The EMBO Journal Vol.17 No.24 pp.7430–7441, 1998

The role of exportin-t in selective nuclear export of mature tRNAs

Gert-Jan Arts, Scott Kuersten, Pascale Romby[1], Bernard Ehresmann[1] and Iain W.Mattaj[2]

European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117 Heidelberg, Germany and [1]IBMC, UPR 9002 du CNRS, 15 rue René Déscartes, 67084 Strasbourg Cedex, France

[2]Corresponding author
e-mail: Mattaj@embl-heidelberg.de

Exportin-t (Xpo-t) is a vertebrate nuclear export receptor for tRNAs that binds tRNA cooperatively with GTP-loaded Ran. Xpo-t antibodies are shown to efficiently block tRNA export from *Xenopus* oocyte nuclei suggesting that it is responsible for at least the majority of tRNA export in these cells. We examine the mechanism by which Xpo-t–RanGTP specifically exports mature tRNAs rather than other forms of nuclear RNA, including tRNA precursors. Chemical and enzymatic footprinting together with phosphate modification interference reveals an extensive interaction between the backbone of the TΨC and acceptor arms of $tRNA^{Phe}$ and Xpo-t–RanGTP. Analysis of mutant or precursor tRNA forms demonstrates that, aside from these recognition elements, accurate 5′ and 3′ end-processing of tRNA affects Xpo-t–RanGTP interaction and nuclear export, while aminoacylation is not essential. Intron-containing, end-processed, pre-tRNAs can be bound by Xpo-t–RanGTP and are rapidly exported from the nucleus if Xpo-t is present in excess. These results suggest that at least two mechanisms are involved in discrimination of pre-tRNAs and mature tRNAs prior to nuclear export.

Keywords: exportin-t/nuclear export/nucleocytoplasmic transport/RNA–protein interaction/tRNA

Introduction

RNA transcription occurs largely within the nucleus of a eukaryotic cell. Since most RNAs either function in the cytoplasm, or have to pass through the cytoplasm during maturation, the vast majority of cellular RNAs must be exported from the nucleus to the cytoplasm (reviewed by Izaurralde and Mattaj, 1995; Izaurralde and Adam, 1998). A number of RNA-binding proteins that are thought to be mediators of export of particular RNAs have been identified (Piñol-Roma and Dreyfuss, 1993; Izaurralde *et al*., 1995, 1997; Lee *et al*., 1996; Segref *et al*., 1997; Grüter *et al*., 1998). In addition, the nucleocytoplasmic transport receptor CRM1/Xpo1 is known to function in the export of specific cellular RNAs from the nucleus (Fornerod *et al*., 1997a; Stade *et al*., 1997). The relationship between CRM1 and other known nucleocytoplasmic transport receptors, all members of the importin-β family (Fornerod *et al*., 1997b; Görlich *et al*., 1997) has led to the expectation that RNA export from the nucleus will generally occur in a manner analogous to the importin-β mediated nuclear import of proteins (Görlich, 1997).

In general it is thought that RNA-binding proteins involved in export are recognized in the nucleus by export receptor molecules, together with the RanGTPase in its GTP-bound form (Fornerod *et al*., 1997a; Kutay *et al*., 1997), and the resultant complex is translocated to the cytoplasm through a nuclear pore complex (Corbett and Silver, 1997; Cole and Hammell, 1998; Dahlberg and Lund, 1998; Izaurralde and Adam, 1998; Mattaj and Englmeier, 1998; Ohno *et al*., 1998 for review). However, the second characterized export receptor involved in the transport of cellular RNAs, the tRNA export receptor [exportin(tRNA), exportin-t or Xpo-t], binds directly to tRNA in the presence of RanGTP, and does not require an adaptor protein to mediate its interaction with the substrate RNA (Arts *et al*., 1998; Kutay *et al*., 1998). Xpo-t was found to bind to many, and perhaps all, different tRNAs (Arts *et al*., 1998; Kutay *et al*., 1998).

Other tRNA-binding proteins that recognize common features of multiple different tRNAs include the bacterial translation elongation factor EF-Tu (Nissen *et al*., 1995) and its eukaryotic equivalent eEF1 alpha (Negrutskii and El'skaya, 1998); the CCA-adding enzyme (tRNA nucleotidyl transferase) that creates mature tRNA 3′ ends (Shi *et al*., 1998); ribonuclease P, which creates the mature 5′ end on all tRNAs by endonucleolytic cleavage (Altman *et al*., 1993) and the ribosome (Nazarenko *et al*., 1994). These proteins recognize universally conserved structural and sequence features of tRNAs (Nazarenko *et al*., 1994; Liu *et al*., 1998; Shi *et al*., 1998, and references therein). Since Xpo-t is unrelated to these proteins it was of interest to determine which features of mature tRNAs are recognized by the Xpo-t–RanGTP complex and mediate its binding. Note that there are many more examples of proteins, like the aminoacyl-tRNA synthetases or tRNA modifying enzymes (Edqvist *et al*., 1993; Giegé *et al*., 1993), that recognize specific tRNA species and discriminate between them.

A further aspect of the Xpo-t–RanGTP interaction relevant to tRNA export is that tRNAs, like almost all cellular RNAs, are transcribed in the form of precursors. The maturation of all tRNAs involves removal of 5′ and 3′ extensions, addition of the mature CCA 3′ end and both base and sugar modifications. In addition, many pre-tRNAs contain introns that must be removed by splicing. Almost all of these processing steps occur in the nucleus prior to tRNA export (Melton *et al*., 1980; De Robertis *et al*., 1981; Nishikura and De Robertis, 1981). This raises the question of how pre-tRNAs and mature tRNAs are discriminated so that only the latter are efficiently exported and, in particular, what is the role of Xpo-t in this process.

In this study three aspects of Xpo-t function in tRNA export are investigated. First, how Xpo-t, together with RanGTP, recognizes and binds to tRNAs. Secondly, the involvement of Xpo-t in discriminating between pre-tRNAs and mature tRNAs with regard to nuclear export. Thirdly, we investigate the quantitative importance of Xpo-t for tRNA export from the nuclei of *Xenopus* oocytes.

Results

It was previously established that Xpo-t can bind to many different tRNAs (Arts *et al*., 1998; Kutay *et al*., 1998). This suggested that Xpo-t recognizes conserved structural features rather than individual tRNA sequences. For this reason, Xpo-t–tRNA interactions were analysed via enzymatic and chemical protection and interference analyses utilising reagents that modify the ribose–phosphate backbone of the tRNA rather than reagents that target nucleotide bases. As a substrate tRNA we chose *Saccharomyces cerevisiae* $tRNA^{Phe}$. This tRNA has been extensively studied by structural, biochemical and mutagenic methods (Kim *et al*., 1974; Ladner *et al*., 1975; Romby *et al*., 1987; Nazarenko *et al*., 1994) and this background information facilitated interpretation of the analyses presented here.

Footprinting of tRNA in the Xpo-t complex

Fe/EDTA results in cleavage of ribose sugar rings through the local production of hydroxyl radicals, and has been used to probe aspects of $tRNA^{Phe}$ structure (Latham and Cech, 1989). Extensive protection of $tRNA^{Phe}$ against Fe/EDTA cleavage was seen on addition of Xpo-t and RanGTP, which bind cooperatively to the tRNA (Arts *et al*., 1998; Kutay *et al*., 1998). The positions of protected riboses was determined using both 5′- and 3′ end-labelled $tRNA^{Phe}$ (Figure 1A). Equal cleavage was seen in the presence and absence of the proteins at the resolved positions between 7–16 and 19–46 (Figure 1A, lanes 4–6, 11–13; note that since the T1-cleaved markers carry an intact ribose ring they migrate above the corresponding band in the experimental lanes). Riboses at positions 18, 47–49 and 72 exhibited mild protection against cleavage whereas the riboses between positions 50 and 71 were significantly protected in the presence of Xpo-t–RanGTP (Figure 1A, compare lanes 1–4 with 5 and 6). In addition, better seen with 5′ end-labelled RNA, positions 4–6 and 17 were also protected against Fe/EDTA attack by Xpo-t–RanGTP (Figure 1A, lanes 11–13). For technical reasons, positions 1–3 and 73–76 cannot be examined by this method.

The next probing agent used was ribonuclease V1, which cuts accessible double-stranded helical regions in RNA. RNase V1 cuts several positions in the stems of the anticodon and acceptor arms of free $tRNA^{Phe}$ (Figure 1B, lane 3; Lockard and Kumar, 1981). When preformed $tRNA^{Phe}$–Xpo-t–RanGTP complex is probed with V1 nuclease, the majority of the cleaved positions in the acceptor stem (positions 67–70, see also Figure 3) were protected to some extent against cleavage while cleavages at most of the anticodon stem positions (positions 28–30) as well as a site in the D arm (position 22) were enhanced and the cleavage at 41 was little affected (Figure 1B, lanes 1–4, Figure 3). This suggested that Xpo-t–RanGTP would interact with the acceptor arm and that binding would result in a conformational change in the tRNA resulting in increased accessibility of parts of the anticodon and D arms to V1 nuclease. Note that, as for Fe/EDTA-induced cleavage, the protection afforded by Xpo-t–RanGTP was incomplete. Whether this is due to partial accessibility to the cleaving reagents in the presence of bound Xpo-t–RanGTP or to some displacement of Xpo-t–RanGTP in the cleavage conditions is not known.

Pb^{2+} ions have been shown to cleave $tRNA^{Phe}$ between positions D17 and G18 (Werner *et al*., 1976; Brown *et al*., 1983; Behlen *et al*., 1990). Since Fe/EDTA-induced cleavage at position 17 was reduced in the presence of Xpo-t–RanGTP (Figure 1A, lanes 11–13), the effect of these proteins on lead cleavage was tested. Addition of Xpo-t–RanGTP essentially blocked lead cleavage (Figure 1B, lanes 8–10).

Phosphate ethylation-binding interference

To obtain further information on likely points of contact between Xpo-t–RanGTP and $tRNA^{Phe}$, ethylnitrosourea (ENU) modified tRNA was prepared. ENU ethylates phosphate groups. In this experiment, free $tRNA^{Phe}$ was ethylated at a ratio of approximately one ethyl group per tRNA molecule (Romby *et al*., 1985) then bound to immobilized Xpo-t–RanGTP (Arts *et al*., 1998). Bound and free RNA populations were separated, cleaved at ethylated phosphate positions by high pH treatment, and fractionated by denaturing gel electrophoresis. Bands resulting from phosphates whose ethylation interfered with Xpo-t–RanGTP binding should be under-represented in the bound (P) as compared with the input and non-bound (IN,S) fractions (Figure 2). The experiment is carried out under conditions of tRNA excess (Arts *et al*., 1998) and there is thus no enrichment of bands in the non-bound versus input lanes at interfering positions. The results are shown in Figure 2, lanes 4–6, with positions whose ethylation interfered significantly with Xpo-t–RanGTP binding indicated on the left. The strongest interference with binding was seen on ethylation of phosphates at positions 49, 56, 57, 59 and 62, making some of these positions good candidates for close contact with Xpo-t–RanGTP (see Discussion).

