BIOCHEMISTRY

 Volume 47, Number 34 August 26, 2008

Articles

A Study of Binding Features between Exportin-t and *Thermus thermophilus* $tRNA^{Phe}$ Using a Photo-Cross-Linking Method[†]

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Received January 3, 2008; Revised Manuscript Received July 2, 2008

ABSTRACT: The interaction of tRNA and its carrier, exportin-t, was studied by photoaffinity cross-linking of randomly s^4U-substituted $tRNA^{Phe}_{T.th}$ in vitro transcript that was able to form complex with exportin-t and ran·GTP. We found that in the ternary complex, $tRNA^{Phe}_{T.th}$ was cross-linked only to exportin-t, not to ran·GppNHp (an analogue of ran·GTP), suggesting that tRNA had much stronger contact with exportin-t than with ran·GTP. The contact sites between $tRNA^{Phe}_{T.th}$ and exportin-t were supposed to be the cross-linking sites in this study and further determined by primer extension analysis. The major cross-linking sites were U55, U47, U20, and U33. Apart from the footprinting data on the contact sites between exportin-t and tRNA, which are mainly on the acceptor arm and TΨC region, our results showed that exportin-t bound tRNA in a very extensive way, and the anticodon loop and D loop might also contact, though weakly, the tRNA carrier. Using the site-directed mutagenesis method, we also found that while position 47 in $tRNA^{Phe}_{T.th}$ is important for the binding interaction, the identity of the nucleotide at this position is not.

Transcribed in nucleus, eukaryotic tRNAs have to pass the nuclear envelope to reach their destination in cytosol. Though tRNAs with their small size can diffuse across the nuclear envelope theoretically, it was found that tRNA export is a saturable and, thus, carrier-mediated process (*1*). Injection of excess tRNA into nucleus only saturated tRNA export but not those of other RNAs, suggesting that the tRNA export was mediated by a class specific carrier (*2*). Later, it was observed that microinjection of ran·GAP, a protein accelerating GTP hydrolysis normally localized in the cytoplasm, into the nucleus depleted nuclear ran·GTP and blocked tRNA export, indicating that the protein responsible for tRNA export may be a member of the importin β family (*3*). This suggestion was confirmed by the discovery of the tRNA specific transport protein, exportin-t (*4, 5*).

tRNA genes in eukaryotes are transcribed by RNA polymerase III to functionless pre-tRNAs with extra 5′- and 3′-tails. In some cases, tRNA transcripts contain introns. Maturation of eukaryotic tRNAs is a multistep process involving the removal of 5′- and 3′-tail sequences, modification of specific nucleotide residues, excision of introns, and addition of 3′-CCA ends (*6, 7*). Therefore, there are many kinds of tRNA intermediates in the nucleus. Exportin-t in complex with ran·GTP preferentially binds mature tRNA, as tRNA with 5′- and 3′-extension exhibited much less affinity for the exportin-t·ran·GTP complex in vitro (*8*). The presence of an intron, on the other hand, did not affect

[†] This work was supported by grants from Deutsche Forschungsgemeinschaft.

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10.1021/bi800008x CCC: $40.75
Published on Web 07/30/2008

exportin-t binding in vitro (*8, 9*). Neither aminoacylation nor modification is an absolute requirement for tRNA transport (*10*).

Exportin-t binds tRNAs from *Escherichia coli* and *Saccharomyces cerevisiae* with the same affinity as calf liver tRNAs (*8*). It was therefore concluded that exportin-t recognizes the conserved structural features of tRNA. Enzymatic and chemical footprinting together with phosphate modification interference were applied in studying the contact regions between tRNA and exportin-t. The TΨC arm and acceptor arm of tRNA were significantly protected by the exportin-t·ran·GTP complex, and these regions are supposed to be the major interaction sites between tRNA and the proteins (*9*). A series of exportin-t variants with N- and C-terminal deletions were constructed to interact with ran·GTP and tRNA. Removal of the C-terminus of exportin-t eliminated the tRNA interaction but not the ability to bind ran·GTP. Removal of the N-terminus affected both binding activities. Thus, it is likely that tRNA binds to the C-segment of exportin-t (*11*).

In this work, photo-cross-linker 4-thiouridine (s^4U) was randomly incorporated into the transcript of $tRNA^{Phe}$ from *Thermus thermophilus* to study the characteristics for binding of tRNA to the exportin-t·ran·GTP complex. Primer extension analysis was used to identify the cross-linking sites.

