Utp8p Is an Essential Intranuclear Component of the Nuclear tRNA Export Machinery of Saccharomyces cerevisiae

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A yeast tRNA three-hybrid interaction approach and an in vivo nuclear tRNA export assay based on amber suppression was used to identify proteins that participate in the nuclear tRNA export process in Saccharomyces cerevisiae. One of the proteins identified by this strategy is Utp8p, an essential 80-kDa nucleolar protein that has been implicated in 18 S ribosomal RNA biogenesis. Our characterization indicated that the major function of Utp8p is in nuclear tRNA export. Like the S. cerevisiae Los1p and the mammalian exportin-t, which are proteins known to facilitate nuclear tRNA export, overexpression of Utp8p restored export of tRNA-am mutants defective in nuclear export. Furthermore, depletion of Utp8p blocked nuclear export of mature tRNAs derived from both intronless and intron-containing pre-tRNAs but did not affect tRNA and rRNA maturation, nuclear export of mRNA and ribosomes, or nuclear tRNA aminoacylation. Overexpression of Utp8p also alleviated nuclear retention of non-aminoacylated tRNA\(^{\text{Ty}}\) in a tyrosyl-tRNA synthetase mutant strain. Utp8p binds tRNA directly and saturably, indicating that it has a tRNA-binding site. Utp8p does not appear to function as a tRNA export receptor, because it does not shuttle between the nucleus and the cytoplasm. Taken together, the results suggest that Utp8p is an essential intranuclear component of the nuclear tRNA export machinery, which may channel tRNA to the various tRNA export pathways operating in S. cerevisiae.

Translocation of macromolecules between the nucleus and cytoplasm occurs through the nuclear pore complex (NPC)\(^{1}\) located in the nuclear envelope (for reviews see Refs. 1–4). Many of the components of the NPC, known as nucleoporins, as well as the soluble transporters that participate in nuclear transport of proteins and nucleic acids have been identified in both mammalian and yeast cells (1–5). The transport proteins are frequently members of the β-karyopherin family of nuclear import/export receptors, which translocate macromolecules through the NPC by interacting with specific nucleoporins, and their function is regulated by RanGTPase, a small Ras-like protein (2, 3).

Nuclear tRNA export is facilitated by exportin-t and exportin-5 in mammalian cells, and Los1p, the orthologue of exportin-t, in Saccharomyces cerevisiae (6–10). These proteins are members of the β-karyopherin family of nucleocytoplasmic transport factors and bind the tRNA cargo directly in a RanGTP-dependent manner in vitro (6–8). Exportin-t and exportin-5 are nucleoplasmic proteins, whereas Los1p is found associated with the NPC (6, 8–11). The function of Los1p is not essential, because disruption of the chromosomal LOS1 gene did not affect growth or viability of S. cerevisiae. This finding suggests that in addition to Los1p another receptor is required for nuclear tRNA export in S. cerevisiae.

Early studies in Xenopus laevis suggest that aminoacylation of tRNAs in the nucleus plays a role in nuclear tRNA export (12). This conclusion is based on the observation that tRNA\(^{\text{Ty}}\) or tRNA\(^{\text{Met}}\) microinjected in the nucleus of X. laevis is aminoacylated, and loss of nuclear aminoacylation of tRNA\(^{\text{Ty}}\) in oocytes by inhibition of the tyrosyl-tRNA synthetase (TyrRS) (12) led to a significant decrease in the efficiency of nuclear export of the RNA. Moreover, aminoacyl-tRNA synthetases have been detected in the nucleus of mammalian cells (13). Mutants of tRNA\(^{\text{Tyr}}\) that are defective in aminoacylation, however, were found to be exported to the cytoplasm after injection into the nucleus of X. laevis oocytes (14). This finding suggests that nuclear tRNA aminoacylation is not absolutely required for tRNA export in mammalian cells. Aminoacylated tRNAs were also detected in the nucleus of an S. cerevisiae nup116 mutant strain defective in nuclear export (15). In addition, nuclear retention of tRNA was observed in several aminoacyl-tRNA synthetase mutant strains and in wild type strains when aminoacylation was blocked by amino acid starvation (16, 17). Subcellular fractionation detected TyrRS in the nucleus of S. cerevisiae (18). In addition, the enzyme was shown to contain a nuclear localization signal (18). Mutation of the nuclear localization signal caused a reduction in the nuclear pool of the protein as well as a block in nuclear export of tRNA\(^{\text{Ty}}\) (18). However, this mutation did not affect the aminoacylation activity of the enzyme or the viability of the cells (18). These results provided very good evidence that nuclear aminoacylation also plays a role in nuclear tRNA export in S. cerevisiae, but it is not absolutely required. Nuclear tRNA aminoacylation may constitute a Los1p-independent export pathway, because overexpression of the methionyl-tRNA synthetase restored export of tRNA\(^{\text{Met}}\) but not tRNA\(^{\text{Tyr}}\) in the los1 mutant strain (19).
The receptor that facilitates nuclear export of aminoacylated tRNAs in *S. cerevisiae* is not known.

The ATP (CTP):nucleotidyltransferase (Cca1p) is 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′-terminal ends of tRNAs. This maturation step, but not aminoacylation itself, appears to be absolutely required for tRNA export in both mammalian cells and *S. cerevisiae*, and because it has been shown that exportin-t preferentially binds tRNAs with the 3′,3′-diadenylate structure, the loss of aminoacylation function of Cca1p is not known.