The data from Figures 1 and 2, as well as the results of RNase T1 protection experiments (data not shown) are summarized in Figure 3. Protection or interference is seen over much of the acceptor and TΨC arms, indicative of these structures being directly involved in Xpo-t–RanGTP interaction. Two phosphates at the anticodon stem–extra arm junction interfere with Xpo-t–RanGTP binding (positions 43 and 44; Figure 2) making interaction here also likely. Other changes seen in the anticodon arm are generally enhancements of nuclease cleavage on binding, suggesting that this region of the tRNA is not in contact with Xpo-t. The results from the D arm are more varied, and will be discussed in detail below.

Changes in the acceptor arm and in $tRNA^{Phe}$ tertiary structure affect functional interaction with Xpo-t

To obtain evidence that the interaction study presented above was related to Xpo-t function, i.e. to $tRNA^{Phe}$ nuclear export, we examined various mutant forms of yeast $tRNA^{Phe}$ with respect to their nuclear export and

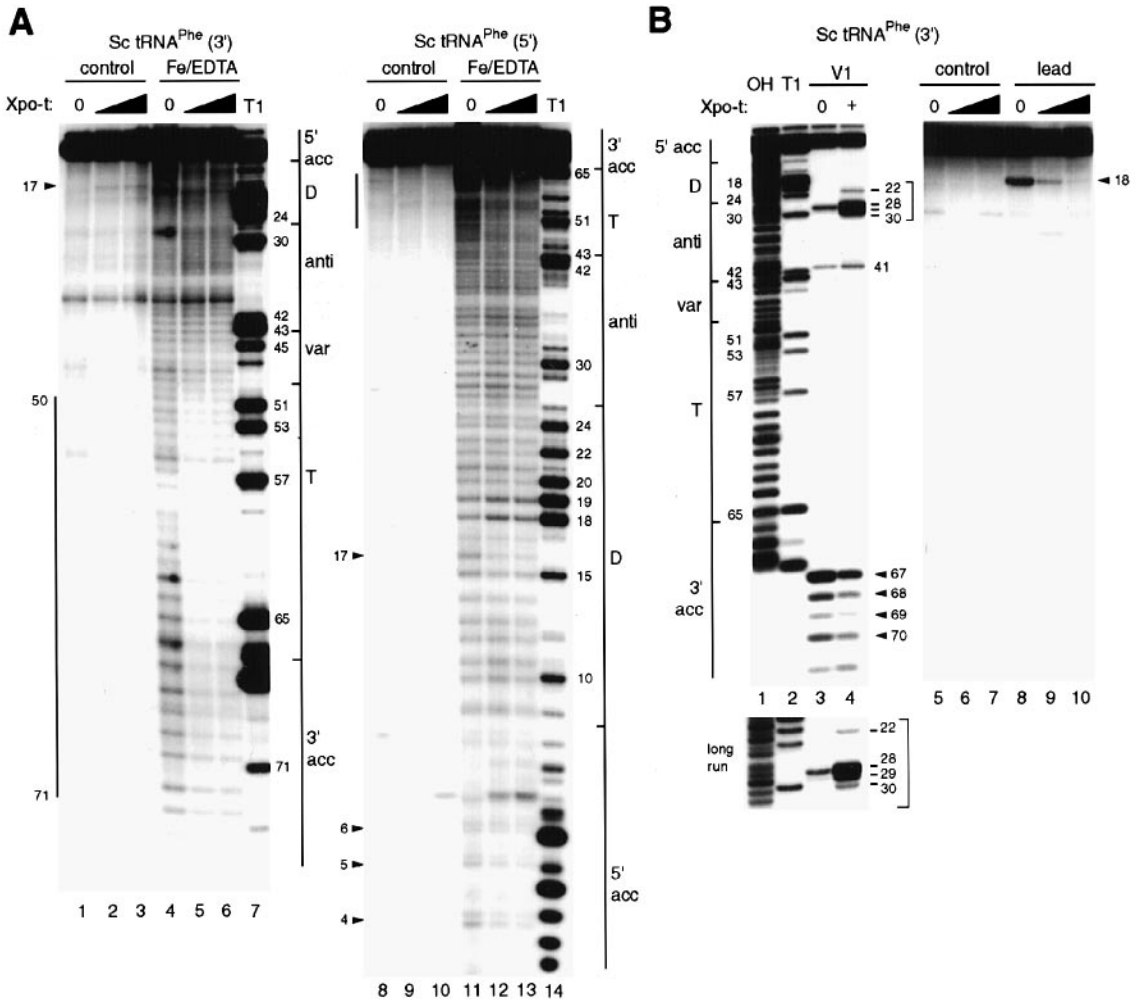

Fig. 1. Protection of *S.cerevisiae* tRNA^{Phe} against endonuclease activity and ribose modification by the Xpo-t–RanGTP complex. (**A**) Hydroxyl radical footprint analysis of 3′- or 5′ end-labelled yeast tRNA^{Phe}. The domains of the tRNA are indicated on the right: 5′ acceptor stem (5′ acc), D arm (D), anticodon arm (anti), variable region (var), TΨC arm (T) and 3′ acceptor stem (3′ acc). RNase T1 digests of either 5′ or 3′ end-labelled denatured tRNA^{Phe} (T1, lanes 7 and 14) were used as size references. Some T1 cleavage sites are indicated. See Figure 3 for the secondary structure of yeast tRNA^{Phe} and nucleotide numbering. The 3′ (lanes 1–7) or 5′ (lanes 8–14) labelled tRNA was incubated in the absence (control, lanes 1–3, 8–10) or presence (Fe/EDTA, lanes 4–6, 11–13) of iron(II)-EDTA induced hydroxyl radicals. Xpo-t and RanGTP were added in increasing concentrations (1.5 and 3 μM) (lanes 2, 3, 5, 6, 9, 10, 12 and 13) or were omitted (lanes 1, 4, 8 and 11). The regions showing strong protection against cleavage are indicated on the left of each panel with arrowheads or lines in grey. (**B**) Enzymatic (RNase V1) and lead(II) probing of 3′ end-labelled yeast tRNA^{Phe}. Lanes 1 and 2 are size markers, an alkaline ladder and an RNase T1 digest, respectively. In lanes 3 and 4 cleavage by RNase V1 is shown in the absence (lane 3) or presence of 2 μM (lane 4) of both Xpo-t and RanGTP. Positions of RNase V1 cleavages are given on the right. Protected positions are indicated with grey arrowheads, enhanced cuts with dashes. At the bottom, a better resolved longer run of the D and anticodon arm region is indicated with a bracket. On the right, lead(II) catalysed hydrolysis of 3′ end-labelled yeast tRNA^{Phe} is shown. The tRNA was incubated in the absence (control, lanes 5–7) or presence (lead, lanes 8–10) of lead(II). Xpo-t and RanGTP were added in increasing concentrations (1 and 2 μM) (lanes 6, 7, 9 and 10) or were omitted (lanes 5 and 8). The cleavage site at G18 is indicated on the right.

interaction with Xpo-t. As expected, wild-type tRNA^{Phe} bound specifically to Xpo-t in the presence of RanGTP, but did not bind in the presence of RanGDP (Figure 4A, lanes 1–3). tRNA^{Phe} also did not bind to importin-β in the presence of RanGTP (Figure 4A, lane 4), and neither U1 nor U6 bound specifically to either Xpo-t or importin-β (Figure 4A, lanes 1–4). Yeast tRNA^{Phe} was exported from the nucleus of *Xenopus laevis* oocytes after microinjection (Figure 4A, lanes 5–10) at a rate similar to the export of *X.laevis* tRNA^{Phe} (Figure 5A, lanes 1–6). Increasing the nuclear concentration of Xpo-t by microinjection into oocytes increases the rate of tRNA export from the nucleus (Arts *et al*., 1998; Kutay *et al*., 1998) and this effect was also seen with yeast tRNA^{Phe} (Figure 4A, lanes 11–14).

The interaction data presented in Figures 1–3 suggested that a ‘minihelix’ consisting of only the acceptor arm and TΨC arm might be a substrate for Xpo-t–RanGTP binding and export. However, this was not the case. The minihelix construct showed no detectable binding to Xpo-t–RanGTP *in vitro* (Figure 4F, lanes 1–4) and was too unstable on microinjection to assess whether it could be exported from the nucleus (data not shown).

Since the acceptor arm and TΨC arm minihelix was not sufficient for binding, the effect of removal of specific structural features from the tRNA was tested. Removal of the entire acceptor arm prevented Xpo-t–RanGTP binding (Figure 4B, lanes 1–4) and nuclear export, even in the presence of excess Xpo-t (Figure 4B, lanes 5–14). A much smaller deletion, removal of the CCA 3′ end, consistent with previous data on tRNA^{Ser} (Kutay *et al*., 1998), also

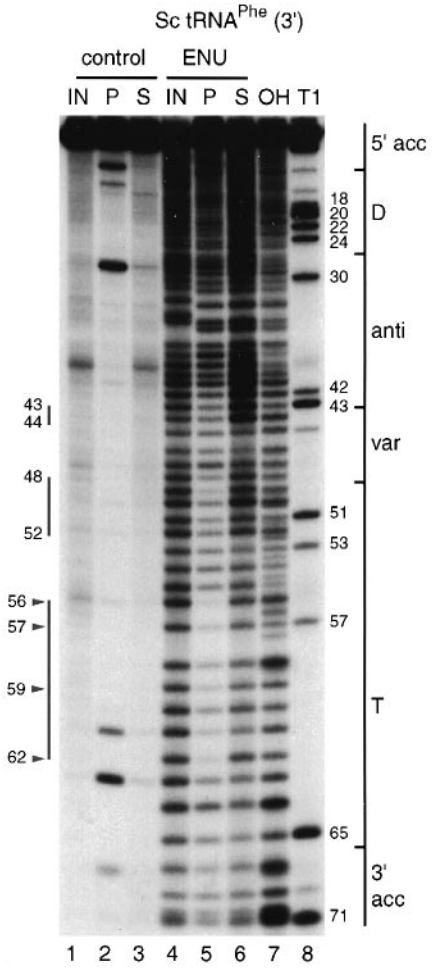

Fig. 2. Ethylation interference analysis of *S.cerevisiae* tRNA^{Phe} complex formation with Xpo-t and RanGTP. 3′ end-labelled untreated tRNA (control, lanes 1–3, note that some endonuclease contamination is present in lane 2) or tRNA that was pretreated with ethylnitrosourea (ENU, lanes 4–6) was used in an *in vitro*-binding assay with immobilized Xpo-t and RanGTP. The input RNAs (IN) are shown in lanes 1 and 4. The bound fractions (P, lanes 2 and 5) were separated from the unbound supernatants (S, lanes 3 and 6) and the RNAs were recovered. Subsequently, cleavage of the tRNA at the modified positions was induced by high pH and the fragments were analysed by gel electrophoresis. The annotation of the tRNA domains is as in Figure 1. Lanes 7 and 8 show the alkaline and RNase T1 ladders. The positions in the tRNA whose modification strongly interferes with binding to Xpo-t–RanGTP are indicated on the left.