MATERIALS AND METHODS

Materials. Pure chemicals and protease K were from Merck (Darmstadt, Germany). ATP, CTP, GTP, GppNHp, and UTP were purchased from Roche (Mannheim, Germany). T7 RNA polymerase was purified from *E. coli* (*12*). Nuclease P1 (from *Penicillium citrinum*), DNase I (from calf pancreas, RNase free), and alkaline phosphatase from *E. coli* were purchased from Roche. M-MLV (H^-) reverse transcriptase was from Promega (Mannheim, Germany). The Supelcosil LC-18S column (25 cm × 4.6 mm) was from Bellefonte. Resctriction endonucleases were purchased from New England Biolab (Frankfurt am Main, Germany). Radioactive [^{32}P]ATP (3000 Ci/mmol) was obtained from Hartmann Analytic (Braunschweig, Germany). 4-Thiouridine triphosphate was prepared according to the method published in ref *13*.

The gene of $tRNA^{Phe}_{T.th}$ under T7 promoter was constructed into pUC19 by the standard methods.

Preparation of Exportin-t and ran·GppNHp. Exportin-t with an N-terminal His tag was expressed and purified as described in ref *5*. p24Ran was expressed and purified as described in ref *14*. In a 0.8 mL sample, 670 μM ran·GDP and 3.4 mM GppNHp (Roche) were mixed together in 50 mM potassium phosphate (pH 7.0), 200 mM $(NH_4)_2SO_4$, 150 mM KCl, 10 mM EDTA, 5 mM $MgCl_2$, and 5 mM dithiothreitol. The reaction was started by adding shrimp alkaline phosphatase (Roche) and carried out at room temperature for 2 h. ran·GppNHp was purified by gel permeation chromatography on a Sephacyl S200 HR column (Pharmacia).

In Vitro Transcription. All in vitro transcriptions were performed using T7 RNA polymerase. s^4U and ^{32}P were incorporated into tRNA transcripts as described in ref *15*. Templates were either plasmid DNA linearized with BstNI or gel-purified PCR products. For transcription of ^{32}P-labeled

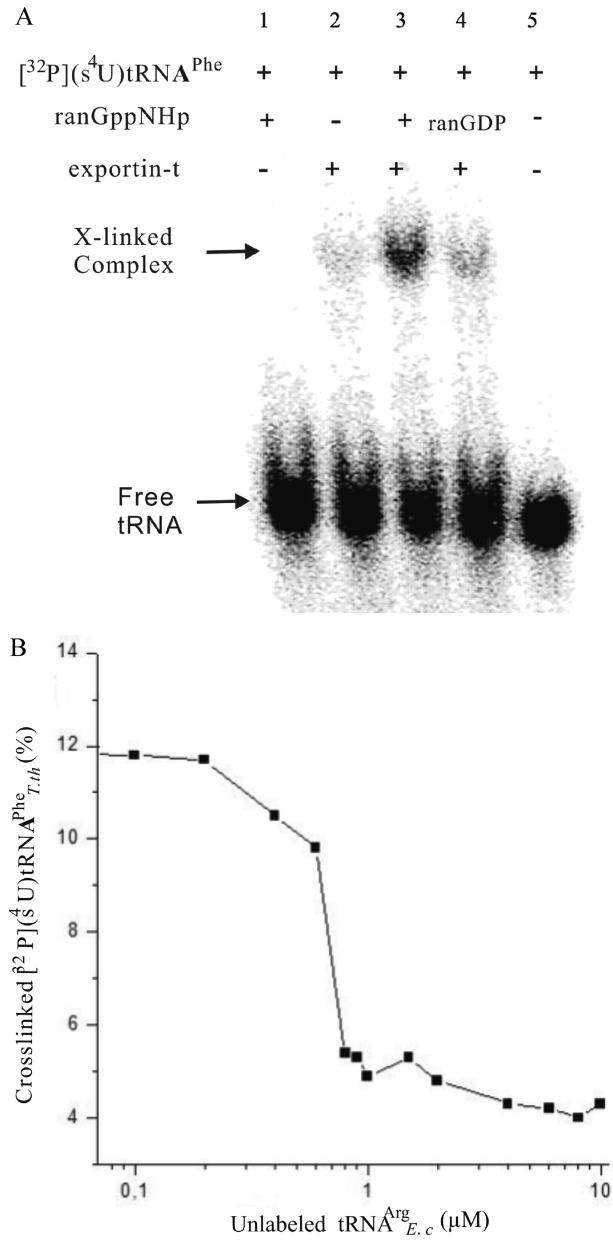

FIGURE 1: UV cross-linking of [^{32}P](s^4U)$tRNA^{Phe}_{T.th}$ with exportin-t. (A). PAGE analysis of cross-linking products. The composition of the reaction mixture is indicated at the top of respective lane. (B). Inhibition of cross-linking of [^{32}P](s^4U)$tRNA^{Phe}_{T.th}$ to the exportin-t·ran·GppNHp complex by unlabeled $tRNA^{Arg}_{E.c}$. [^{32}P]tRNA were visualized in Instant Imager 2024.