Experimental Procedures

**Strains and Plasmids**—The 2-μm *p*YX242 vector with a *LEU2* selection marker and *p*ET19b were obtained from Novagen. The plasmid *p*II-MS2-1 was provided by Dr. M. Wickens, Department of Biochemistry, University of Wisconsin (23). The *p*IIex242-*tRNA*<sub>Tyr</sub> plasmid containing the *URA3* selection marker was obtained from Dr. P. Good, Department of Biological Chemistry, University of Michigan (24). The *p*RS416-*CEN-URA3* vector was purchased from Invitrogen and converted to plasmids (pACT2 carrying the *S. cerevisiae* UTP8 is a gift of Dr. M. Wickens, Department of Biochemistry, University of Wisconsin (23). The *p*RS416-CA1p plasmid containing the *URA3* selection marker was obtained from Dr. P. Good, Department of Biological Chemistry, University of Michigan (24). The *p*RS416-*CEN-URA3* vector was purchased from Invitrogen and converted to plasmids (pACT2 carrying the *S. cerevisiae* UTP8 was a gift of Dr. M. Wickens, Department of Biochemistry, University of Wisconsin (23). The *p*RS416-CA1p plasmid containing the *URA3* selection marker was obtained from Dr. P. Good, Department of Biological Chemistry, University of Michigan (24). The *p*RS416-*CEN-URA3* vector was purchased from Invitrogen and converted to plasmids (pACT2 carrying the *S. cerevisiae* UTP8 was a gift of Dr. M. Wickens, Department of Biochemistry, University of Wisconsin (23).

**List of yeast strains**

| Strain | Genotype | Source |
|--------|----------|--------|
| HEY301–129 | MATa, met8–1, trp1–1, his4–580, leu2–3,112, ural3–1, ade1 | Dr. M. Wickens, Department of Biochemistry, University of Wisconsin (23) |
| L40<sub>mut</sub> | MATa, ura3–2, leu2–3,112, his3Δ200, trp1Δ1, ade2, lys2Δ200, His3, ura3::LexA<sub>op</sub>–LexZ, LexA-MS2 coat (TRP1) | Dr. E. Hurt, University of Heidelberg (11) |
| kar1–1 | MATa | Dr. E. Hurt, University of Heidelberg (11) |
| BY4743 | MATa, MATa, his3Δ1, his3Δ1, leu2Δ0, leu2Δ0, MET15ΔD0, lys2Δ200, His3, ura3Δ0, ura3Δ0, Utp8::KAN<sup>R</sup>/UTP8 | Research Genetics |
| BY4742 | MATa, his3, leu2, lys2, ura3 | Dr. E. Hurt, University of Heidelberg (11) |
| los1 | MATa, MATa, his3Δ, lys3Δ1, his3–1, trp1–1, leu2–3,112, can100 | This study |
| pus1 | MATa, MATa, his3Δ, lys3Δ1, his3–1, trp1–1, leu2–3,112, can100 | This study |
| BYU8 | pU8::KAN<sup>R</sup>, pCEN-URA-GAL1-UTP8, MATa | American Type Culture Collection |
| BYU | pU8::KAN<sup>R</sup>, pCEN-URA-GAL1-UTP8, pCEN-URA-GAL1-UTP8 | American Type Culture Collection |
| W303 | MATa, ade2–1, his3Δ200, tyr1, ura3–52, tly1–1 | Dr. A. K. Hopper, Pennsylvania State University (18) |
| ts2 | MATa, ade2–101, his3Δ200, tyr1, ura3–52, tly1–1 | Dr. A. K. Hopper, Pennsylvania State University (18) |

**Table I**

| Strain | Genotype | Source |
|--------|----------|--------|
| HEY301–129 | MATa, met8–1, trp1–1, his4–580, leu2–3,112, ural3–1, ade1 | Dr. M. Wickens, Department of Biochemistry, University of Wisconsin (23) |
| L40<sub>mut</sub> | MATa, ura3–2, leu2–3,112, his3Δ200, trp1Δ1, ade2, lys2Δ200, His3, ura3::LexA<sub>op</sub>–LexZ, LexA-MS2 coat (TRP1) | Dr. E. Hurt, University of Heidelberg (11) |
| kar1–1 | MATa | Dr. E. Hurt, University of Heidelberg (11) |
| BY4743 | MATa, MATa, his3Δ1, his3Δ1, leu2Δ0, leu2Δ0, MET15ΔD0, lys2Δ200, His3, ura3Δ0, ura3Δ0, Utp8::KAN<sup>R</sup>/UTP8 | Research Genetics |
| BY4742 | MATa, his3, leu2, lys2, ura3 | Dr. E. Hurt, University of Heidelberg (11) |
| los1 | MATa, MATa, his3Δ, lys3Δ1, his3–1, trp1–1, leu2–3,112, can100 | This study |
| pus1 | MATa, MATa, his3Δ, lys3Δ1, his3–1, trp1–1, leu2–3,112, can100 | This study |
| BYU8 | pU8::KAN<sup>R</sup>, pCEN-URA-GAL1-UTP8, MATa | American Type Culture Collection |
| BYU | pU8::KAN<sup>R</sup>, pCEN-URA-GAL1-UTP8, pCEN-URA-GAL1-UTP8 | American Type Culture Collection |
| W303 | MATa, ade2–1, his3Δ200, tyr1, ura3–52, tly1–1 | Dr. A. K. Hopper, Pennsylvania State University (18) |
| ts2 | MATa, ade2–101, his3Δ200, tyr1, ura3–52, tly1–1 | Dr. A. K. Hopper, Pennsylvania State University (18) |
pACT2 (29). The transformed cells were plated on complete synthetic dextrose (CSD) medium lacking uracil, adenine, and histidine (CSD-Ura-Leu-His) and containing 10 mM 3-aminotriazol. Transformants appeared within 7 days of incubation at 30 °C. The His+ transformants were tested for lacZ expression using the colony lift assay as specified by Clontech.

Isolation of the pACT2 Library Plasmid from His+: The L40mut Transformants—The transformants were grown at 30 °C on CSD medium to select for the pACT plasmid. A single colony was streaked on CSD-Leu medium containing 0.1% 5-fluoro-uracil acid to select for cells lacking the pII vector carrying the tRNA-MS2 fusion gene. The pACT2 plasmid was isolated from the L40mut transformant and amplified in DH5α. DNA sequencing was performed by a BLAST search of the S. cerevisiae genome database to provide the complete DNA sequence and identity of the cloned genes.