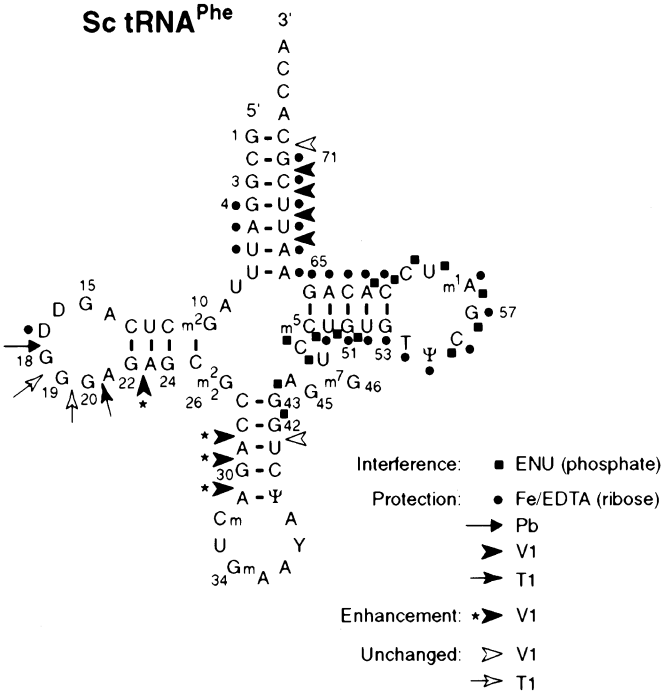

Fig. 3. Summary of the results of the protection and the interference experiments on the secondary structure of *S.cerevisiae* tRNA^{Phe}. G residues are indicated by number. Modified bases are represented by conventional symbols. Phosphates that interfere with binding to the Xpo-t–RanGTP complex after ENU treatment are represented by squares. The riboses protected against hydroxyl radical probing are indicated by circles. The results of cleavage protection are indicated as follows: the lead cleavage site by a filled triangular arrow, the RNase V1 cuts by arrowheads and the RNase T1 cut by a filled arrow. The enhanced RNase V1 cleavages are marked with an asterisk. Cleavage sites which are not affected are shown by an empty arrow or arrowhead. The arrowhead is for V1 and the arrows T1.

had a drastic effect on Xpo-t–RanGTP interaction (Figure 4C, lanes 1–4). However, since the CCA end was re-added to this mutant RNA very rapidly following injection into oocyte nuclei (Figure 4C, compare the injected RNA in lane 5 with the uninjected sample in the IN lane), the effect of CCA deletion on export could not be assessed.

The loop of the TΨC arm is essential for tRNA tertiary folding, mainly due to interactions between this loop and the D loop (Kim *et al*., 1974; Ladner *et al*., 1975). Before examining the contribution of the TΨC arm to binding of Xpo-t–RanGTP it was therefore essential to determine whether tertiary folding was important for Xpo-t–RanGTP interaction. To this end, a C56G mutant of yeast tRNA^{Phe} was used. This mutation removes the C56–G19 base pair and thereby disrupts tRNA^{Phe} tertiary folding (Nazarenko *et al*., 1994). This mutation prevented Xpo-t–RanGTP binding and nuclear export (Figure 4D, lanes 1–4, 5–14).

Taken together, the above data demonstrate that the acceptor arm and interaction between the D- and TΨC-loops of tRNA^{Phe} are requirements for both Xpo-t binding and export.

Discrimination between pre-tRNA and tRNA

Pre-tRNA processing, i.e. intron removal and mature 5′- and 3′ end-formation, occurs in the nucleus of *Xenopus* oocytes (Melton *et al*., 1980; De Robertis *et al*., 1981). tRNAs carrying 5′- and 3′ end-extensions do not leave the nucleus even in the presence of excess Xpo-t (Melton *et al*., 1980; Arts *et al*., 1998) and do not detectably bind to Xpo-t–RanGTP (data not shown). Melton *et al*. (1980) did however observe export of an intron-containing, end-matured version of yeast tRNA^{Tyr} in *Xenopus* oocytes. This prompted us to determine whether yeast tRNA^{Phe} with its intron would interact with Xpo-t–RanGTP.

This pre-tRNA bound to Xpo-t–RanGTP as well as wild-type tRNA^{Phe} (Figure 4E, lanes 1–4). On injection into oocyte nuclei, however, very little export of the intron-containing species was seen, whereas the small amount of mature tRNA^{Phe} produced was exported at a rate similar to injected tRNA^{Phe} (Figure 4E, lanes 5–10). When excess Xpo-t was coinjected with the pre-tRNA (Figure 4E, lanes 11–14) the intron-containing tRNA was exported at a rate similar to wild-type tRNA^{Phe} with excess Xpo-t (Figure 4A, lanes 11–14). The same result was obtained whether RNA and Xpo-t were coinjected or

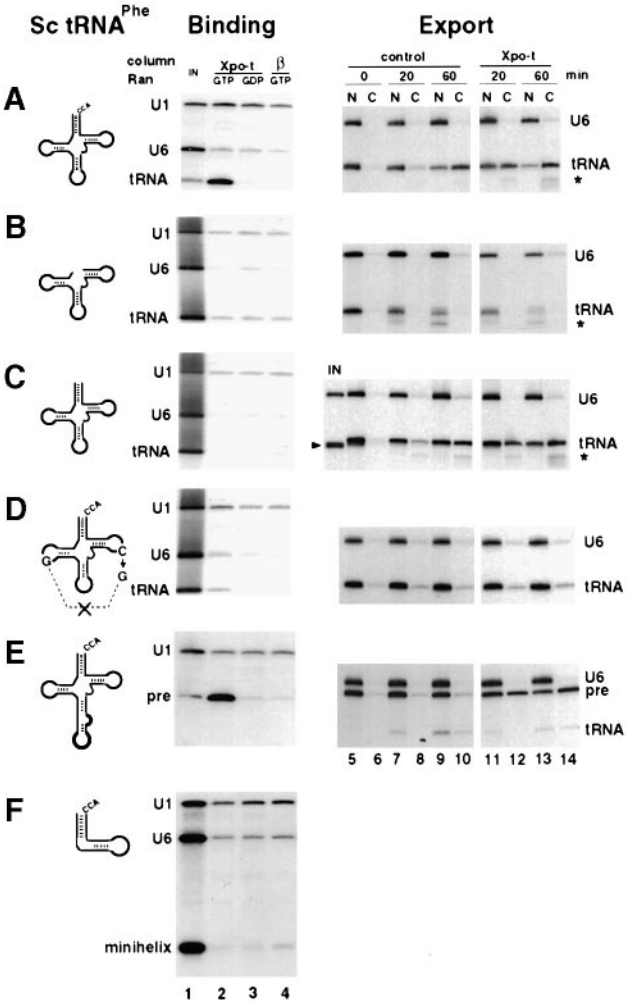

Fig. 4. *In vitro* binding to Xpo-t–RanGTP and export from *Xenopus* oocyte nuclei of *S.cerevisiae* tRNA^{Phe} variants. (**A**) On the left is the secondary structure of wild-type *S.cerevisiae* (Sc) tRNA^{Phe}. The result of an *in vitro*-binding experiment with this tRNA, mixed with U1ΔSm snRNA (U1) and U6Δss snRNA (U6) as internal controls, is shown in the middle (Binding, lanes 1–4). The RNA mixture was incubated either with immobilized exportin-t (Xpo-t, lanes 2 and 3) or with immobilized importin-β as a negative control (β, lane 4) in the presence of RanGTP (lanes 2 and 4) or RanGDP (lane 3). The bound RNA fractions were recovered and analysed by denaturing PAGE together with 10% of the input RNA mixture (IN, lane 1). On the right is a microinjection experiment in *Xenopus* oocytes (Export, lanes 5–14). A mixture of the tRNA and U6Δss snRNA was co-injected into oocyte nuclei with either buffer (control, lanes 5–10) or recombinant exportin-t (Xpo-t, lanes 11–14). The oocytes were dissected into nuclear (N) and cytoplasmic (C) fractions either immediately (lanes 5–6), after 20 min (lanes 7–8 and 11–12), or after 60 min (lanes 9–10 and 13–14) and extracted RNAs analysed by denaturing gel electrophoresis. The asterisk indicates degradation products that arise in the oocytes. For reasons that are not known, but that may be due to the export of incompletely modified or non-aminoacylated tRNAs in the presence of excess Xpo-t, these degradation products increase on injection of Xpo-t. (B–F) As for (A) but with the following tRNA variants: (**B**) Sc tRNA^{Phe}Δacc, a mutant lacking the acceptor stem. (**C**) Sc tRNA^{Phe}ΔCCA, a mutant lacking the 3′ terminal CCA. The lane preceding lane 5 labelled with IN shows the uninjected RNA mixture. The arrowhead indicates the size of the tRNA lacking the terminal CCA sequence. (**D**) Sc tRNA^{Phe}C56G, a mutant with C56 mutated to G that has a disrupted tertiary structure. (**E**) Sc pre-tRNA^{Phe}, a precursor tRNA with matured 5′ and 3′ ends but containing a 19 nucleotide intron. In the export panel the precursor tRNA (pre) as well as the tRNA that has been spliced in the oocyte (tRNA) are indicated. (**F**) Sc tRNA^{Phe} minihelix, a 35 nt RNA consisting of the acceptor stem and the TΨC arm.