$tRNA^{Phe}_{T.th}$, 1 mM ATP, 11 nM [α-^{32}P]ATP (3000 Ci/mmol), three other NTPs (4 mM), 130 nM tDNA template, 40 mM Tris-HCl (pH 8.1), 25 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine, 0.16 unit/μL RNasIn, and 1.92 μM T7 RNA polymerase were incubated at 37 °C for 4 h. For transcription of [^{32}P](s^4U)$tRNA^{Phe}_{T.th}$,[1] 2 mM s^4UTP, 0.5 mM UTP, 1 mM ATP, and 11 nM [α-^{32}P]ATP (3000 Ci/mmol) were present, while other conditions remained the same. Two units of DNase I (RNase free, Roche) was then added, and the samples were kept at 37 °C for an additional 1 h to hydrolyze the DNA template. The tRNA transcripts were purified by electrophoresis on polyacrylamide gels containing 7 M urea.

[1] Abbreviations: Xpo-t, exportin-t; (s^4U)tRNA, tRNA transcript containing s^4U residues; [^{32}P]tRNA, tRNA labeled with ^{32}P; [^{32}P](s^4U)tRNA, tRNA labeled with ^{32}P containing s^4U residues.

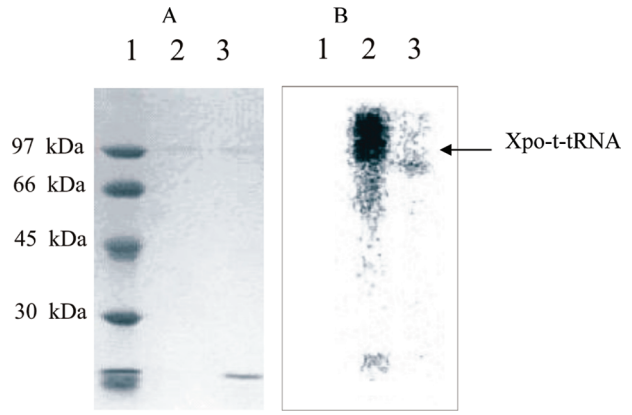

FIGURE 2: SDS−PAGE analysis of the purified cross-linked complex on a 15% polyacrylamide gel by Coomassie brilliant blue staining (A) and ^{32}P detection (B). Protein standards are shown in lane 1. The purified cross-linked complex without (lane 2) or with RNase digestion (lane 3) was used.