Amber Suppression Analysis of the Effect of Overproduction of Utp8p on Nuclear Export of tRNA^Ser_3 Mutants Defective in Export in S. cerevisiae—The yEFL1C195 plasmid with and without the gene for the wild type tRNA^Ser_3 or the G11.C24 tRNA^Ser_3 mutant was electroporated into a HEY301-129 transformant carrying pXY242-UTP8 or pXY242-LOSI, and transformants were selected on CSD-Leu-Ura medium. The transformants were grown at 30 °C in CSD-Leu-Ura medium and suppression of amber codons in the trp1 gene in HEY301-129 was assessed by growth of the transformants on CSD-Leu-Ura-Trp (21).

Overproduction of a Wild Type Utp8p Mutant Strain—The BY4743 (MATα his3Δ2Δ1 leu2Δ0 ura3Δ0) and mutant pACT2-His+ transformant carrying pXY242-Utp8p or pXY242-LOSI, and transformants were selected on CSD-Leu-Ura medium. The transformants were grown at 30 °C on CSD-Leu-Ura medium and suppression of amber codons in the trp1 gene in HEY301-129 was assessed by growth of the transformants on CSD-Leu-Ura-Trp (21). The His+ transformants were streaked on CSD-Leu-Ura medium and suppression of amber codons in the trp1 gene in HEY301-129 was assessed by growth of the transformants on CSD-Leu-Ura-Trp (21).

Fluorescence in Situ Hybridization (FISH) Analysis of the Nuclearcytoplasmic Distribution of tRNAs—The utp8 strain (BY21C) carrying the pCEN-URA-GAL1-UTP8 plasmid was grown overnight at 30 °C in CS medium lacking Ura and containing 2% raffinose and 200 μg/ml G418. The cells were diluted to an A600 of 0.1 in CS medium lacking Ura and containing 2% raffinose, 2% galactose, and 200 μg/ml G418.

Overproduction and Purification of Utp8p Containing an N-terminal His Tag—The His6-tagged Utp8p was overproduced in E. coli BL21 (DE3) pLysS (PAM Systems) at 30 °C (200 × g for 10 min) using the pET6a-His plasmid. A transformant carrying the pET6a-His-UTP8 vector was grown overnight at 37 °C in Luria-Bertani medium containing 100 μg/ml ampicillin and 30 μg/ml chloramphenicol. The culture was diluted 50-fold into 2 liters of 2YT containing the antibiotics and grown at 37 °C until the culture reached an A600 of 0.6. Expression of the His6-tagged Utp8p was induced for 20 min using 0.02 μM isopropyl-1-thio-galactopyranoside (IPTG) and the aqueous extract was precipitated by addition of 3 volumes of 10% TCA and resuspended in 40 ml of 20 mM Tris-HCl, pH 7.5, buffer containing 15 mM imidazole, 100 mM NaCl, and a mixture of protease inhibitors (Roche Applied Science) (binding buffer). The cells were lysed at 70,000 kPa using a French press, and unlysed cells and debris were removed by centrifugation at 10,000 × g for 10 min at 4 °C (30). The supernatant was clarified by Falcon® A30 centrifugation at 3000 × g (bed volume). The clarified supernatant was pre-equilibrated with binding buffer. The column was washed with 20 ml of binding buffer, followed by 20 ml of binding buffer containing 50 mM imidazole. The bound protein was eluted from the column with binding buffer containing 300 mM imidazole and dialyzed against 20 mM HEPES buffer, pH 7.6, containing 150 mM NaCl at 4 °C. Immunoprecipitation antibodies against purified Utp8p were prepared by ResGen, Huntsville, AL.

Western Blot Analysis of Utp8p Expression—The utp8 mutant strain harboring the pCEN-URA-GAL1-UTP8 vector was grown overnight at 30 °C in CS medium lacking Ura and containing 2% raffinose and 200 μg/ml G418. An aliquot of the culture was diluted to an A600 of 0.1 in CS medium lacking Ura and containing 2% raffinose and 2% galactose at 30 °C. At the required times, an aliquot of the culture corresponding to the same number of cells, based on A600, was pelleted and washed with water. The cells were lysed in 7.4% β-mercaptoethanol and 1.5% NaOH as described (27), and the proteins were precipitated with 10% trichloroacetic acid. The protein precipitate was washed three times with water by boiling for 5 min in 62.5 mM Tris-HCl, pH 6.8, buffer containing 5% SDS (w/v), 10% glycerol (v/v), and 0.02% bromphenol (v/v). The proteins were separated on a 10% PAGE and transferred electrophoretically to Protran nitrocellulose membrane. Utp8p was detected with a rabbit anti-Utp8p antibody using the ECL detection system (Amersham Biosciences).

Northern Blot Analysis of the State of tRNA Processing and Maturat—The utp8 and UTP8 (BYU) strains carrying the pCEN-URA-GAL1-UTP8 plasmid were grown overnight at 30 °C in CS medium lacking Ura and containing 2% raffinose and 200 μg/ml G418. The cells were diluted to an A600 of 0.1 in CS medium lacking Ura and containing 2% raffinose and 2% galactose and grown at 30 °C for 6 h. The cells were washed with 40 ml of 2% galactose and grown at 30 °C for 6 h. The haploids were screened for Ura7 by growth on CSD-Leu-Ura-Ura-Trp (21). The transformed cells were plated on complete synthetic media containing 8 μg/ml 1X TBE at room temperature (21) or on a 6.5% polyacrylamide gel containing 8 μg/ml at 4 °C using 100 mM sodium acetate buffer, pH 5.0 (21, 31–34). The separated RNAs were transferred electrophoretically onto Nytran membrane. The membranes were incubated at 37 °C for 4 h in pre-hybridization solution containing 4X SSPE (1X SSPE = 0.18 M NaCl, 10 mM NaH2PO4, 1 mM Na EDTA), 250 μl/ml sheared and denatured salmon sperm DNA, 0.1% SDS, and 1X Denhardt’s solution (1X Denhardt’s = 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone 40, and 0.02% Ficoll; Hybridization was performed at 37 °C overnight in pre-hybridization solution containing 5-end 32P-labeled oligonucleotide (1–2 × 106 cpm/ml). The membranes were washed twice for 30 min at room temperature and once for 30 min at 38 °C with 1X SSPE and 0.1% SDS andsubject to autoradiography.