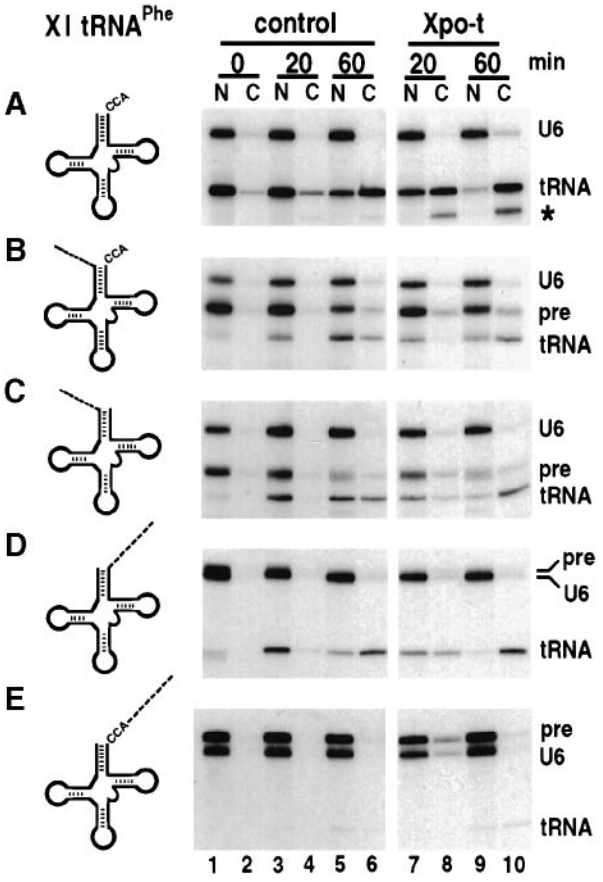

Fig. 5. Export from *Xenopus* oocyte nuclei of *X.laevis* pre-tRNA^{Phe}. (**A**) On the left is the secondary structure of the wildtype *X.laevis* (Xl) tRNA^{Phe} with its 5′ and 3′ ends completely matured. On the right is the result of a microinjection experiment into the nuclei of *Xenopus* oocytes (lanes 1–10) using this tRNA and U6Δss. The RNA mixture was co-injected either with buffer (lanes 1–6) or with Xpo-t (lanes 7–10). The annotations are as in Figure 4. (B–E) As in (A) but with the following tRNA variants: (**B**) Xl tRNA^{Phe}-B, a precursor variant containing the unprocessed 5′ extension, but the mature 3′ end including the terminal CCA sequence. (**C**) Xl tRNA^{Phe}-C, a precursor variant containing the unprocessed 5′ extension and a processed 3′ end lacking the terminal CCA sequence. (**D**) Xl tRNA^{Phe}-D, a precursor variant containing the mature 5′ end and the unprocessed 3′ end. (**E**) Xl tRNA^{Phe}-E, a variant containing the mature 5′ end and an unprocessed 3′ end into which the CCA sequence has been introduced at positions 73–76 of the RNA.

Xpo-t was injected 30 min after the RNA (data not shown). These results demonstrate that intron-containing tRNA^{Phe} binds well to Xpo-t–RanGTP, and suggest strongly that the reason that intron-containing tRNA is not normally exported is due to competition between Xpo-t and other nuclear proteins that recognize intron-containing tRNAs and retain them in the nucleus (see Discussion).

As mentioned above, tRNA precursors without introns but carrying both 5′ and 3′ extensions and lacking the post-transcriptionally added CCA end are not exported and do not bind Xpo-t. To study the ‘end’ requirements for export in more detail, we made a series of tRNA constructs as illustrated in Figure 5A–E. These were based on *X.laevis* pre-tRNA^{Phe} (Lin-Marq and Clarkson, 1995) since this pre-tRNA has extensions of defined sequence and lacks an intron. tRNAs carrying a 5′ extension and having either a mature CCA end or an immature 3′ end were not substrates for export even in the presence of

excess Xpo-t (Figure 5B and C), and these RNAs did not bind to Xpo-t–RanGTP *in vitro* (data not shown). Mature tRNA^{Phe} derived from the artificial precursors by processing in the injected oocytes was however efficiently exported (Figure 5B and C). Similarly, a 5′ end-mature but 3′ end-extended precursor was not exported until processed (Figure 5D). An artificial construct that was 5′ end-mature, included the 'mature' CCA residues, but also carried a 3′ extension attached to the CCA, was neither exported nor matured to a significant extent (Figure 5E). The 3′ extended RNAs also did not bind Xpo-t–RanGTP *in vitro* (data not shown). These experiments show that the possession of either a 5′- or a 3′ end-extension interferes with Xpo-t binding and nuclear export. An RNA that has the mature sequence but that does not end at the conserved CCA (Figure 5E) is not an export substrate. The requirement for correct ends for Xpo-t binding is not absolute, as at least some tRNAs lacking the 3′ CCA or carrying a few extra nucleotides at their 5′ ends do bind detectably to Xpo-t *in vitro* (Arts *et al*., 1998; Kutay *et al*., 1998).

Aminoacylation is not required for tRNA^{Phe} export

Recently it has been discovered that tRNA aminoacylation occurs in the nucleus of *Xenopus* oocytes as well as in the cytoplasm (Lund and Dahlberg, 1998). Since the identity elements of yeast tRNA^{Phe} are G20, G34, A35, A36 and A73 (Sampson *et al*., 1989) the intron-containing version of tRNA^{Phe} (Figure 4E) should not be aminoacylated. It therefore seemed unlikely that aminoacylation would be an absolute requirement for export. Further, many of the binding studies reported here and previously (Arts *et al*., 1998; Kutay *et al*., 1998) were carried out with non-aminoacylated, and indeed non-modified, *in vitro* transcripts, demonstrating that neither aminoacylation nor modification is a requirement for Xpo-t–RanGTP binding.

To examine the relationship between aminoacylation and export more definitively, we made use of two mutant forms of tRNA^{Phe} that are known to be extremely poor substrates for aminoacylation by yeast phenylalanyl-tRNA synthetase (Sampson *et al*., 1992). One is a double mutant in which G34 and A35 are both mutated to U, the second a triple mutant with these two changes plus an A73U mutation (Figure 6A and B). Both displayed specific binding to Xpo-t–RanGTP *in vitro* (Figure 6A and B, lanes 1–4). The binding of the double mutant was indistinguishable from wild-type tRNA^{Phe} (data not shown) while the triple mutant bound less strongly (Figure 6A and B). The double mutant was exported at a readily detectable rate, although more slowly than wild-type tRNA^{Phe} (Figure 6A, lanes 5–10 and Figure 4A). The weaker binding of the triple mutant to Xpo-t–RanGTP correlated with a lower export rate (Figure 6B, lanes 5–10). Export of both mutants was significantly increased by microinjection of excess Xpo-t (Figure 6A and B, lanes 11–14).

To demonstrate that the mutants were not subject to aminoacylation in *Xenopus* oocytes, we analysed microinjected wild-type and mutant tRNA^{Phe} on acid gels that separate aminoacylated and non-aminoacylated tRNA species (Varshney *et al*., 1991). As markers we used mature tRNA^{Phe} purified from yeast before and after aminoacylation with purified yeast phenylalanyl-tRNA synthetase (a gift of D.Kern, UPR 9002, Strasbourg). The

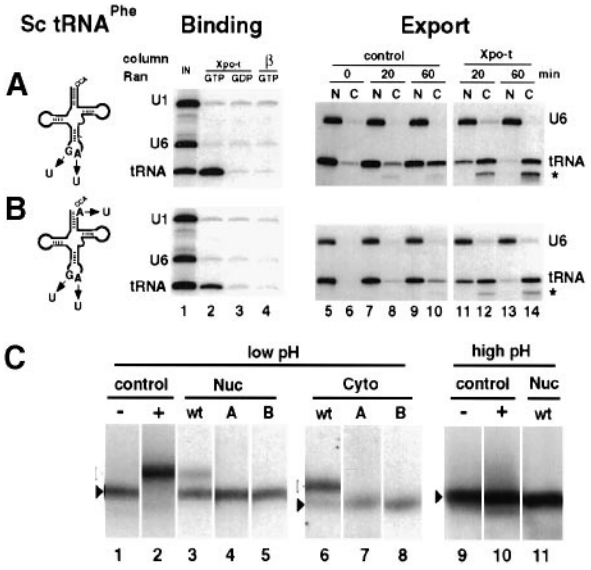

Fig. 6. *In vitro* binding to exportin-t and export from *Xenopus* oocyte nuclei of *S.cerevisiae* tRNA^{Phe} mutants defective in aminoacylation. (**A**) Sc tRNA^{Phe}G34U,A35U double mutant. Shown is the secondary structure of the tRNA with the positions of the mutations indicated (left), the *in vitro* binding to Xpo-t (lanes 1–4) and its export behaviour on microinjection into *Xenopus* oocyte nuclei (lanes 5–14). Other conditions are as in Figure 4. (**B**) Sc tRNA^{Phe}G34U,A35U, A73U triple mutant. Other conditions are as in (A). (**C**) Analysis of aminoacylation of Sc tRNA^{Phe} and mutants after microinjection into *Xenopus* oocytes. Purified Sc tRNA^{Phe} before (lane 1) and after (lane 2) *in vitro* aminoacylation. Lanes 3–8, wild-type or mutant Sc tRNA^{Phe} was microinjected either into the nucleus or cytoplasm of *Xenopus* oocytes as indicated, extracted 30 min later, and separated on a denaturing gel at acid pH. Slow migrating species in lanes 3 and 6 correspond to aminoacylated tRNA, the G34U, A35U (A) and G34U, A35U, A73U (B) mutants do not give rise to aminoacylated species (lanes 4–8). On treatment with alkali to remove the bound amino acid, the migration of *in vitro* and *in vivo* aminoacylated tRNA^{Phe} (lanes 10 and 11) is identical to that of control tRNA^{Phe} (lane 9). Aminoacylated tRNAs are indicated with open triangles, non-aminoacylated tRNAs with filled triangles.

aminoacylated tRNA migrates more slowly (Figure 6C, lanes 1 and 2). After microinjection into the nucleus or cytoplasm of oocytes and 30 min incubation, aminoacylation of tRNA^{Phe} was examined. In both cases, two bands were seen, representing aminoacylated and non-aminoacylated tRNA^{Phe} in both samples (Figure 6C, lanes 3 and 6). Note that the nuclear- and cytoplasmic-injected tRNAs migrate differently, probably due to their being differentially modified in the nucleus and cytoplasm (Nishikura and De Robertis, 1981). To demonstrate further that the upper bands (open triangles) represented aminoacylated tRNA^{Phe}, the control RNAs and the wild-type tRNA injected into the nucleus were subjected to alkaline pH to deacylate the tRNAs. After this treatment, the aminoacylated control tRNA^{Phe} and the microinjected wild-type tRNA^{Phe} co-migrated with non-aminoacylated tRNA^{Phe} (Figure 6C, lanes 9–11). In contrast to the wild-type, both mutant forms of tRNA^{Phe} were present as single species after either cytoplasmic or nuclear injection, equivalent to the faster-migrating non-aminoacylated form of the wild-type tRNA (Figure 6C, lanes 4, 5, 7 and 8). Thus, these mutant tRNAs are not subject to aminoacylation, although they are exported from the nucleus. The difference in the rate of export between the double mutant and wild-type tRNA^{Phe} suggests that aminoacylation may

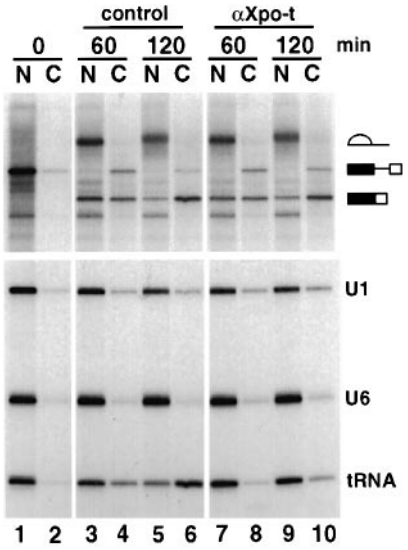

Fig. 7. Inhibition of tRNA export by Xpo-t antibodies. tRNA export in *Xenopus* oocytes is inhibited by the pre-injection of antibodies raised against Xpo-t. *Xenopus* oocytes were pre-injected into the nucleus with either buffer (control, lanes 1–6) or affinity purified antibodies raised against Xpo-t (lanes 7–10) and incubated for 30 min In a second nuclear injection, a mixture of $tRNA^{Phe}$, U6Δss, U1ΔSm and ftz pre-mRNA was injected. The oocytes were dissected into nuclear (N) and cytoplasmic (C) fractions either immediately (lanes 1–2), after 60 min (lanes 3–4, 7–8) or 120 min (lanes 5–6, 9–10). RNA was recovered and analysed by denaturing gel electrophoresis. Two different autoradiographic exposures of parts of the same gel are shown.

increase export efficiency (see also Lund and Dahlberg, 1998).

