA^{Tyr}_{am} used in this study

Nucleotides	Mutations
C11	A11, U11, G11
C12	U12
D17	G17
D17a	G17a
G23	A23
G24	A24, U24, C24
G53	A53
G57	U57, C57
C61	U61
C11/G24	A11/U24, U11/A24, G11/C24
C12/G23	U12/A23
G53/C61	A53/U61

Growth of transformants expressing the tRNA^{Tyr}_{am} mutants on amber selection medium

The nucleotides mutated in the tRNA^{Tyr}_{am} are shown in Figure 2, and a list of the tRNA mutants is provided in Table 1. These nucleotides were chosen because they have been shown to be important for nuclear export of the human initiator tRNA in *X. laevis* oocytes [25]. Transformants expressing the G11, A11 and U11 tRNA mutant genes from the high copy number 2μ yEPLAC195 vector did not grow on medium lacking Trp or His (Figure 3A). Similarly, no growth was observed for transformants expressing the A24, C24 and U24 tRNA mutants. In contrast, the U12, G17, G17a and A23 tRNA mutants were as active in

Figure 3 Growth of *S. cerevisiae* HEY301-129 expressing mutants of tRNA^{Tyr}_{am} on amber-suppression medium

HEY301-129 transformants harbouring the yEPLAC195 vector without or with the wild-type (Wt) or mutant tRNA gene were streaked on CSDD medium lacking Ura, or Ura and His, or Ura and Trp, and incubated at 30 °C for 5 days.

suppression as the wild-type tRNA^{Tyr}_{am}. Suppression was also not observed when G53 was changed to A, G57 to U or C, C61 to U and G53/C61 to A53/U61 (Figure 3B). Transformants expressing the A11/U24, U11/A24 and U12/A23 tRNA mutants but not the G11/C24 tRNA mutant were capable of growth on medium lacking His or Trp (Figure 3B). The transformant

Figure 4 Northern-blot analysis of the level of tRNA^{Tyr_{am}} mutants *in vivo*

Total RNA was isolated from HEY301-129 transformants harbouring the yEPLAC195 vector without or with the wild-type or mutant tRNA gene. An aliquot containing 15 µg of total RNA was subjected to electrophoresis on a 10% polyacrylamide gel containing 8 M urea. The separated RNAs were transferred to Nytran membrane, and the tRNA^{Tyr_{am}} was detected with ³²P-labelled oligonucleotide probe complementary to nucleotides 27–43. The blot was also probed with an oligonucleotide complementary to the endogenous tRNA^{Leu}.

expressing the A11/U24 tRNA mutant appeared to grow more slowly than the others.

Level of the tRNA^{Tyr_{am}} mutants *in vivo*

The level *in vivo* of the tRNA mutants in transformants found to be incapable of growth on amber selection medium was determined by Northern-blot analysis. The probe used to detect the

tRNA^{Tyr_{am}} mutants is specific, since it did not detect the endogenous tRNA^{Tyr} in total RNA isolated from a transformant carrying the vector without the tRNA^{Tyr_{am}} gene (Figure 4, compare lanes 1 and 2). A probe for the endogenous yeast tRNA^{Leu} was used as a control to ensure that approximately the same amount of total RNA was analysed. The U57 (Figure 4, lane 13) and A53/U61 (lane 12) tRNA^{Tyr_{am}} mutants were not detected. The level of the U11 (lane 4), G11 (lane 5) and A24

Figure 5 Analysis of the extent of distribution of the tRNA^{Tyr_{am}} mutants between the nucleus and cytoplasm

RNA was isolated from nuclear and post-nuclear fractions prepared from HEY301-129 transformants carrying yEPLAC195 alone, or the vector with the wild-type or mutant tRNA gene. An aliquot containing 15–30 µg of RNA was separated by electrophoresis on a 10% polyacrylamide gel containing 8 M urea and transferred to Nytran membrane. The blot was probed with an oligonucleotide complementary to tRNA^{Tyr_{am}}. The U18 snoRNA was used to monitor the extent of breakage of the nucleus.