Analysis of the State of tRNA Accumulation in the Nucleus—The utp8 strain carrying the pCEN-URA-GAL1-UTP8 plasmid was grown overnight at 30 °C in CS medium lacking Ura and containing 2% raffinose and 200 μg/ml G418. The cells were diluted to an A600 of 0.1 in CS medium lacking Ura and containing 2% raffinose and 2% galactose and grown at 30 °C for 6 h. The nuclear and post-nuclear fractions were isolated as described (21). The cells were washed with 40 ml of 0.5% β-mercaptoethanol and resuspended in 5B (1.2 μM sorbitol, 10 mM EDTA, pH 8.0, 10 mM KPO4, pH 7.5, 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. All subsequent steps were performed at 4 °C. The cells were washed with 15 ml of AME (300 mM sodium acetate, pH 5.0, 5 mM magnesium acetate, 0.5 μM succrose) and resuspended in 25 ml of AMS (300 mM sodium acetate, pH 5.0, 5 mM magnesium acetate, 0.25 μM succrose) 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 lysed cells were removed by centrifugation at 100 × g for 10 min. The supernatant resulting after centrifugation was extracted by addition of 3 volumes of phenol/chloroform, followed by a final extraction with an equal volume of chloroform. To the aqueous phase was added 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 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 μl at 4 °C using 100 mM sodium acetate buffer, pH 5.0, and transferred onto Nytran Plus membranes. Northern analysis was performed as described above. 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. Purification of E. coli 5S rRNA—Total RNA was isolated from E. coli as described (32). A 500-μl aliquot of the RNA was mixed with an equal volume of loading buffer (8 M urea, 0.05% bromphenol blue, 0.05% xylene cyanol in 1× TBE) and subjected to electrophoresis on an 8% polyacrylamide gel containing 8 M urea (700 V, 3 h). The RNA bands were visualized by UV shadowing, and the band corresponding to the 5S RNA was excised. The RNA was extracted from the gel with TE, pH 8.0, by shaking at 30 °C. The RNA was precipitated and dissolved in TE, pH 8.0.

Utp8p-RNA Interaction—Substrate-induced intrinsic fluorescence quenching was used to determine whether Utp8p binds tRNA. The reaction mixtures contain 20 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 0.25 μM Utp8p, and a varying amount of a mixture of mature yeast tRNA (Sigma) or E. coli 5S rRNA (0.25, 0.5, 1, 2, 4, 6.25, 8, 12.5, 16, and 18.75 μM) and incubated for 1 h at 4 °C. Control reactions containing tRNA alone were prepared as above. Trp and Tyr fluorescence was measured using a Photon Technology International spectrofluorimeter (London, Ontario, Canada) with excitation and emission slits set to 4 nm, and excitation and emission wavelengths of 280 and 318 nm, respectively (30). The fluorescence intensity of each sample was subtracted from that of the appropriate control reaction and expressed as a percent reduction of the fluorescence intensity obtained with Utp8p alone.

\[ \text{Fluorescence}_{\text{sample}} - \text{Fluorescence}_{\text{control}} \]

RESULTS

Identification of Utp8p Using a Yeast tRNA Three-Hybrid Selection System—To identify proteins that participate in the nuclear tRNA export process in S. cerevisiae, a yeast tRNA three-hybrid selection approach was used to screen a cDNA library for genes encoding proteins that interact with tRNA (Fig. 1). The hybrid RNA gene consists of the S. cerevisiae tRNA\text{AM} \text{Tyr} gene lacking the intron sequence fused to the 5′ end of the MS2 RNA gene. The RNA polymerase III promoter of the MS2 RNA gene behind the tRNAoch gene was fused to the GAL4 activation domain (AD) was carried on pACT2. The HIS3 and lacZ reporter genes are under the control of LEXA operator (LexA op). Interaction between the LexA DBD-MS2 coat protein, the hybrid RNA, and a prospective AD-tRNA-binding protein forms a functional transcriptional factor, which activates expression of HIS3 and lacZ. Expression of HIS3 is detected by the ability of transformants to grow on minimal medium lacking His, whereas β-galactosidase activity is used to monitor lacZ expression. Transformants are unable to grow in minimal medium lacking His if the ternary complex is not formed.

S. cerevisiae. This defect is not due to lack of maturation of the tRNA and can be rescued by overexpression of Los1p or exportin-1 but not by Arc1p, the Tfc5p subunit of transcription factor TFIIB, or elongation factor eEF3 (21). To ascertain whether Utp8p could be involved in nuclear tRNA export, the effect of overexpression of Utp8p on nuclear export of the G11:C42 tRNA\text{AM}Tyr mutant was investigated. Export of the G11:C42 tRNA\text{AM}Tyr mutant was detected by growth of the transformants on Trp amber medium, which selects for amber suppression (Fig. 2A). Transformants harboring the pYX242 vector without (sector 1) or with the UTP8 (sector 4) or LOS1 (sector 7) gene and lacking the tRNA\text{AM}Tyr were unable to grow on the Trp amber medium. As expected, cells expressing the wild type tRNA\text{AM}Tyr alone (sector 2) or with Utp8p (sector 5) or Los1p (sector 8) grew on the Trp amber medium. In contrast, no amber suppression was observed for the transformant expressing the G11:C42 mutant tRNA\text{AM}Tyr alone (sector 3). However, amber suppression was observed when the G11:C42 tRNA\text{AM}Tyr mutant and Utp8p (sector 6) or Los1p (sector 9) were co-expressed.