Xpo-t antibodies drastically reduce tRNA export

As shown using HeLa cell tRNA (Kutay *et al*., 1998), Xpo-t does not seem to be specific for a subset of tRNAs, but rather to bind most or all of the population of tRNAs. This is also true of the tRNAs present in *Xenopus* oocyte extracts (data not shown) and suggests that Xpo-t can in principle be the export receptor for all nuclear tRNAs. We wished to inhibit Xpo-t function and assay the effect on tRNA export. To this end a rabbit antiserum was raised against recombinant human Xpo-t. This antiserum specifically recognizes both human Xpo-t and, although more weakly, a single band corresponding to the expected size of *Xenopus* Xpo-t on Western blots (data not shown). The antiserum was affinity-purified and injected into the nucleus of *Xenopus* oocytes. After 30 min incubation, a mixture of four RNAs was injected into the same nuclei. U6Δss, a control for nuclear integrity and injection accuracy, U1ΔSm to assay U snRNA export, a pre-mRNA derived from the *fushi-tarazu* (*ftz*) gene that is efficiently spliced in oocytes and yeast $tRNA^{Phe}$. On injection, ftz pre-mRNA is spliced and the resultant mRNA exported to the cytoplasm (Figure 8, lanes 1–6). U6 RNA remains largely nuclear while U1ΔSm and $tRNA^{Phe}$ are exported at different rates (Figure 7, lanes 1–6). Note that the figure shown is of an experiment where overall RNA export rates were slower than average, the result of variation between batches of oocytes. On injection of affinity-purified Xpo-t antibodies neither pre-mRNA splicing, mRNA export nor U snRNA export were affected. In contrast, $tRNA^{Phe}$ export was reduced by 99% after 60 min and by 78% after 120 min (Figure 7, lanes 7–10). The percentage inhibition was derived by quantitation of the bands seen in a phosphoimager followed by subtraction of the percentage of the negative control, U6 RNA, that had leaked into the cytoplasm. Antibodies purified from pre-immune or non-immune sera did not affect tRNA export (data not shown). The inhibitory effect was not specific for $tRNA^{Phe}$. Xpo-t antibodies had a similar quantitative effect on the export rate of $tRNA_i^{Met}$ when it was injected alone and on the entire population of yeast tRNAs when they were labelled by CCA transfer and injected together.

Discussion

Three aspects of tRNA export from the nucleus, and the role of Xpo-t in this process, were investigated in this study. First, detailed biochemical probing of the interaction between Xpo-t–RanGTP and tRNA was presented. Analysis of this interaction was then pursued making use of mutant and variant forms of tRNAs and pre-tRNAs. These studies provide evidence for the functional relevance of the interaction data, and suggest mechanisms by which pre-tRNAs and mature tRNAs are discriminated by the export machinery such that only the latter are transported to the cytoplasm. Finally, evidence that Xpo-t is the major tRNA export receptor in *Xenopus* oocytes was presented.

Extensive interaction between Xpo-t–RanGTP and tRNA

Xpo-t binds tRNA cooperatively with RanGTP (Arts *et al*., 1998; Kutay *et al*., 1998). Binding of the Xpo-t–RanGTP complex to yeast $tRNA^{Phe}$ was examined by protection and interference analysis using a variety of probing reagents. These data are displayed on a representation of the 3-D structure of $tRNA^{Phe}$ in Figure 8. It is immediately obvious that Xpo-t–RanGTP interacts mainly with the upper half of the tRNA, composed of the acceptor and TΨC arms. The interactions presented in this figure are incomplete since analysis of tRNA variants indicates that the mature 5′ and 3′ ends of the tRNA are also important for Xpo-t–RanGTP binding (Kutay *et al*., 1998 and this study). Further, mutation of the conserved purine (A) residue that precedes the mature CCA 3′ end to U also decreased Xpo-t–RanGTP interaction, probably by disrupting stacking of the ACCA end on the acceptor helix.

Remarkably, ribose groups located much of the way along both strands of the acceptor and TΨC stems as well as riboses located in the TΨC loop are protected against modification by Xpo-t–RanGTP. Further, in the TΨC arm, phosphates along both strands of the stem and in the loop, when modified, interfere with Xpo-t–RanGTP binding. These data, together with ribonuclease V1 protection studies, support the hypothesis that Xpo-t–RanGTP makes extensive interactions with this part of the tRNA, and essentially envelopes the upper part of the tRNA structure.

The fact that ethylation of several individual phosphate groups, those at positions 56, 57, 59 and 62, abolishes binding suggests that Xpo-t–RanGTP may directly contact the backbone at these positions. The strong effect of ethylation at phosphate 49 on Xpo-t–RanGTP binding is, on the other hand, not likely to be due to direct interaction. This phosphate interacts with the 2′ hydroxyl of ribose 7

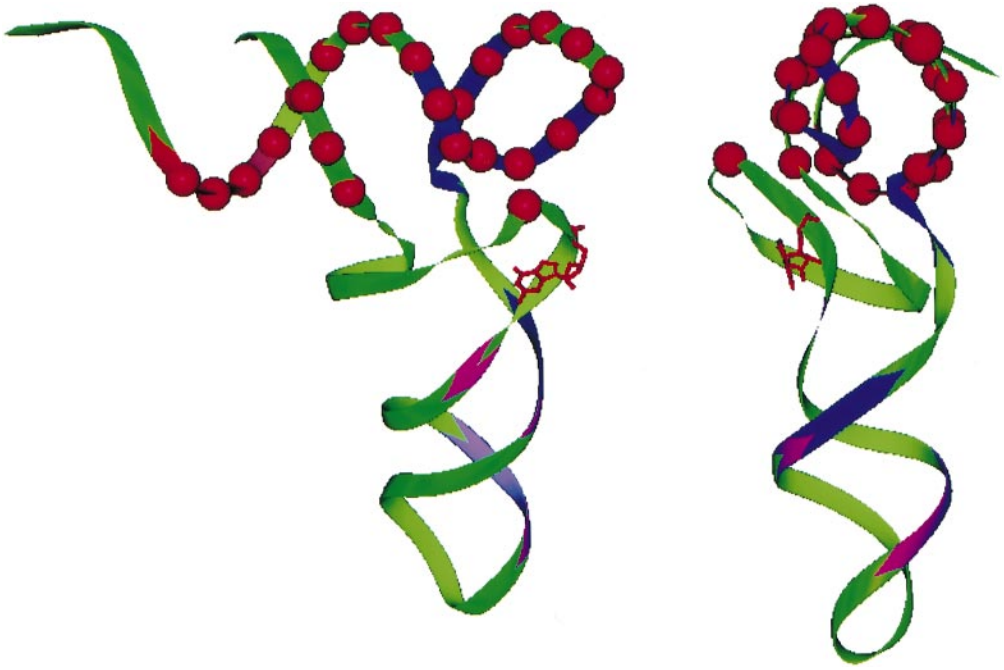

Fig. 8. Three-dimensional model of yeast $tRNA^{Phe}$ summarizing the protection and interference data for the Xpo-t–RanGTP/tRNA complex. The figure was created with DRAWNA (Massire *et al*., 1994) with coordinates from Quigley *et al*. (1975). Riboses protected by Xpo-t–RanGTP are red spheres, phosphates whose modification interferes with their binding are blue ribbons. The G at position 20 protected against RNase T1 digestion is red, and purple ribbon represents increased accessibility to RNase V1.

(Ladner *et al*., 1975). Modification of phosphate 49 will therefore alter the conformation of $tRNA^{Phe}$, and this is the more likely cause of binding inhibition.

Other factors that recognize most or all tRNAs have related modes of recognition. CCA-adding enzyme (Shi *et al*., 1998), elongation factor-Tu in the GTP-bound state (Nissen *et al*., 1995) and RNase P (McClain *et al*., 1987) all bind to this region of the tRNA, although it is not thought that these enzymes make such extensive interactions as the Xpo-t–RanGTP complex. A further difference between the interactions is that a minihelix consisting solely of the acceptor and TΨC arms is not a substrate for Xpo-t–RanGTP binding (Figure 4F) while all three of the above-mentioned tRNA-binding enzymes recognize the minihelix and can use it as a substrate (McClain *et al*., 1987; Rudinger *et al*., 1994; Li *et al*., 1997). These three enzymes are all ancient components of the protein synthetic machinery, and their ability to bind the minihelix may be a reflection of tRNA evolution. It has been proposed that minihelices are the oldest remaining aspect of tRNA structure, with the D loop, anticodon arm and extra arm being later additions (Schimmel *et al*., 1993). Xpo-t, being a more recent addition to the tRNA-binding protein family, might have evolved at a later date, after tRNA structure had become more complex, and thus has evolved to recognize additional structural features.

This reasoning is supported by analysis of the basis of the difference in binding behaviour between Xpo-t–RanGTP and the enzymes. Xpo-t–RanGTP requires aspects of tRNA structure for binding that are not present in the minihelix. This can be deduced from the loss of binding to a mutant tRNA where the TΨC loop–D loop interaction is disrupted and is further supported by the protection by Xpo-t–RanGTP of the ribose of D17. Other changes in accessibility of the D arm on Xpo-t–RanGTP binding are suggestive of some alteration of the structure of this region, including the TΨC loop–D loop interaction. The nuclease T1 cleavages after G18 and G19 are not affected on binding while cleavage after G20 is reduced. In contrast, V1 cleavage after G22 increases on Xpo-t–RanGTP binding (Figure 3).

The non-uniformity of these changes suggests they may not be due to Xpo-t–RanGTP interaction with the affected residue. Rather, they may reflect a change in tRNA structure induced by Xpo-t–RanGTP. Similarly, since Pb^{2+} cleavage after D17 requires tertiary interaction between the D and TΨC loops, with the TΨC loop forming the Pb^{2+}-binding site (Brown *et al*., 1985; Behlen *et al*., 1990) the loss of lead cleavage on Xpo-t–RanGTP binding may reflect a structural change in either loop rather than direct protection against cleavage. Finally, the increase in cleavage by V1 of the 5′ side of the anticodon stem is again suggestive of some conformational change in this area that increases accessibility towards the enzyme.