Figure 6 Suppression analysis of the effect of overproduction of (A) Los1p and exportin-t or (B) Los1p Δ N150 on rescue of tRNA^{Tyr}_{am} mutants defective in nuclear export

The HEY301-129 transformants were streaked on CSDD medium lacking Ura and Leu, or Ura, Leu and His, or Ura, Leu and Trp and grown at 30 °C for 5 days.

(lane 6) tRNA^{Tyr}_{am} mutants was almost comparable with that of the wild-type tRNA^{Tyr}_{am} (lane 2). The quantity of the tRNA^{Tyr}_{am} carrying A11 (lane 3) or C24 (lane 8) was much higher compared with that of the tRNA^{Tyr}_{am}. The amount of the U24 (lane 7), G11/C24 (lane 9), U11/A24 (lane 10) and A11/U24 (lane 11)

tRNA^{Tyr}_{am} mutants was significantly reduced. Therefore, the inability of transformants harbouring the genes for the U57 and A53/U61 tRNA mutants to grow on amber selection medium was due to unavailability of the suppressor tRNA. This could be due a defect in transcription, or instability of the tRNA mutants. Consequently, these tRNA mutants were not characterized further. The reduced level of the G11/C24, U11, G11, C24 and U24 tRNA^{Tyr}_{am} mutants could account for the lack of growth of the HEY301-129 strain. However, this does not appear to be the reason for the inability of transformants harbouring the genes for the A11 and C24 tRNA^{Tyr}_{am} mutants to grow on amber selection medium.

Nucleocytoplasmic distribution of tRNA^{Tyr}_{am} mutants

The nucleocytoplasmic distribution of tRNA^{Tyr}_{am} carrying changes at positions 11 and 24 was investigated to ascertain whether these mutants could also be defective in nuclear export. The RNAs prepared from nuclear and post-nuclear fractions of the various transformants were subjected to Northern-blot analyses. The blots were probed with the tRNA^{Tyr}_{am}-specific oligonucleotide and an oligonucleotide complementary to the U18 small-nucleolar RNA (snoRNA) to monitor the extent of lysis of the nucleus. The analysis showed that wild-type tRNA^{Tyr}_{am} was entirely in the cytoplasmic fraction whereas U18 snoRNA was in the nuclear fraction (Figure 5A), indicating that the cellular separation procedure results in very little lysis of the nuclei (less than 5 % lysis is typically observed). The A11 and G11 tRNA^{Tyr}_{am} mutants were retained in the nucleus (Figure 5B) whereas the A24 and C24 tRNA^{Tyr}_{am} mutants were entirely in the cytoplasm (Figure 5C). The double mutant A11/U24, which was found to allow the HEY301-129 strain to grow on amber selection medium, was entirely in the cytoplasm (Figure 5D). A longer exposure indicated that a small amount of the tRNA^{Tyr}_{am} mutant was retained in the nucleus. In contrast, a large amount of the G11/C24 tRNA^{Tyr}_{am} mutant was found in both the nucleus and the cytoplasm (Figure 5D).

Rescue of the export-defective tRNA^{Tyr}_{am} mutants by overproduction of Los1p, exportin-t and Los1p Δ N150

Rescue of nuclear export of the G11 and G11/C24 tRNA mutants by overproduction of the nuclear-tRNA-export receptors Los1p and exportin-t was investigated by monitoring the ability of HEY301-129 to grow on amber selection medium (Figure 6A). The transformant harbouring the pYX242 and yEPLAC195 vectors alone did not grow on the selection medium. Transformants harbouring the yEPLAC195 vector and pYX242 with the gene for Los1p or exportin-t also did not grow. The transformants expressing the wild-type tRNA^{Tyr}_{am} and harbouring pYX242 alone or the vector with the Los1p or exportin-t gene grew on selection medium lacking His or Trp. However, no growth was observed for transformants harbouring the yEPLAC195 vector with the G11 mutant tRNA^{Tyr}_{am} gene, and the pYX242 vector without or with the gene for Los1p or exportin-t. Nucleocytoplasmic distribution analysis clearly indicated that overexpression of Los1p significantly increased the amount of the G11 tRNA^{Tyr}_{am} mutant in the cytoplasm (Figure 7). Transformants expressing the G11/C24 tRNA^{Tyr}_{am} mutant and Los1p or exportin-t grew on medium lacking Trp but not on medium lacking His. In contrast, the transformant expressing the G11/C24 tRNA^{Tyr}_{am} mutant but not the receptors did not grow. Furthermore, deletion of the first 150 amino acids of the RBD motif of Los1p (Los1p Δ N150) resulted in a reduction in the