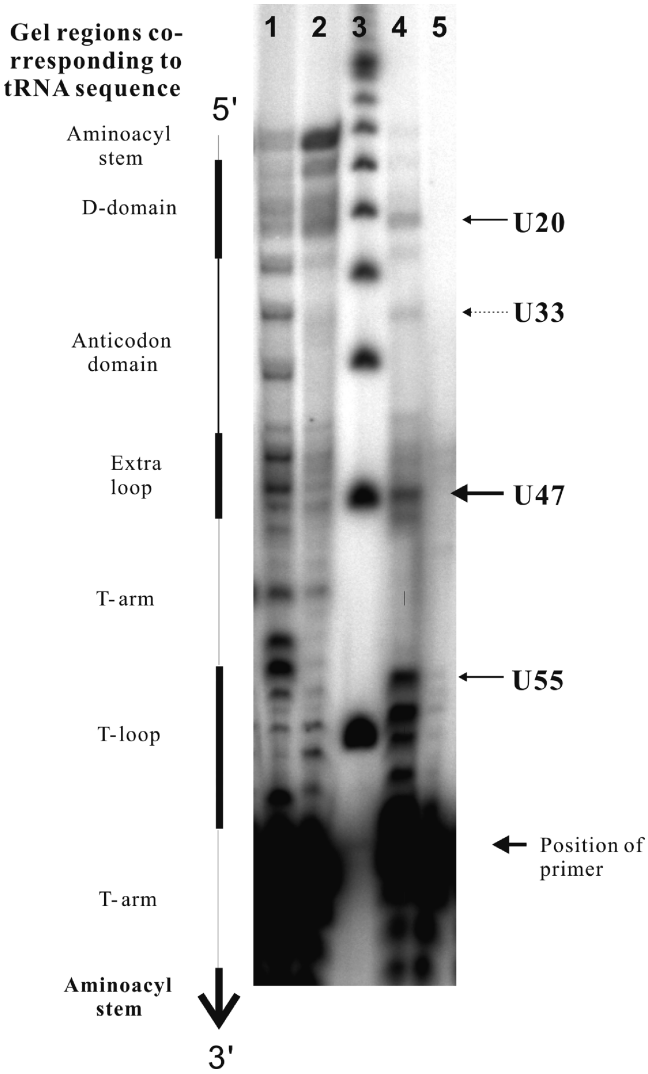

FIGURE 3: Sequencing gel analysis of primer extension products with 5′-labeled [^{32}P]DNA primer. (s^{4}U)tRNA$^{Phe}_{T.th}$ was used as the template in the presence (lane 1) or absence (lane 2) of ddATP. Lane 3 contained DNA length standards. In lane 4, purified cross-linked (s^{4}U)tRNA$^{Phe}_{T.th}$ was used as the template. Lane 5 contained the [^{32}P]DNA primer. Radioactive DNA bands were visualized in Instant Imager 2024.

Nucleoside Analysis by HPLC. For analysis of nucleotide composition, the tRNA transcripts were digested to nucleoside by nuclease P1 and bacterial alkaline phosphatase as described in ref *16*. The samples were applied to a Supelcosil LC-18S column (25 cm × 4.6 mm) (Bellefonte). The nucleosides were eluted at room temperature using 100 mM NH_4OAc (pH 5.3) with a methanol gradient from 2.5 to 70% over 70 min. The flow rate was 0.5 mL/min; absorbance of eluate was monitored at 254 and 330 nm (*17*).

Complex Formation and Cross-Linking. Formation of a complex among tRNA, exportin-t, and ran·GppNHp was achieved in 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 5% glycerol. Ten picomoles of tRNA was incubated with a 5-fold molar excess of exportin-t and ran·GppNHp in a total volume of 10 μL at 0 °C for 15 min. The complex was separated from free tRNA by gel electrophoresis on a 5% polyacrylamide gel precooled to 4 °C. The electrophoresis was conducted at 4 °C and 12.5 V/cm.

For cross-linking, samples containing the complex (s^{4}U)tRNA$^{Phe}_{T.th}$·exportin-t·ran·GppNHp were transferred to a glass plate cooled on ice and were irradiated at a distance of 10 cm by a UV light source composed of four 15 W mercury lamps (4.5 mW/cm^2) (F8T5, Sankyo). In competition experiments, each 10 μL sample containing 0.4 μM tRNA, 2 μM exportin-t, 2 μM ran·GppNHp, and a series of tRNA$^{Arg}_{E.c}$ concentrations (from 0 to 10 μM) was mixed in 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 5% glycerol and irradiated as described above for 1 min. For analyses, irradiated samples were heated in 1% SDS in 50 mM Tris-HCl (pH 6.8) at 60 °C for 5 min to disassemble the uncross-linked complex and loaded on a 5% polyacrylamide gel. The electrophoresis was conducted at room temperature and 12.5 V/cm. The radioactive bands in polyacrylamide gels were visualized in Instant Imager 2024 (Packard).

Primer Extension Analysis. The oligonucleotide DNA primer (5′-TGGTGCCGAGGAGCGG-3′) complementary to the 3′-end of tRNA$^{Phe}_{T.th}$ was purchased from Biomers.net GmbH (Ulm, Germany) and labeled with T4 polynucleotide kinase (Fermentas) in the presence of [γ-^{32}P]ATP (3000 Ci/mmol). For preparation of the cross-linked tRNA template, 1.2 nmol of s^{4}U-containing tRNA$^{Phe}_{T.th}$, 6 nmol of exportin-t, and 6 nmol of ran·GppNHp were transferred to 200 μL of buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 5% glycerol, irradiated, and subjected to native PAGE as described above. The upper band containing the cross-linked complex was cut out and incubated in 5 volumes of 0.3 M NaOAc (pH 6.0) at 4 °C. After centrifugation, the cross-linked complex in the supernatant was ethanol-precipitated and dissolved in 150 μL of 10 mM Tris-HCl (pH 7.8), 5 mM EDTA, and 0.5% SDS. Approximately 0.25 unit of protease K was added to digest the protein moiety in the complex. The reaction was performed at 37 °C for 30 min.