In summary, Xpo-t–RanGTP binds mainly to the upper half of the tRNA and probably also contacts the backbone of the D loop and the anticodon stem–extra arm junction (Figure 8). Other changes in accessibility to enzymes and modifiers are likely to be due to conformational alteration in the tRNA induced by Xpo-t–RanGTP binding.

Discrimination between pre-tRNA and tRNA

The enzymes responsible for pre-tRNA processing are located in the oocyte nucleus (Melton *et al*., 1980; De Robertis *et al*., 1981). In general, pre-tRNA processing is completed in the nucleus before export (Figures 4E and 5; Melton *et al*., 1980). How does the cell discriminate between pre-tRNA and tRNA, and only export the latter? Our data provide evidence for at least two separate

mechanisms. First, tRNAs carrying the natural 5′ or 3′ extensions do not bind Xpo-t–RanGTP *in vitro* and are not substrates for export *in vivo*. Thus, end processing is clearly a prerequisite for efficient interaction of a tRNA with the export machinery. It appears that the exact mature 5′ and 3′ ends are not essential for the interaction of all tRNAs with Xpo-t, since, for example, $tRNA^{Ser}$ lacking the CCA showed reduced, rather than no, binding to Xpo-t *in vitro* (Kutay *et al*., 1998).

The presence of an intron, on the other hand, did not affect Xpo-t interaction *in vitro*. Melton *et al*. (1980) observed that intron-containing $tRNA^{Tyr}$ could be exported. Although intron-containing $tRNA^{Phe}$ was only exported to a very minor extent under normal conditions (Figure 4E), this pre-tRNA was efficiently exported either on coinjection of excess Xpo-t (Figure 4E) or if Xpo-t was injected in excess subsequent to injection of the pre-tRNA (data not shown). This suggests strongly that there is a competition between Xpo-t–RanGTP and other nuclear proteins for binding to intron-containing tRNA, and that, in this case, discrimination between the mature and immature species with regard to export is simply the result of competition between proteins that retain the pre-tRNA in the nucleus and those that export it. The most obvious possible competitors of Xpo-t–RanGTP are the proteins that constitute the tRNA splicing machinery (Di Nicola Negri *et al*., 1997; Fabbri *et al*., 1998 and references therein).

Xpo-t and redundancy in export pathways

It is likely, but not certain (Arts *et al*., 1998), that Xpo-t is the vertebrate homologue of the *S.cerevisiae* Los1p protein. The *LOS1* gene was discovered in two different genetic screens, one for mutations that affected tRNA processing (Hopper *et al*., 1980; Hurt *et al*., 1987) and one for mutations that produced synthetic lethality together with a gene encoding a defective nuclear pore complex protein, or nucleoporin (Simos *et al*., 1996a). Subsequent analysis demonstrated interactions between *LOS1* and several genes encoding proteins involved in various aspects of tRNA metabolism (Simos *et al*., 1996a,b; Hellmuth *et al*., 1998). Los1p is a member of the importin-β family of proteins (Fornerod *et al*., 1997b; Görlich *et al*., 1997) which includes all currently known nucleocytoplasmic transport receptors (Ohno *et al*., 1998). These data are all consistent with the hypothesis that Los1p is, like Xpo-t, a tRNA export receptor. However, *LOS1* is not an essential gene (Hopper *et al*., 1980; Hurt *et al*., 1987) suggesting either that tRNA export can still occur in the absence of a dedicated receptor or that there is a redundant pathway of tRNA export in yeast. The fact that no other member of the yeast importin-β family is closely related to Los1p (Fornerod *et al*., 1997b; Görlich *et al*., 1997; Ohno *et al*., 1998) suggests that none of these genes is likely to encode a protein that is functionally identical to Los1p. Thus, if there is a redundant tRNA export mediator in yeast, it is not closely related to Los1p.

Our analysis shows that there is a strong correlation between Xpo-t–RanGTP binding and tRNA nuclear export. That is, for the RNAs tested, those that bound tightly to Xpo-t–RanGTP *in vitro* were exported efficiently from the nucleus *in vivo*, those that did not bind were not exported, and those that bound with intermediate affinity were exported slowly in the presence of endogenous levels of Xpo-t. The only exception to this correlation was intron-containing pre-tRNA, as discussed above. This correlation between binding and export would be consistent with the hypothesis that Xpo-t could be the major, or indeed the only, mediator of tRNA export in *Xenopus* oocyte nuclei.

Further evidence on this topic came from antibody inhibition experiments. Antibodies against Xpo-t, when injected into oocyte nuclei, had a strong and specific inhibitory effect on export of $tRNA^{Phe}$ and $tRNA_i^{Met}$ when they were injected individually, or on the whole population of yeast tRNAs when they were injected together (Figure 7; data not shown). Injection of lower concentrations of antibody resulted in reduced inhibition, but it proved technically impossible to further increase the antibody concentration. We cannot therefore say if the minor amount of tRNA export seen in the conditions used is due to incomplete inhibition of Xpo-t due to insufficient antibody or if it is due to export by some other mechanism. However, even in the latter case, if there is a redundant mediator of tRNA export in *Xenopus* oocytes that is not recognized by the Xpo-t antibodies, it functions very inefficiently. The binding and antibody inhibition data would therefore suggest that Xpo-t and other related proteins recognized by the antibody are either the major or the only tRNA export receptors in *Xenopus* oocytes.

Materials and methods

Recombinant proteins

Expression and purification of the histidine-tagged recombinant proteins exportin-t, zz-exportin-t, z-importin-β, RanGTP, RanGDP and RanQ69LGTP was performed as described previously (Arts *et al*., 1998).

End labelling of yeast $tRNA^{Phe}$

Purified $tRNA^{Phe}$ from yeast (Sigma) was either 5′- or 3′ end-labelled. For the 3′ end-labelling (Vlassov *et al*., 1981), 3′ terminal nucleotides were removed by incubating 10 µg $tRNA^{Phe}$ for 10 min at 20°C in 25 µl containing 0.1 µg phosphodiesterase (Boehringer), 50 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$. The reaction was stopped by phenol–chloroform extraction and ethanol precipitation. Three micrograms of the pretreated tRNA was 3′ end-labelled by incubating for 45 min at 37°C in 25 µl containing 0.5 mg/ml yeast CCA adding enzyme (Rether *et al*., 1974), 50 mM Tris–glycine pH 8.5, 10 mM $MgCl_2$, 8 mM DTT, 50 µM rCTP and 60 µCi [α-^{32}P]ATP (3000 Ci/mmol, Amersham). For 5′ end-labelling, the tRNA was dephosphorylated by bacterial alkaline phosphatase (Amersham) and subsequently 5′ labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Gibco-BRL) according to the manufacturer's instructions. Both end-labelled tRNAs were purified from an 8% polyacrylamide–7 M urea denaturing gel.

RNA protection assays

In the enzymatic and lead-induced cleavage assays 50 000 c.p.m. of either 3′ or 5′ end-labelled tRNA was used per reaction. For the hydroxyl radical protection experiments double this amount was used. In all footprinting assays, the RNA was mixed in buffer A (50 mM HEPES–KOH pH 7.5, 5 mM Mg acetate, 200 mM K acetate) and incubated for 15 min at 20°C. Recombinant exportin-t and GTP-loaded Ran were then both added to a final concentration of 0.4 to 3 µM, or omitted. Complex formation was allowed by incubating the reaction at 20°C for 30 min. Fe(II)EDTA reactions were as described (Hüttenhofer and Noller, 1992; Lentzen *et al*., 1996) in 25 µl buffer A containing 4 mM EDTA, 10 mM DTT, 2 mM $(NH_4)_2Fe(SO_4)_2$, 0.02% H_2O_2 for 2 min at 20°C.

RNase V1 probing was performed in 10 µl buffer A containing 2 µg carrier 5S RNA and 0.05 units of RNase V1 (Pharmacia) for 5 min at 20°C. Lead(II) induced hydrolysis (Behlen *et al*., 1990) was in 10 µl buffer A with 1 mM Pb(II) acetate for 5 min at 20°C and was stopped by addition of 5 µl 50 mM EDTA. Incubation controls were done in parallel without probing reagent. All reactions were stopped by the

addition of 50 µl 0.3 M NaAc pH 5.0 and phenol–chloroform extraction. The RNAs were ethanol precipitated with 2 µg of 5S RNA as carrier (if not already present) and the pellets were washed with 80% ethanol, dried, and resuspended in 6 µl RNA-loading dye. Equal counts were loaded in each lane for 15% denaturing PAGE. As standards, an alkaline ladder and an RNase T1 ladder were also loaded onto the gel. For the alkaline ladder, 100 000 c.p.m. of 3′- or 5′ end-labelled tRNA mixed with 1 µg carrier RNA was hydrolysed in 70 mM sodium carbonate pH 9.0 for 5 min at 90°C. For the T1 ladder, 50 000 c.p.m. mixed with carrier was first denatured in 20 mM sodium citrate pH 4.5, 1 mM EDTA, 7 M urea and gel-loading dyes for 5 min at 50°C. On ice, 0.005 U RNase T1 was added and the reaction was performed for 5 min at 50°C, stopped and kept on ice until loading.

Ethylation interference assay

Phosphate ethylation by ethylnitrosourea (ENU) was performed under denaturing conditions as described (Romby *et al*., 1985). Briefly, 2×10^6 c.p.m. of 3′ end-labelled yeast $tRNA^{Phe}$ was incubated for 2 min at 90°C in 25 µl containing 100 mM sodium cacodylate pH 8.0, 1 mM EDTA, and 20% ENU which was added to the reaction as a saturated solution in ethanol. In the control reaction ethanol was added to 20%. The reactions were stopped by the addition of 50 µl 0.3 M Na acetate pH 5.0 and ethanol precipitated. The pellets were resuspended in 10 µl of buffer B (50 mM HEPES–KOH pH 7.9, 200 mM NaCl, 5 mM $MgCl_2$) of which 7 µl was used for the *in vitro*-binding assay and the other 3 µl was kept as the input fraction. The binding reaction, the separation of the bound and unbound fractions and the RNA extractions were performed as descibed (Arts *et al*., 1998) and in the section '*in vitro* binding', 2 µg 5S RNA were used as carrier RNA. The washed and dried RNA pellets were dissolved in 10 µl 0.1 M Tris–HCl pH 9.0 and incubated for 10 min at 50°C to hydrolyse the RNA backbone at ethylated phosphates. The reaction was stopped by the addition of 50 µl 0.3 M Na acetate pH 5.0, ethanol precipitated and the washed pellet resuspended in 6 µl RNA-loading dye. Again, equal counts were loaded on a 15% polyacrylamide-7 M urea denaturing gel.