Figure 7 Northern-blot analysis of rescue of the export-defective G11 tRNA^{Tyr}_{am} mutant by overexpression of Los1p

RNA was isolated from nuclear and post-nuclear fractions prepared from a HEY301-129 transformant expressing the G11 tRNA^{Tyr}_{am} mutant and Los1p. An aliquot containing 30 µg of RNA was separated by electrophoresis on a 10% polyacrylamide gel containing 8 M urea, and transferred to Nytran membrane. The blot was probed with an oligonucleotide complementary to tRNA^{Tyr}_{am}. The U18 snoRNA (U18) was used as a marker to monitor the extent of breakage of the nucleus.

growth rate of the transformant expressing the G11/C24 tRNA mutant (Figure 6B). These findings suggest that rescue of export of the tRNA^{Tyr}_{am} mutants was due specifically to the overexpression of the export receptors.

DISCUSSION

Mutation of C11 of the C11/G24 base pair of the D-stem of tRNA^{Tyr}_{am} to U, G or A rendered the tRNA defective in nuclear export (Figure 5D). This effect was not due to lack of processing, since only the mature forms of the tRNA mutants were detected by Northern-blot analysis (results not shown). The fact that changing G24 to U, A or C did not affect export of the tRNA implies that the defect of the tRNA with changes at C11 was not due to gross alteration of the tRNA tertiary structure resulting from disruption of the base pair (Figure 5C). This conclusion is consistent with the observation that disruption of the adjacent C12/G23 base pair, which is also involved in base pairing with nucleotide 9, had no detectable effect on nuclear export of the tRNA^{Tyr}_{am} (Figure 3A). These results suggest that C11 is important for nuclear export of tRNA in *S. cerevisiae*, and that this nucleotide is a tRNA nuclear-export-recognition signal. This interpretation is in accordance with other studies showing that the D-stem of mammalian tRNAs contains nucleotides that are important for their nuclear export [6,7,25].

Several lines of evidence suggest that mutation of C11 is blocking binding of the tRNA to the nuclear-export receptor: (i) overexpression of both Los1p and exportin-t rescued export of the G11/C24 and G11 tRNA^{Tyr}_{am} mutants (Figure 6A and Figure 7); (ii) deletion of a segment of the RBD motif to reduce the activity of Los1p also resulted in a decrease in the level of rescue of the G11/C24 tRNA^{Tyr}_{am} mutant (Figure 6B); (iii) mutation of nucleotides U17, U17a and C12, and G23 of the C12/G23 base pair of the D-stem of the tRNA^{Tyr}_{am} had no effect on export of the tRNA, based on suppression analysis (Figure 3A); (iv) overproduction of transcription factor TFIIC, trans-

lation elongation factor eEF3, or Arc1p did not rescue export of the defective tRNA mutants (results not shown); and (v) recent evidence from studies *in vitro* suggests that nucleotides of the D-stem of a mammalian tRNA interact directly with exportin-t [7]. These data support the conclusion that C11 is involved in tRNA binding to the *S. cerevisiae* export receptor.