FISH was used to determine whether overproduction of Utp8p would also facilitate export of the G11 tRNA\text{AM}Tyr mutant, which is primarily retained in the nucleus (21). Export of this tRNA mutant is also rescued by overexpression of Los1p (21). However, the G11 tRNA\text{AM}Tyr mutant does not suppress amber codons due to an additional defect, which prevents the tRNA from participating in protein synthesis (21). The G11 tRNA\text{AM}Tyr mutant was detected with a 5′-fluorescein-labeled oligonucleotide complementary to nucleotides 29–47 of the anticodon stem and loop of tRNA\text{AM}Tyr. We have shown previously (25) by Northern blot analysis that this probe is specific for the tRNA\text{AM}Tyr, and FISH analysis indicated that it did not hybridize to any significant extent to the endogenous tRNA\text{AM}Tyr in vivo (data not shown). The endogenous U18 snoRNA was used as a nuclear marker (Fig. 2B) (21). The wild type tRNA\text{AM}Tyr was found in the nucleus and cytoplasm of the cells. In transformants expressing the G11 tRNA\text{AM}Tyr mutant alone, the tRNA was found predominantly in the nucleus, as reported previously (Fig. 2B).
An Intracellular Component Involved in Nuclear tRNA Export

(21). In contrast, overproduction of Utp8p or Los1p shifted accumulation of the G11 tRNA\textsubscript{am} mutant toward the cytoplasm (Fig. 2B). Taken together, these results suggest that Utp8p plays a role in nuclear tRNA export in S. cerevisiae.

Depletion of Utp8p Blocked Nuclear Export of tRNAs Derived from Intronless and Intron-containing Pre-tRNAs but Not mRNA and Ribosomal Export or Ribosomal RNA and tRNA Maturation—Previous studies (37) have shown that disruption of the YGR128c ORF encoding Utp8p is lethal. To investigate whether depletion of Utp8p caused a block in nuclear tRNA export, a conditional utp8 mutant strain was produced by tetrad dissection of a heterozygote harboring the pCEN-URA vector containing the UTP8 gene under the control of the inducible GAL1 promoter. The utp8 strain grew when expression of Utp8p was induced by galactose (Fig. 3A). However, when expression of Utp8p was repressed by glucose, the mutant strain grew very poorly (Fig. 3A). The use of 5-fluoroorotic acid to select for cells lacking the pCEN-URA-GAL1-UTP8 vector resulted in no cell growth, even after 6 days of incubation at 30 °C (data not shown). These results confirm that the function of Utp8p is essential. The growth observed for the utp8 strain on glucose is most likely due to a low level of expression of the RUNA\textsubscript{am} gene when Utp8p was expressed (Fig. 4B, lower panel). FISH analysis showed that both tRNA\textsubscript{am} and tRNA\textsubscript{am} were detected in the cytoplasm (Fig. 3C). A low amount of Utp8p was observed after 6 h of glucose repression (Fig. 3B, upper panel, compare lanes b and c). This amount of Utp8p did not change even after an 8-h incubation of the cells in medium containing glucose (data not shown). The level of Utp8p after 6 h of depletion was considerably lower than that of the endogenous protein in the wild type UTP8 strain (Fig. 3B, lower panel). FISH analysis showed that both tRNA\textsubscript{am} and tRNA\textsubscript{am} were predominantly in the nucleus of glucose-repressed cells (Fig. 3C). In contrast, the cellular distribution of mRNA was not affected by the loss of Utp8p function (Fig. 4A). Furthermore, the large ribosomal subunits (rpL3 and rpL25) tagged with GFP were detected in the cytoplasm of the utp8 strain when Utp8p expression was repressed for 6 h (Fig. 4B, lower panel). The same localization pattern was observed prior to glucose repression and after galactose induction of Utp8p expression (Fig. 4B, upper panel). Although the overall levels of the proteins decreased, only a modest amount of rpL3 and rpL25 was observed in the nucleus when depletion of Utp8p was carried out for 24 h (Fig. 4B, lower panel). In addition, the levels of 25 S, 18 S, and 5 S rRNAs did not change significantly during the 24-h depletion period (Fig. 4C, left panel). Northern blot analyses also showed that the level of the 25 S and 18 S rRNAs did not decrease significantly during the 24-h period.
An Intranuclear Component Involved in Nuclear tRNA Export

Fig. 4. Loss of Utp8p function does not affect mRNA export or ribosome biogenesis and export. A, FISH analysis of the cellular location of mRNA. The *utp8:KAN* strain harboring the pCEN-URA-GAL1-UTP8 plasmid was grown for 6 h at 30 °C in CS medium lacking Ura and Leu and containing 2% raffinose and 2% galactose or 2% glucose. The cellular location of mRNA was detected by FISH using a 5′-end fluorescein-labeled oligonucleotide consisting of 30 dT. B, cellular location of proteins of the large ribosomal subunit. The *utp8:KAN* strains harboring pCEN-URA-GAL1-UTP8 and pRS-CEN-LEU carrying the ribosomal protein genes were grown as described above. At the times specified, the cellular location of the proteins was determined by direct fluorescence microscopy using a ×100 objective lens, analysis of ribosomal RNA processing. The *utp8:KAN* strain harboring the pCEN-URA-GAL1-UTP8 plasmid were grown at 30 °C in CS medium lacking Ura and containing 2% raffinose and 2% glucose or 2% galactose. At the specified times, total RNA was isolated, and an aliquot (2.5 μg) was subjected to 1 (upper panel) or 2% agarose gel electrophoresis, and the RNAs were visualized by staining the gels with ethidium bromide (left panel). The level of 18 S and 25 S rRNAs was assessed by Northern blot analysis after the RNAs were separated on a 1.2% agarose gel under denaturing conditions (right panel). The 18 S and 25 S rRNAs were detected with 5′-CATGCGTAAACCTTGGAGAC-3′ and 5′-CTTGGCTATTGATATGC-3′, respectively. The strain was induced (lanes 1–4). This result showed that loss of *utp8* function did not affect modification of uridine in the mature form of tRNA Trp was detected in the Utp8p wild mutant strain when expression of the *utp8* gene was repressed (lanes 1 and 3) or induced (lanes 2 and 4).