In vitro-binding assay

In vitro-binding assays of internally labelled, *in vitro* transcribed tRNA variants using immoblized zz-exportin-t or z-importin-β were as described previously (Arts *et al*., 1998). The bound and unbound fractions were incubated for 30 min at 37°C in 300 µl homogenization mixture containing 50 mM Tris–HCl pH 7.5, 5 mM EDTA, 1.5% SDS, 300 mM NaCl and 1.5 mg/ml proteinase K. Samples were phenol–chloroform extracted and ethanol precipitated with 10 µg glycogen as a carrier. RNAs were analysed on a 10% polyacrylamide-7 M urea denaturing gel.

Antibody purification

An affinity column carrying 1.5 mg recombinant human exportin-t was prepared, using 500 µl of Affigel-15 beads (Bio-Rad) that had been quickly washed twice with ice cold deionized water. The beads were rotated for 4 h at 4°C in a total volume of 4 ml Transport buffer (20 mM HEPES–KOH pH 7.5, 140 mM K acetate, 5 mM Mg acetate, 1 mM DTT and 250 mM sucrose) containing the protein. The beads were spun down at 1000 r.p.m. for 5 min at 4°C and the supernatant removed. Three millilitres of 0.1 M ethanolamine pH 8.0 were added to the beads, and the mixture was rotated overnight at 4°C. The blocked beads were washed with 20 ml PBS and rotated with 10 ml immune serum, from a rabbit injected with recombinant human exportin-t, for 3 h at 4°C. The column was allowed to settle and the supernatant drained. The column was washed with 20 ml of PBS and eluted with 10 ml of 0.2 M glycine pH 2.2, 0.5 M NaCl. Fractions of 1 ml were collected in tubes containing 0.5 ml 1 M Tris–HCl pH 8.0. The flow-through fraction was collected and the binding and elution cycle repeated twice more. The OD_{280} of the fractions was measured and the most concentrated fractions pooled. Protein was precipitated with 60% (final) ammonium sulfate by stirring for 2 h at 4°C and spun at 20 000 r.p.m. at 4°C for 30 min. The pellet was resuspended in 600 µl PBS and dialysed overnight against PBS (final protein concentration 4 mg/ml). This affinity purified fraction was concentrated a further 10× with centrifugal concentrators (Nanosep, Pall Filtron Corporation) and used for micro-injection experiments in *Xenopus* oocytes.

Oocyte injections

Microinjections of RNA into *X.laevis* oocytes, incubations, extractions and analyses were performed as described previously (Jarmolowski *et al*., 1994). In experiments where recombinant exportin-t was co-injected with the RNA mixtures, 0.15 pmol exportin-t in Transport buffer was injected per oocyte. In control injections Transport buffer was used. In the double nuclear injection experiments with the affinity purified antibody raised against human exportin-t, ~0.7 µg antibody was injected into each oocyte nucleus. Oocytes were then incubated for 30 min at 19°C before injection of RNA mix. Both injection mixtures contained dye to allow selection for double-injected nuclei.

Cloning of tRNA genes

The tRNA genes described in this paper were obtained as follows: two oligonucleotides were synthesized; (i) a sense oligo containing at the 5′ end a cloning site (*Hin*dIII) and the T7 promoter sequence followed by the 5′ half of the desired tRNA sequence, (ii) an antisense oligo containing at its 5′ end a cloning site (*Bam*HI or *Xba*I), a restriction site for linearization followed by the antisense sequence of the 3′ half of the desired tRNA. The oligos were designed so that they could base pair with one another at their 3′ ends for ~15 nucleotides. The sense and antisense oligos (15 µM of each) in annealing buffer (50 mM Tris–HCl pH 7.9, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT) were denatured for 5 min at 95°C and allowed to anneal by incubation for 3 min at 65°C then slowly cooled to room temperature. From this mixture, 37.5 fmoles annealed oligos were extended by incubating for 5 min at 37°C, 10 min at 20°C in a 30 µl reaction containing 2.5 U Klenow fragment (Gibco-BRL) and 0.2 mM of each dNTP in the buffer recommended by the manufacturer. The double-stranded fragment was digested with *Bam*HI (or *Xba*I) and *Hin*dIII and cloned into the appropriate cloning sites of pUC19. The inserts were completely sequenced. Details of the oligonucleotides used are available on request (Mattaj@embl-heidelberg.de).

^{32}P-labelled RNAs were synthesized according to Jarmolowski *et al*. (1994). Templates which are described elsewhere are: T7-U1ΔSm, T7-U6Δss, T7-ftz (Hamm *et al*., 1987; Rio, 1988; Hamm and Mattaj, 1989; Jarmolowski *et al*., 1994; Boelens *et al*., 1995).

The minihelix RNA was obtained as a ribo-oligonucleotide that was fractionated on a 10% polyacrylamide denaturing gel, visualized by UV shadowing and extracted. The RNA was labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase.

Analysis of aminoacylated tRNAs

Labelled tRNAs were injected into either the nucleus or the cytoplasm of *Xenopus* oocytes. After 30 min incubation, RNA was extracted from total oocytes. Samples were extracted under acid conditions, using buffer I (8 M Guanidine-HCl, 0.1 M Na acetate pH 4.5, 0.5% Sarkosyl, 10 mM EDTA, 5 mM DTT), followed by phenol–chloroform extraction (equilibrated with 0.3 M Na acetate pH 4.5, 10 mM EDTA) and then precipitated with ethanol. The pellet was resuspended in buffer II (8 M urea, 0.3 M Na acetate pH 4.5, 0.2% Sarkosyl, 10 mM EDTA, 1 mM DTT) and extracted twice with phenol–chloroform prior to ethanol precipitation. The pellet was resuspended in 0.3 M Na acetate pH 4.5, 0.1 mM EDTA, and re-precipitated with ethanol. To deacylate the tRNAs, extraction was performed at pH 7.9. The samples were then treated as described above and ethanol precipitated. Pellets were washed, dried and resuspended in acid dyes (0.4 M Na acetate pH 5.0, 50% urea, 30% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol). The samples were loaded on a 10% polyacrylamide (ratio of acrylamide to bisacrylamide, 19:1), 8 M urea, 0.1 M Na acetate pH 5.0, denaturing gel. The 20 cm long gel was run for 20 h at 9 V/cm at 4°C using 0.1 M Na acetate pH 5.0 as running buffer and dried before exposure.

Acknowledgements

We thank D.Kern for the gift of phenylalanyl-tRNA synthetase. We are also grateful to Matthias Hentze, David Tollervey and the members of our laboratory, in particular Maarten Fornerod, for critical comments on the manuscript and discussion of the project and Elsebet Lund for pointing out that the methionyl tRNA T7 transcript carries a short 5′ extension. G.-J.A. and S.K. hold a TMR Marie Curie and an NIH research training fellowship, respectively.

References

Altman,S., Kirsebom,L. and Talbot,S. (1993) Recent studies of ribonuclease P. *FASEB J*., **7**, 7–14.

Arts,G.-J., Fornerod,M. and Mattaj,I.W. (1998) Identification of a nuclear export receptor for tRNA. *Curr. Biol*., **8**, 305–314.

Behlen,L.S., Sampson,J.R., DiRenzo,A.B. and Uhlenbeck,O.C. (1990) Lead-catalyzed cleavage of yeast tRNA^{Phe} mutants. *Biochemistry*, **29**, 2515–2523.

Boelens,W.C., Palacios,I. and Mattaj,I.W. (1995) Nuclear retention of RNA as a mechanism for localization. *RNA*, **1**, 273–283.

Brown,R.S., Hingerty,B.E., Dewan,J.C. and Klug,A. (1983) Pb (II)-catalysed cleavage of the sugar-phosphate backbone of yeast tRNAPhe-implications for lead toxicity and self-splicing RNA. *Nature*, **30**, 543–546.

Brown,R.S., Dewan,J.C. and Klug,A. (1985) Crystallographic and biochemical investigation of the lead (II)-catalyzed hydrolysis of yeast phenylalanine tRNA. *Biochemistry*, **24**, 4785–4801.

Cole,C.N. and Hammell,C.M. (1998) Nucleocytoplasmic transport: driving and directing transport. *Curr. Biol.*, **8**, 368–372.

Corbett,A.H. and Silver,P.A. (1997) Nucleocytoplasmic transport of macromolecules. *Microbiol. Mol. Biol. Rev.*, **61**, 193–211.

Dahlberg,J.E. and Lund,E. (1998) Functions of the GTPase Ran in RNA export from the nucleus. *Curr. Opin. Cell Biol.*, **10**, 400–408.

De Robertis,E.M., Black,P. and Nishikura,K. (1981) Intranuclear location of the tRNA splicing enzymes. *Cell*, **23**, 89–93.

Di Nicola Negri,E., Fabbri,S., Bufardeci,E., Baldi,M.I., Gandini,A.D., Mattoccia,E. and Tocchini-Valentini,G.P. (1997) The eucaryal tRNA splicing endonuclease recognizes a tripartite set of RNA elements. *Cell*, **89**, 859–866.

Edqvist,J., Straby,K.B. and Grosjean,H. (1993) Pleiotrophic effects of point mutations in yeast tRNA (Asp) on the base modification pattern. *Nucleic Acids Res.*, **21**, 413–417.

Fabbri,S., Fruscoloni,P., Bufardeci,E., Di Nicola Negri,E., Baldi,M.I., Attardi,D.G., Mattoccia,E. and Tocchini-Valentini,G.P. (1998) Conservation of substrate recognition mechanisms by tRNA splicing endonucleases. *Science*, **280**, 284–286.

Fornerod,M., Ohno,M., Yoshida,M. and Mattaj,I.W. (1997a) CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell*, **90**, 1051–1060.

Fornerod,M., van Deursen,J., van Baal,S., Reynolds,A., Davis,D., Murti,K.G., Fransen,J. and Grosveld,G. (1997b) The human homologue of yeast CRM1 is in a dynamic subcomplex with CAN/Nup214 and a novel nuclear pore component Nup88. *EMBO J.*, **16**, 807–816.

Giegé,R., Puglisi,J.D. and Florentz,C. (1993) tRNA structure and aminoacylation efficiency. *Prog. Nucleic Acid Res. Mol. Biol.*, **45**, 129–206.

Görlich,D. (1997) Nuclear protein import. *Curr. Opin. Cell Biol.*, **9**, 412–419.

Görlich,D., Dabrowski,M., Bischoff,F.R., Kutay,U., Bork,P., Hartmann,E., Prehn,S. and Izaurralde,E. (1997) A novel class of RanGTP binding proteins. *J. Cell Biol.*, **138**, 65–80.

Grüter,P., Tabernero,C., von Kobbe,C., Schmitt,C., Saavedra,C., Bachi,A., Wilm,M., Felber,B.K. and Izaurralde,E. (1998) TAP, the human homolog of Mex67p, mediates CTE-dependent RNA export from the nucleus. *Mol. Cell*, **1**, 649–659.

Hamm,J. and Mattaj,I.W. (1989) An abundant U6 snRNP found in germ cells and embryos of *Xenopus laevis*. *EMBO J.*, **8**, 4179–4187.