Nucleocytoplasmic distribution analysis showed that essentially all of the A11/U24 (Figure 5D) and U11/A24 (results not shown) tRNA^{Tyr}_{am} mutants were located in the cytoplasm, whereas the G11/C24 tRNA^{Tyr}_{am} mutant was distributed between both compartments (Figure 5D). The fact that the transformant expressing the A11/U24 or U11/A24 tRNA^{Tyr}_{am} mutant grew on suppression selection medium also confirmed that the mutants were exported to the cytoplasm (Figure 3B). Therefore, the export-defective mutation of the G11, A11 and U11 tRNA^{Tyr}_{am} mutants could be suppressed by the introduction of the complementary base at position 24. This finding suggests that binding of the tRNA^{Tyr}_{am} mutant to the nuclear-tRNA-export receptor can be restored by coupling the mutation at position 11 to a complementary mutation at position 24. Moreover, the results imply that the nature of the base of nucleotide 11 is not the important factor. The evidence suggests that the conformation of the base of nucleotide 11 is the critical determinant in the interaction of the tRNA with the nuclear-tRNA-export receptor. The particular conformation adopted by the base of nucleotide 11 is assisted by the formation of the base pair between the nucleotides at positions 11 and 24. Further studies are required to establish whether C11 contributes directly or indirectly to the tRNA-receptor interaction.

Previous studies showed that mutation of nucleotide 24 of the human initiator tRNA significantly affected nuclear export of the tRNA in oocytes [25]. Changing this nucleotide in the yeast tRNA^{Tyr}_{am} had no effect on export, irrespective of the nature of the nucleotide used (Figure 5C). The reason for this discrepancy between mammalian and *S. cerevisiae* tRNA export is not clear. A possible explanation is that there are some intrinsic differences in the nuclear-tRNA export mechanism between mammalian cells and *S. cerevisiae*. For instance, nuclear-tRNA export in mammalian cells may employ additional components that are not required in lower eukaryotes such as *S. cerevisiae*. The component(s) may also interact directly with the tRNA during the export process. Nucleotide 24 of mammalian tRNAs could play a role in this interaction. Alternatively, the mechanism of action of the mammalian and yeast nuclear export receptors may be different. The lack of significant identity between Los1p and exportin-t and the fact that exportin-t did not complement a *los1* deletion strain favour this possibility (results not shown).

A significant portion of the G11/C24 tRNA^{Tyr}_{am} mutant, and essentially all of the tRNA^{Tyr}_{am} with G24 changed to C or A were found in the cytoplasm (Figure 5C and 5D). However, these tRNA mutants did not participate in protein synthesis based on the inability of transformants to grow on amber selection medium (Figure 3). The G11/C24 tRNA^{Tyr}_{am} mutant, however, was found to participate in translation when the amount in the cytoplasm was increased by overexpression of Los1p or exportin-t (Figure 6). This implies that the G11/C24, C24 and A24 tRNA^{Tyr}_{am} mutants were also defective in a step subsequent to export. It is unlikely that this step is aminoacylation, since nucleotide 24 is not an identity element for recognition of tRNA^{Tyr} by the tyrosyl-tRNA synthetase [31]. A plausible explanation is that the mutations are affecting an important interaction before or after the aminoacylation step. This interaction could be with an elongation factor or a component of the ribosome, or an unidentified protein involved in delivering

the tRNA exiting the nucleus to its cognate synthetase. Further investigation is required to ascertain the step affected. Nevertheless, the important finding is that C11 but not G24 plays a critical role in nuclear-tRNA export in *S. cerevisiae*.

Investigation of the effects of mutations in tRNA on their nuclear export in mammalian cells were conducted by micro-injection of radiolabelled mutant RNAs into the nucleus of *X. laevis* oocytes and quantification of their nuclear and cytoplasmic distribution. The distribution analysis we have established for *S. cerevisiae* can now be used to investigate nucleotides in *S. cerevisiae* tRNAs that are important for their export. For instance, nucleotides of the acceptor stem have been shown to be involved in tRNA binding to exportin-t. The significance of these nucleotides on tRNA export in *S. cerevisiae* is not known, and therefore their effects can be studied *in vivo* in *S. cerevisiae* using our methodology. Furthermore, the use of our export-defective tRNA^{Tyr_{am}} mutants and the amber-suppression phenotypic assay, as well as the *in situ* hybridization method reported [9], will greatly facilitate the identification of unknown proteins involved in the nuclear-tRNA export process in mammalian cells and yeast. In addition, the tRNA mutants can be used to map the tRNA-binding site of known receptors such as Los1p and exportin-t by screening for mutations in the proteins that can suppress the export-defective mutation in the tRNA^{Tyr_{am}}. Studies along these lines are currently being conducted in our laboratory.

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