Previous studies (32, 33) have shown that an E. coli initiator tRNA mutant or the *E. coli* tRNA* 3′p* lacking a 2-methylthio-N^9-(α,2-isopentenyl)-N^1-(2-isopentenyl) modification can be easily separated from the fully modified tRNA using PAGE at pH 5.0. Therefore, this approach was also used to investigate whether Utp8p may play a role in tRNA modification. To verify that this method can discriminate between modified and unmodified yeast tRNA, we compared the electrophoretic behavior of the minor tRNA* 3′p* (UAU) present in total RNA isolated from wild type and mutant Pus1p S. cerevisiae strains (Fig. 5B, left panel). Pus1p is an intron-dependent tRNA pseudouridine synthetase, which has been shown to convert U34, U35, U36, and U27 of the minor intron-containing tRNA* 3′p* (UAU) to pseudouridine (11, 41, 42).

This modification results in CH at position 3 changed to NH. tRNA* 3′p* (UAU) from the PUS1 strain (lane 1) migrated slightly faster than the tRNA from the pus1 mutant (lane 2), showing that this electrophoretic system can separate unmodified from modified yeast tRNAs. The increased mobility of the fully modified tRNA is due to the amino group at position 3 in pseudouridine (PU^3′p = 7.5) being positively charged under the acidic conditions used.

The most frequently found modification in tRNAs is conversion of uridine to pseudouridine. To determine whether pseudouridinylation of tRNA was affected in the *utp8* strain, the electrophoretic system described above was used to assess the state of modification of tRNA* 3′p* (Fig. 5B, right panel). A single form of tRNA* 3′p* was observed in the UTP8 strain (Fig. 5B, lanes 1 and 2). This form of tRNA* 3′p* was detected in the *utp8* mutant strain when expression of the UTP8 gene under the control of the GAL1 promoter in pCEN-URA was repressed (lane 3) or induced (lane 4). This result showed that loss of Utp8p function did not affect modification of uridine in tRNA* 3′p* to pseudouridine and implies that Utp8p is not playing a role in tRNA modification. However, we cannot exclude the possibility that another type of tRNA modification is affected, because it is not known whether other types of modification can influence the mobility of tRNA during electrophoresis under acidic conditions. Nonetheless, the notion that Utp8p is not required for tRNA maturation is consistent with the observation that nuclear tRNA aminoacylation was not affected (discussed below).

Loss of Utp8p Function Did Not Affect Nuclear tRNA Aminoacylation—Nuclear tRNA aminoacylation is a requirement for certain tRNA export pathways in *S. cerevisiae*. Therefore, the effect of depletion of Utp8p on nuclear tRNA aminoacylation was investigated by Northern blot analysis of the aminoacylation status of tRNA* 3′p* and tRNA* 3′p* in total RNA obtained from nuclei prepared from the *utp8* strain when expression of
tRNA<sup>Tyr</sup> in both fractions was aminoacylated. Both tRNA<sup>Tyr</sup> (middle panel, lanes 6 and 7) and tRNA<sup>Gly</sup> (right panel, lanes 9 and 10) were present in the aminoacylated form in the nuclear (lanes 7 and 10) and cytosolic fractions (lanes 6 and 9) isolated from the utp8 strain depleted of Utp8p. These results showed that loss of Utp8p function did not significantly affect nuclear aminoacylation of tRNAs derived from intronless (tRNA<sup>Tyr</sup>) and intron-containing (tRNA<sup>Tyr</sup>) pre-tRNAs. In addition, they suggest that depletion of Utp8p did not affect tRNA maturational. The amount of tRNA<sup>Tyr</sup> in the cytoplasm was considerably lower than that in the nucleus when Utp8p expression was turned off (compare lanes 6 and 7). A significant reduction in the level of tRNA<sup>Tyr</sup> in the cytoplasm was also observed after a short exposure time. Quantification of Northern blots by PhosphorImager analyses indicated that the level of nuclear tRNA after depletion of Utp8p is ~4–5 times higher compared with that before repression of Utp8p expression (data not shown). These findings confirm that loss of Utp8p function caused a block in tRNA export from the nucleus.

**Utp8p Increased the Efficiency of Nuclear Export of tRNA<sup>Tyr</sup> in a TyrRS Mutant Strain—**TyrRS has been shown to contain a nuclear localization signal (18). Mutation of this signal reduced the nuclear level of TyrRS but did not affect the catalytic activity of the enzyme. A yeast strain (ts2) harboring a chromosomal temperature-sensitive pys<sup>1</sup> allele and expressing the mutant TyrRS protein (PYS1-nls1) was viable at 37°C, because non-aminoacylated tRNA<sup>Tyr</sup> is exported from the nucleus by another pathway (18). However, the efficiency of export of tRNA<sup>Tyr</sup> is sufficiently reduced that FISH could detect accumulation of the RNA in the nucleus of the mutant strain (18). Therefore, to obtain further proof that Utp8p is involved in nuclear tRNA export, FISH was used to investigate whether overexpression of Utp8p would improve the efficiency of nuclear export of tRNA<sup>Tyr</sup> in the ts2 TYS-nls<sup>1</sup> strain at 37°C (Fig. 7A). Consistent with previous studies, nuclear retention of tRNA<sup>Tyr</sup> was observed in the ts2 TYS-nls1 strain. Retention of the tRNA was overcome by overexpression of wild type TyrRS or Utp8p but not by Cca1p.