For annealing, 2 pmol of (s^{4}U)tRNA$^{Phe}_{T.th}$ or cross-linked tRNA$^{Phe}_{T.th}$ and 10 pmol of 5′-labeled primer were incubated in a 10 μL solution containing 1 mM EDTA and 1 mM sodium phosphate buffer (pH 7.0) at 80 °C for 5 min and then slowly cooled. After the tRNA had been annealed with the primer, 1 mM dNTP, 50 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 75 mM KCl, 5 mM dithiothreitol, 40 units of RNasIn (Promega), and 200 units of M-MLV (H$^-$) reverse tran-

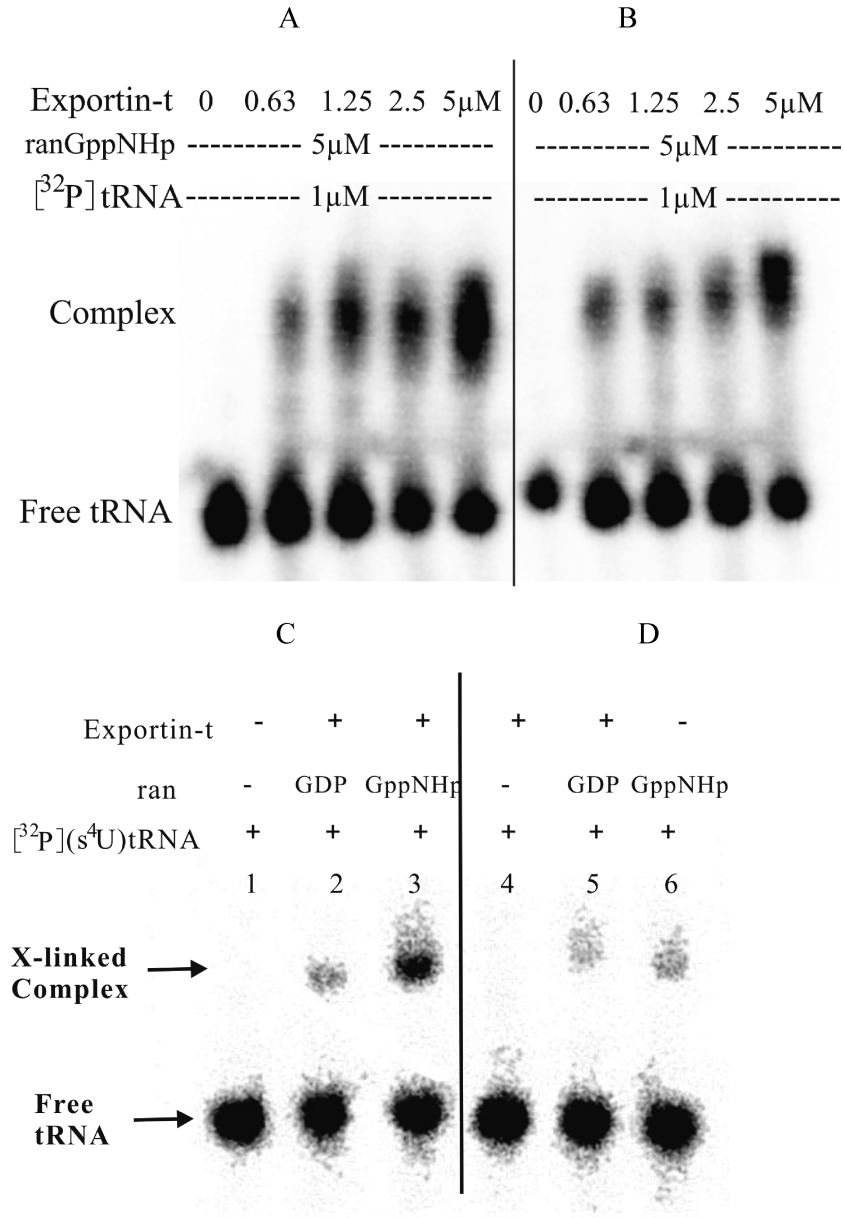

FIGURE 4: Native PAGE analysis of the interaction between [^{32}P]tRNA$^{Phe}_{T.th}$ U47A and the exportin-t·ran·GppNHp complex. In an electrophoretic mobility shift assay, tRNA$^{Phe}_{T.th}$ transcript (A) and tRNA$^{Phe}_{T.th}$U47A (B) formed a complex with exportin-t in the presence of ran·GppNHp. In cross-linking experiments, (s^{4}U) tRNA$^{Phe}_{T.th}$ (C) and (s^{4}U)tRNA$^{Phe}_{T.th}$U47A (D) and exportin-t in the presence of ran·GppNHp and ran·GDP were irradiated at 312 nm.

scriptase (Promega) were added. When ddATP was used, 12.5 μM dATP and 988.5 μM ddATP were added to the sample. The reactions were performed at 50 °C for 1 h.