Hamm,J., Kazmaier,M. and Mattaj,I.W. (1987) *In vitro* assembly of U1 snRNPs. *EMBO J.*, **6**, 3479–3485.

Hellmuth,K., Lau,D.M., Bischoff,R., Künzler,M., Hurt,E. and Simos,G. (1998) Yeast Los1p has properties of an exportin-like nucleocytoplasmic transport factor for tRNA. *Mol. Cell. Biol.*, **18**, 6374–6386.

Hopper,A.K., Schultz,L.D. and Shapiro,R.A. (1980) Processing of intervening sequences: a new yeast mutant which fails to excise intervening sequences from precursor tRNAs. *Cell*, **19**, 741–751.

Hurt,D.J., Wang,S.S., Yu-Huei,L. and Hopper,A.K. (1987) Cloning and characterization of *LOS1*, a *Saccharomyces cerevisiae* gene that affects tRNA splicing. *Mol. Cell Biol.*, **7**, 1208–1216.

Hüttenhofer,A. and Noller,H.F. (1992) Hydroxyl radical cleavage of tRNA in the ribosomal P site. *Proc. Natl Acad. Sci. USA*, **89**, 7851–7855.

Izaurralde,E. and Mattaj,I.W. (1995) RNA export. *Cell*, **81**, 153–159.

Izaurralde,E. and Adam,S. (1998) Transport of macromolecules between the nucleus and the cytoplasm. *RNA*, **4**, 351–364.

Izaurralde,E., Lewis,J., Gamberi,C., Jarmolowski,A., McGuigan,C. and Mattaj,I.W. (1995) A cap-binding protein complex mediating U snRNA export. *Nature*, **376**, 709–712.

Izaurralde,E., Jarmolowski,A., Beisel,C., Mattaj,I.W., Dreyfuss,G. and Fischer,U. (1997) A role for the M9 transport signal of hnRNP A1 in mRNA nuclear export. *J. Cell Biol.*, **137**, 27–35.

Jarmolowski,A., Boelens,W.C., Izaurralde,E. and Mattaj,I.W. (1994) Nuclear export of different classes of RNA is mediated by specific factors. *J. Cell Biol.*, **124**, 627–635.

Kim,S.H., Suddath,F.L., Quigley,G.J., McPherson,A., Sussman,J.L., Wang,A.H., Seeman,N.C. and Rich,A. (1974) Three-dimensional tertiary structure of yeast phenylalanine transfer RNA. *Science*,**185**, 435–440.

Kutay,U., Bischoff,F.R., Kostka,S., Kraft,R. and Görlich,D. (1997) Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. *Cell*, **90**, 1061–1071.

Kutay,U., Liposwky,G., Izaurralde,E., Bischoff,F.R., Schwarzmaier,P., Hartmann,E. and Görlich,D. (1998) Identification of a tRNA-specific nuclear export receptor. *Mol. Cell*, **1**, 359–369.

Ladner,J.E., Jack,A., Robertus,J.D., Brown,R.S., Rhodes,D., Clark,B.F. and Klug,A. (1975) Structure of yeast phenylalanine transfer RNA at 2.5 Å resolution. *Proc. Natl Acad. Sci. USA*, **72**, 4414–4418.

Latham,J.A. and Cech,T.R. (1989) Defining the inside and outside of a catalytic RNA molecule. *Science*, **245**, 276–282.

Lee,M.S., Henry,M. and Silver,P.A. (1996) A protein that shuttles between the nucleus and the cytoplasm is an important mediator of RNA export. *Genes Dev.*, **10**, 1233–1246.

Lentzen,G., Moine,H., Ehresmann,C., Ehresmann,B. and Wintermeyer,W. (1996) Structure of 4.5S RNA in the signal recognition particle of *Escherichia coli* as studied by enzymatic and chemical probing. *RNA*, **2**, 244–253.

Li,Z., Sun,Y. and Thurlow,D.L. (1997) RNA minihelices as model substrates for ATP/CTP:tRNA nucleotidyltransferase. *Biochem. J.*, **327**, 847–851.

Lin-Marq,N. and Clarkson,S.G. (1995) A yeast RNA binding protein that resembles the human autoantigen La. *J. Mol. Biol.*, **245**, 81–85.

Liu,J.C., Liu,M. and Horowitz,J. (1998) Recognition of the universally conserved 3′-CCA end of tRNA by elongation factor EF-Tu. *RNA*, **4**, 639–646.

Lockard,R.E. and Kumar,A. (1981) Mapping tRNA structure in solution using double-stranded ribonuclease V1 from cobra venom. *Nucleic Acids Res.*, **9**, 5125–5140.

Lund,E. and Dahlberg,J.E. (1998) Proofreading and aminoacylation of tRNAs prior to export from the nucleus. *Science*, in press.

Massire,C., Gaspin,C. and Westhof,E. (1994) Program for drawings: a program for drawing schematic views of nucleic acids. *J. Mol. Graph.*, **12**, 201–206.

Mattaj,I.W. and Englmeier,L. (1998) Nucleocytoplasmic transport: The soluble phase. *Annu. Rev. Biochem.*, **67**, 265–306.

McClain,W.H., Guerrier-Takada,C. and Altman,S. (1987) Model substrates for an RNA enzyme. *Science*, **238**, 527–530.

Melton,D.A., De Robertis,E.M. and Cortese,R. (1980) Order and intracellular location of the events involved in the maturation of a spliced tRNA. *Nature*, **284**, 143–148.

Nazarenko,I.A., Harrington,K.M. and Uhlenbeck,O.C. (1994) Many of the conserved nucleotides of tRNA^{Phe} are not essential for ternary complex formation and peptide elongation. *EMBO J.*, **13**, 2464–2471.

Negrutskii,B.S. and El'skaya,A.V. (1998) Eukaryotic translation elongation factor 1 alpha: structure, expression, functions and possible role in aminoacyl-tRNA channeling. *Prog. Nucleic Acid Res. Mol. Biol.*, **60**, 47–78.

Nishikura,K. and De Robertis,E.M. (1981) RNA processing in microinjected *Xenopus* oocytes. *J. Mol. Biol.*, **145**, 405–420.

Nissen,P., Kjeldgaard,M., Thirup,S., Polekhina,G., Reshetnikova,L., Clark,B.F. and Nyborg,J. (1995) Crystal structure of the ternary complex of Phe-tRNAPhe, EF-Tu and a GTP analog. *Science*, **270**, 1464–1472.

Ohno,M., Fornerod,M. and Mattaj,I.W. (1998) Nucleocytoplasmic transport: the last 200 nanometers. *Cell*, **92**, 327–336.

Piñol-Roma,S. and Dreyfuss,G. (1993) hnRNP proteins: localization and transport between the nucleus and the cytoplasm. *Trends Cell Biol.*, **3**, 151–155.

Quigley,G.J., Seeman,N.C., Wang,A.H.J., Suddath,F.L. and Rich,A. (1975) Yeast phenylalanyl tRNA: atomic coordinates and torsion angles. *Nucleic Acids Res.*, **2**, 3289–3293.

Rether,B., Bonnet,J. and Ebel,J.P. (1974) Studies on tRNA nucleotidyltransferase from baker's yeast. 1. Purification of the enzyme. Protection against thermal inactivation and inhibition by several substrates. *Eur. J. Biochem.*, **16**, 281–288.

Rio,D.C. (1988) Accurate and efficient pre-mRNA splicing in *Drosophila* cell-free extracts. *Proc. Natl Acad. Sci. USA*, **85**, 2904–2908.

Romby,P., Moras,D., Bergdoll,M., Vlassov,P.D.V.V., Westhof,E., Ebel,J.P. and Giegé,R. (1985) Yeast $tRNA^{Asp}$ tertiary structure in solution and areas of interaction of the tRNA with aspartyl-tRNA synthetase. *J. Mol. Biol*., **184**, 455–471.

Romby,P., Moras,D., Dumas,P., Ebel,J.P. and Giegé,R. (1987) Comparison of the tertiary structure of yeast tRNA (Asp) and tRNA (Phe) in solution. Chemical modification study of the bases. *J. Mol. Biol*., **195**, 193–204.

Rudinger,J., Blechschmidt,B., Ribeiro,S. and Sprinzl,M. (1994) Minimalist aminoacylated RNAs as efficient substrates for elongation factor Tu. *Biochemistry*, **33**, 5682–5688.

Sampson,J.R., Direnzo,A.B., Behlen,L.S. and Uhlenbeck,O.C. (1989) Nucleotides in yeast $tRNA^{Phe}$ required for specific recognition by its cognate synthetase. *Science*, **243**, 1363–1366.

Sampson,J.R., Behlen,L.S., DiRenzo,A.B. and Uhlenbeck,O.C. (1992) Recognition of yeast $tRNA^{Phe}$ by its cognate yeast phenylalanyl-tRNA synthetase: An analysis of specificity. *Biochemistry*, **31**, 4161–4167.

Schimmel,P., Giegé,R., Moras,D. and Yokoyama S. (1993) An operational RNA code for amino acids and possible relationship to genetic code. *Proc. Natl Acad. Sci. USA*, **90**, 8763–8768.

Segref,A., Sharma,K., Doye,V., Hellwig,A., Huber,J., Luhrmann,R. and Hurt,E. (1997) Mex67p, a novel factor for nuclear mRNA export, binds to both poly (A)+ RNA and nuclear pores. *EMBO J*., **16**, 3256–3271.

Shi,P.-Y., Maizels,N. and Weiner,A.M. (1998) CCA addition by tRNA nucleotidyltransferase: polymerization without translocation*? EMBO J*., **17**, 3197–3206.

Simos,G., Tekotte,H., Grosjean,H., Segref,A., Sharma,K., Tollervey,D. and Hurt,E.D. (1996a) Nuclear pore proteins are involved in the biogenesis of functional tRNA. *EMBO J*., **15**, 2270–2284.

Simos,G., Segref,A., Fasiolo,F., Hellmuth,K., Shevchenko,A., Mann,M. and Hurt,E.D. (1996b) The yeast protein Arc1p binds to tRNA and functions as a cofactor for the methionyl- and glutamyl-tRNA synthetases. *EMBO J*., **15**, 5437–5448.

Stade,K., Ford,C.S., Guthrie,C. and Weis,K. (1997) Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell*, **90**, 1041–1050.

Varshney,U., Lee,C.-P. and RajBhandary,U.L. (1991) Direct analysis of aminoacylation levels of tRNAs *in vivo*. *J. Biol. Chem*., **266**, 24712–24718.

Vlassov,V.V., Giegé,R. and Ebel,J.P. (1981) Tertiary structure of tRNAs in solution monitored by phosphodiester modification with ethylnitrosourea. *Eur. J. Biochem*., **119**, 51–59.

Werner,C., Krebs,G. and Dirheimer G. (1976) Specific cleavages of pure tRNAs by plumbous ions. *Biochim. Biophy. Acta*, **432**, 161–175.

Received September 15, 1998; revised October 22, 1998; accepted October 26, 1998