To verify that overexpression of Utp8p facilitates nuclear export of tRNA<sup>Tyr</sup>, Northern blot analysis was used to investigate the level of tRNA<sup>Tyr</sup> in the nuclear and cytosolic fractions prepared from the ts2 TYS-nls1, ts2 TYS, ts2 TYS-nls1 Utp8p,
Utp8p Binds tRNA Directly and Saturably in Vitro—Substrate-induced intrinsic fluorescence quenching of Tyt and Trp residues was used to investigate whether Utp8p can interact with tRNA. The analysis showed that Utp8p binds mature tRNA directly and saturably with a calculated $K_D$ of 11 nM (Fig. 8). This finding indicated that Utp8p has a tRNA-binding site. Utp8p also interacts with the E. coli 5 S rRNA but to a lower extent compared with that observed for tRNA binding. Furthermore, saturable binding to 5 S rRNA could not be achieved under the conditions employed, suggesting that the protein is interacting non-specifically with the 5 S rRNA. This property of Utp8p is not unusual, because it is well established that bona fide eukaryotic and prokaryotic tRNA-binding proteins interact non-specifically with non-cognate RNAs in vitro (43-46). Taken together, the data suggest that Utp8p has an RNA-binding site that is specific for tRNA.

**Utp8p Does Not Appear to Function as a Nuclear tRNA Export Receptor**—To ascertain whether Utp8p is a nuclear tRNA export receptor, a heterokaryon shuttling assay was used to investigate whether Utp8p shuttles between the nucleus and cytoplasm (19, 26). Xpo1p, a nuclear receptor that is involved in nuclear export of proteins containing a leucine-rich nuclear export signal, was used as a control for a protein known to shuttle between the nucleus and cytoplasm (25). The heterokaryon assay involves monitoring the movement of a protein from a donor nucleus to a recipient nucleus in heterokaryons. To avoid nuclear import of cytoplasmic Utp8p and Xpo1p into the recipient nucleus, the donor strain harboring a low copy number plasmid with UTP8-GFP or XPO1-GFP under the control of the GAL1 promoter was first grown in medium containing galactose to induce expression of the fusion proteins, and then briefly in medium containing glucose to repress the GAL1 promoter. The donor strain was then mated with a kar1-1 mutant strain, which is defective in nuclear fusion. Movement of Utp8-GFP and Xpo1p into the recipient nucleus was monitored by direct fluorescence microscopy (Fig. 9). Utp8-GFP was found in a single nucleus in essentially all heterokaryons analyzed over a 5-h period after mating was initiated. In contrast, Xpo1-GFP was present in both nuclei of heterokaryons over the same period.

**DISCUSSION**

Genetic and biochemical evidence suggest that nuclear tRNA export in *S. cerevisiae* involves multiple redundant pathways (7, 15-19, 47, 48). These pathways have been classified into two major groups. The first is Los1p-dependent and the second is Los1p-independent. The Los1p-independent pathway is thought to consist of the nuclear aminoacylation-dependent and nuclear aminoacylation-independent export pathways. The aminoacylation-dependent variant has been proposed to operate through two independent export receptor proteins. The two proposed receptors are aminoacyl-tRNA synthetase and an unidentified protein. Three potential mechanisms have been proposed for the aminoacylation-independent variant pathway. The first involves Cca1p for export of only tRNAs derived from intronless pre-tRNAs; the second involves Cca1p-facilitated export of tRNAs derived from both intronless and intron-containing pre-tRNAs; and the third is the participation of an unidentified protein for export of tRNAs made from intron-containing pre-tRNAs. Currently, the details of these mechanisms as well as their relative contribution and significance to nuclear tRNA export are poorly understood.

To identify components of the *S. cerevisiae* nuclear tRNA export machinery, we used a yeast tRNA three-hybrid selection method to screen a cDNA library for genes of proteins that...
interact with tRNA, and an in vivo nuclear tRNA export assay based on amber suppression to ascertain whether the identified proteins are playing a role in nuclear tRNA export. This strategy resulted in the identification of Utp8p, an essential 80-kDa nuclear protein. Utp8p plays a role in nuclear export of both aminoacylated and non-aminoacylated tRNAs, and it appears to act at a step between tRNA maturation/ aminoacylation and tRNA translocation out of the nucleus.

Utp8p was recently identified as part of a protein complex associated with the U3 small nuclear RNA, which is involved in processing of 18S rRNA (22). Depletion of Utp8p for an extended period was reported to cause a reduction in the amount of 18S rRNA but not 25S rRNA. This finding led to the suggestion that Utp8p may play a role in 18S rRNA biogenesis. Western blot analyses indicated that Utp8p is maximally depleted within 6 h (Fig. 3B), despite carrying out the depletion for 24 h, we could not detect any significant changes in the level of 18S rRNA (Fig. 4C). Furthermore, no defect was observed for nuclear export of rpl3 and rpl25, two ribosomal subunits that associate with the 35S pre-rRNA during the early stages of ribosomal biogenesis in the nucleus (Fig. 4B). However, a block in nuclear tRNA export was observed after depletion of Utp8p for 6 h (Fig. 3C). Although these results do not exclude the involvement of Utp8p in pre-18S rRNA maturation, they strongly suggest that the major function of Utp8p is in nuclear tRNA export and not 18S rRNA biogenesis. The significance of the involvement of Utp8p in both 18S rRNA synthesis and nuclear tRNA export is not understood and will require further studies.