For sequencing, electrophoresis on 12% polyacrylamide gels containing 7 M urea, 90 mM Tris-borate (pH 8.4), and 2.5 mM EDTA were used. The electrophoresis was conducted at 40 V/cm. The gel was dried at 80 °C for 1 h and scanned with a Phosphoimager (Molecular Dynamics).

RESULTS

4-Thiouridines (s^{4}U) were randomly incorporated into tRNA$^{Phe}_{T.th}$ by in vitro transcription using T7 RNA polymerase. As analyzed by HPLC, 41% of uridines were replaced with 4-thiouridines (s^{4}U) under applied conditions (data not shown). The ability of (s^{4}U)tRNA$^{Phe}_{T.th}$ to bind the exportin-t·ran·GppNHp complex was tested with an electrophoretic mobility shift assay. It was found that both tRNA$^{Phe}_{T.th}$ and (s^{4}U)tRNA$^{Phe}_{T.th}$ could bind exportin-t in the presence of ran·GppNHp. The slowly hydrolyzable GTP analogue GppNHp was used to exclude GTPase activity during the experiment.

Samples containing exportin-t, [^{32}P](s^{4}U)tRNA$^{Phe}_{T.th}$, and ran·GppNHp were irradiated at 312 nm. After irradiation, the samples were treated with 1% SDS at 60 °C to disassemble the complex and then subjected to gel electrophoresis (Figure 1A). As shown in the figure, (s^{4}U)tRNA$^{Phe}_{T.th}$ was barely cross-linked to exportin-t in the absence of ran. In the presence of ran·GDP, the cross-linking yield increased somewhat. A strong band of cross-linked complex was observed only in the sample containing [^{32}P](s^{4}U)tRNA$^{Phe}_{T.th}$, exportin-t, and ran·GppNHp. It is evident from these experiments that the cross-linking of tRNA to exportin-t is strongly stimulated by ran·GppNHp. It is noteworthy that UV irradiation of (s^{4}U)tRNA$^{Phe}_{T.th}$ alone

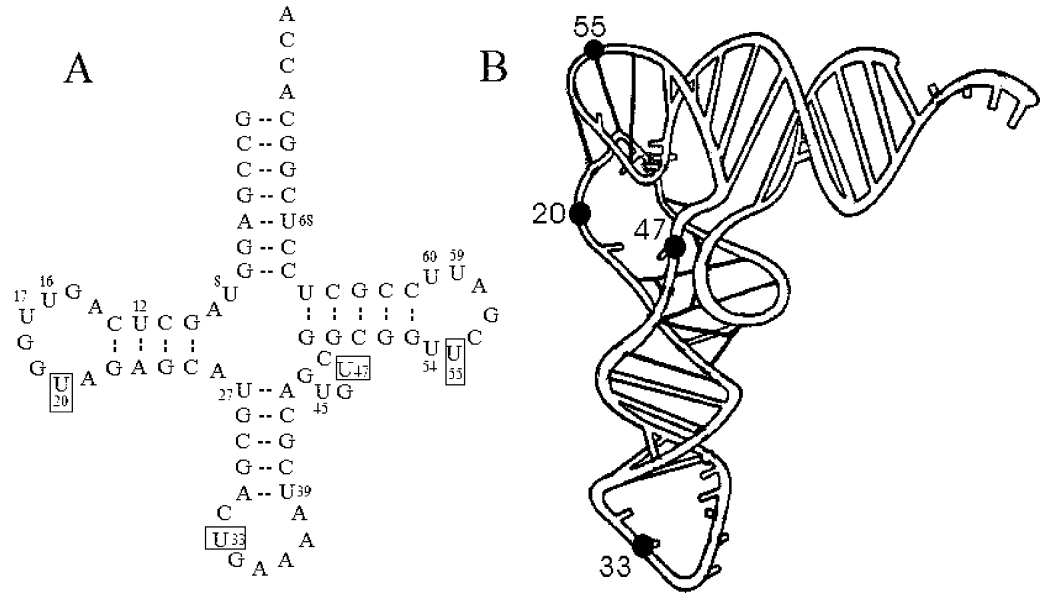

FIGURE 5: Contact sites of tRNA$^{Phe}_{T.th}$ in cross-linking with exportin-t. (A) In the sequence of the tRNA$^{Phe}_{T.th}$ transcript, cross-linked sites are framed. (B) Tertiary structure of yeast tRNA^{Phe} (*21*). Cross-linked sites are represented by dots.

produced intramolecular cross-linked tRNA with the same mobility as intact tRNAs (lane 5), while in the upper band indicated as "X-linked complex", there was tRNA cross-linked with proteins. The yield of photo-cross-linking of [^{32}P](s^{4}U)tRNA$^{Phe}_{T.th}$ was inhibited by unlabeled tRNA$^{Arg}_{E.c}$ (Figure 1B). As the amount of the competitor tRNA$^{Arg}_{E.c}$ increased, the cross-linking yield with [^{32}P](s^{4}U)tRNA$^{Phe}_{T.th}$ decreased. Other unlabeled tRNA species were also tested and produced similar inhibition effects (data not shown). The cross-linking was the consequence of the formation of a stable tRNA·exportin-t·ran·GppNHp complex.

The cross-linked tRNA−protein complex was eluted from the polyacrylamide gel and subjected to PAGE in the presence of 1% SDS. The bands were visualized both by Coomassie brilliant blue staining and by ^{32}P detection (Figure 2). The RNA (detected by radioactivity) was found at the position corresponding to the exportin-t−tRNA conjugate (~125 kDa) but not at the position of tRNA conjugate with ran·GppNHp (~50 kDa). Cross-linking was not observed between ran·GppNHp and (s^{4}U)tRNA$^{Phe}_{T.th}$, suggesting that the intensity of their contact with each other is much lower than that of tRNA and exportin-t in the complex, though ran·GppNHp played a critical role in this interaction.

For detection of the contact sites between (s^{4}U)tRNA$^{Phe}_{T.th}$ and exportin-t, the cross-linked tRNA−protein product was separated from intramolecular cross-linked tRNAs by gel splicing and used as the template for the primer extension reaction. It was expected that tDNAs synthesized with this template would prematurely terminate at the cross-linked sites. Prior to the reverse transcription, the RNA was treated with protease K to remove the bulk of the cross-linked protein. The product of the primer extension was analyzed in a sequencing gel shown in Figure 3. Lane 1 represents the DNA synthesized using the (s^{4}U)tRNA^{Phe} as a matrix in the presence of ddATP. This experiment allows us to locate the positions of uridines. Lane 2 is a control obtained in an experiment similar to that shown in lane 1 but in the absence of ddATP. Though some weak bands were observed, which might be due to accidental stops of the reverse transcriptase, the major product was the full-length tDNA, suggesting that most of the primer extension reactions came to completion and s^{4}U residues in the tRNA template did not interfere with the DNA synthesis. When the cross-linked (s^{4}U)tRNA$^{Phe}_{T.th}$ was used as template (lane 4), no full-length DNA was synthesized. The occurring DNA bands suggest that s^{4}U residues of tRNA$^{Phe}_{T.th}$ were cross-linked at positions 20, 33, 47, and 55, positions 47 and 55 being the most intensive. In the case of intramolecularly cross-linked (s^{4}U)tRNA$^{Phe}_{T.th}$ being used as a template, the main DNA bands were approximately 20−25 nucleotides in length (near the position of the primer) (Supporting Information), implying that (s^{4}U)tRNA in the absence of the exportin-t·ran·GppNHp complex after UV irradiation formed cross-links that could not be overriden by the reverse transcriptase.

To test the significance of U47 for the interaction with exportin-t, replacement of T47 with A47 in the tRNA$^{Phe}_{T.th}$ gene was performed. Electrophoretic mobility shift assay experiments were then used to test the ability of the tRNA$^{Phe}_{T.th}$ U47A transcript to bind exportin-t. Both [^{32}P]tRNA$^{Phe}_{T.th}$ and [^{32}P]tRNA$^{Phe}_{T.th}$ U47A transcripts could bind exportin-t, with similar abilities (Figure 4A,B). Thus, substitution of uridine 47 with adenosine did not substantially affect the tertiary structure of tRNA$^{Phe}_{T.th}$ in the interaction with exportin-t. However, the ability of the [^{32}P](s^{4}U)tRNA$^{Phe}_{T.th}$U47A transcript to cross-link with exportin-t was remarkably weakened (Figure 4D). It is concluded that position 47 is a major site of contact between tRNA$^{Phe}_{T.th}$ and exportin-t, although the structure of this uridine does not play an essential role in the interaction.