Similar to Los1p and exportin-t, overexpression of Utp8p restored export of tRNA\textsuperscript{amts} mutants defective in nuclear export (Fig. 2, A and B) (21). Furthermore, loss of Utp8p function blocked nuclear export of mature tRNAs derived from both intronless and intron-containing pre-tRNAs (Fig. 3C) but not tRNA aminoacylation in the nucleus (Fig. 6). Utp8p provided in trans also alleviated nuclear retention of tRNA\textsuperscript{Tyr} in the tyr1ts mutant strain expressing a catalytically active TyrRS enzyme that is defective in nuclear import (Fig. 7). These results suggest that Utp8p could be functioning as a nuclear tRNA export receptor for both the aminoacylation-dependent and -independent nuclear tRNA export pathways operating in S. cerevisiae. A characteristic of nuclear import/export receptors is that they shuttle between the nucleus and cytoplasm. Thus, a heterokaryon shuttling assay was used to ascertain whether Utp8p shuttles between the nucleus and cytoplasm. This assay is well documented and has been used to show that Cca1p plays a role in nuclear tRNA export in S. cerevisiae (19, 26). Unlike the nuclear export receptor Xpo1p, Utp8p was found in only one nucleus of heterokaryons (Fig. 9). This suggests that either Utp8p does not shuttle between the nucleus and cytoplasm or it shuttles so slowly that the amount of the protein in the recipient nucleus could not be detected. However, the most likely explanation based on the finding that neither Los1p nor exportin-t could compensate for loss of Utp8p function (data not shown) is that Utp8p is not functioning as a nuclear tRNA export receptor. These results combined with the finding that depletion of Utp8p did not affect tRNA processing/modification (Fig. 5) and aminoacylation of tRNAs derived from intronless and intron-containing pre-tRNAs in the nucleus (Fig. 6) suggest that Utp8p acts at a step between tRNA maturation/ aminoacylation and translocation of the tRNA to the cytoplasm. This step appears to be located in the nucleolus, because the protein is found in this compartment at steady state (data not shown (22)).

Maturation of pre-tRNAs is a multistep process that occurs in the nucleolus, nucleoplasm, and nuclear envelope in an order that is undefined (35, 49–51). Recently, FISH analysis detected mature tRNAs derived from intronless and intron-containing pre-tRNAs in the nucleolus of S. cerevisiae defective in nuclear tRNA export because of a block in nuclear tRNA aminoacylation or loss of Los1p function (17). This finding suggests that tRNAs are taken to the nucleolus for final maturation and/or aminoacylation before they are exported to the cytoplasm. This observation also implies that the nucleolus is the starting point for the tRNA export process. It is possible that Utp8p is responsible for initiating tRNA export by enabling the tRNA to gain access to the components involved in translocation of the tRNA across the NPC.

Channeling is a mechanism used to spatially compartmentalize biochemical processes. This is achieved by directly transferring a substrate from one component to another within a multistep biochemical pathway. A channeling mechanism is used in mRNA and ribosome biogenesis and export, tRNA maturation, delivery of cytoplasmic tRNAs to aminoacyl-tRNA synthetases, and transfer of aminoacyl-tRNAs from aminoacyl-tRNA synthetases to ribosomes (4, 35, 48, 49, 52, 53, 54–57). This type of mechanism may be used to link the tRNA maturation system to the nuclear export apparatus. Because Utp8p binds tRNA directly (Fig. 8), we suggest that the protein may link tRNA maturation and export by functioning as an intranuclear factor that picks up both aminoacylated and non-aminoacylated tRNAs from the nucleolus and delivers them directly to the export receptors of the aminoacylation-dependent and -independent pathways, or indirectly by delivering the tRNAs to the next component(s) of the export pathways. To address this possibility, a yeast two-hybrid assay was used to test whether Utp8p interacts with Los1p or TyrRS. However, no interaction between Utp8p, Los1p, or TyrRS was observed (data not shown). This finding does not negate the proposed role of Utp8p, because it is possible that the interaction between Utp8p and Los1p or TyrRS is transient or too weak to be detected by the yeast two-hybrid interaction assay.

Recent studies have shown that Cca1p is directly involved in nuclear tRNA export in S. cerevisiae (19). Like methionyl-tRNA synthetase, overexpression of Cca1p restored nuclear export of tRNA\textsuperscript{amts} in a los1 strain. In addition, Cca1p was shown to shuttle between the nucleus and cytoplasm. The fact that tRNA\textsuperscript{amts} is derived from intronless pre-tRNA led to the suggestion that Cca1p is required for nuclear export of non-aminoacylated tRNAs derived from intronless pre-tRNAs (19). We have shown that overexpression of wild type TyrRS or Utp8p but not Cca1p restored nuclear export of tRNA\textsuperscript{Tyr}, a tRNA made from intron-containing pre-tRNA, in the tyr1ts strain expressing the nuclear import-defective TyrRS mutant protein (Fig. 7). This observation supports the conclusion that Cca1p is
involved in nuclear export of tRNAs from intronless pre-tRNAs and suggests that export of non-aminoacylated tRNAs derived from intron-containing tRNAs is facilitated by an unidentified protein.

Finally, we have established a biochemical method to assess directly the aminoacylation status of tRNAs in the nucleus. This approach is an adaptation of published methods and allows for the preparation of aminoacylated tRNAs from isolated nuclei and separation of the aminoacylated and non-aminoacylated forms of a tRNA by PAGE (21, 31, 32, 34). The method described can be used to ascertain whether all tRNAs or certain tRNAs are aminoacylated in the nucleus. This information will be necessary to establish whether both the aminoacylation-dependent and -independent export pathways are used concurrently in S. cerevisiae. Furthermore, it can be used routinely to ascertain whether a block in nuclear tRNA export resulting from chromosomal mutations is due to a defect in nuclear tRNA aminoacylation. Because this electrophoretic method can distinguish between modified and unmodified tRNA, it will also be useful for analysis of the effect of chromosomal mutations on tRNA modification (Fig. 5B).

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