DISCUSSION

The importin β family is the most popular transport receptor group known in higher eukaryotes. As a hallmark, ran·GTP/ran·GDP is absolutely required in the carrier−-cargo binding of members of this family. Exportin-t was the first member of the importin β family found to bind directly to a RNA cargo. Like other importin β family members, exportin-t was found to bind ran·GTP at its N- terminus, and its cargo of tRNA at the C-terminus (*11*). In our

experiment, only exportin-t was cross-linked to tRNA, indicating that ran·GTP contacts tRNA much more weakly than exportin-t, if there was any contact between tRNA and ran·GTP.

Though a mammalian protein, exportin-t is able to recognize the conservative features of cytoplasmic tRNAs and binds prokaryotic tRNAs as well as eukaryotic tRNAs (*8*). Using a yeast tRNA, Arts et al. undertook a series of biochemical experiments to study the sites of contact between tRNA and exportin-t (*9*). The riboses of residues 4−6, 17, and 47−72 were mildly protected by the exportin-t·ran·GTP complex, and the strongest protection was observed for residues 50−71. Consequently, the TΨC region and acceptor arm were considered as the major regions of interaction between tRNA and the exportin-t·ran·GTP complex. In photoaffinity cross-linking experiments, reported in this work, the major contact sites of $tRNA^{Phe}_{T.th}$ and exportin-t were U55, U47, U20, and U33 (Figure 5). That is to say, except U33, the cross-linking sites of U55, U47, and U20 are in the region where footprinting effects were previously observed. Judged from band intensities, which may roughly correspond to the accessibilities to cross-linking sites, the TΨC loop (U55) and extra loop (U47) are the major regions of contact between tRNA and exportin-t. The acceptor arm could not be analyzed by primer extension analysis, because one-half of it was covered by the primer.

4-Thiouridine 20 in the D loop provides less cross-linking than s^4U at positions 47 and 55. It was reported that tRNAs with a mutated D stem were defective in nuclear export in *S. cerevisiae* (*18*). Since tRNAs with an intron in the anticodon loop were still able to bind to exportin-t in vitro, Lipowsky et al. argued that the anticodon loop does not contribute to exportin-t binding (*8*). In our experiments, U33 of $tRNA^{Phe}_{T.th}$ is a minor site of contact between $tRNA^{Phe}_{T.th}$ and exportin-t. It seems that the anticodon loop interacts with exportin-t. It is noteworthy that Arts et al. observed a low extent of transport of intron-containing $tRNA^{Phe}$ in vivo, and a minihelix composed of the TΨC region and acceptor arm could not be bound by exportin-t at all (*8, 9*). These results suggested that regions of tRNA other than the TΨC region and acceptor arm might be involved in the binding process. Above all, it seems that the area of interaction between tRNA and exportin-t is extensive, and no domain of a tRNA is absolutely excluded in the binding process.

U47 in $tRNA^{Phe}_{T.th}$ is a major cross-linking site in this study. It occupies an interesting position. First, it is situated near the Levitt base pair of G15-C48, which is critical for maintaining tertiary structure of tRNA; however, in the crystal structure of $tRNA^{Phe}$ (*19*), U47 extends outside from the core of $tRNA^{Phe}$ and does not base pair with other nucleotides. Second, an interesting rule has been generalized about the presence of nucleotide 47 in most cytoplasmic tRNAs. There is a correlation between the absence of a nucleotide at position 47 and the presence of a U13·G22 base pair in the D stem. If a Watson−Crick C13·G22 pair is present, the tRNA has to set a nucleotide at position 47 to accommodate this base pair, and to maintain a functional structure (*20*).

Thus, the role of U47 in the interaction of tRNA and the exportin-t·ran·GTP complex was investigated by site-directed mutagenesis. $tRNA^{Phe}_{T.th}$ U47A was able to bind exportin-t, but $(s^4U)tRNA^{Phe}_{T.th}$ U47A does not cross-link efficiently with the protein. These results demonstrate that U47 is a major site of contact between $tRNA^{Phe}_{T.th}$ and exportin-t, and more importantly, it can be replaced with adenosine without disturbing the formation of the tRNA·exportin-t·ran·GTP complex. It is suggested that position 47, not the uridine base at the position, in $tRNA^{Phe}_{T.th}$ is important for the binding interaction, because $tRNA^{Phe}_{T.th}$ has a C13·G22 base pair that requires a nucleotide 47 in its sequence.

ACKNOWLEDGMENT

I thank Prof. Dr. Mathias Sprinzl and Dr. Alexandra Wolfrum for their valuable suggestion and Dr. Petra Hoffmüller for the construction of the plasmid-containing $tRNA^{Phe}_{T.th}$ gene.

SUPPORTING INFORMATION AVAILABLE

Additional electrophoresis results. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI800008X