

tRNAs are imported into mitochondria of *Trypanosoma brucei* independently of their genomic context and genetic origin

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The mitochondrial genome of *Trypanosoma brucei* does not encode any identifiable tRNAs. Instead, mitochondrial tRNAs are synthesized in the nucleus and subsequently imported into mitochondria. In order to analyse the signals which target the tRNAs into the mitochondria, an *in vivo* import system has been developed: tRNA variants were expressed episomally and their import into mitochondria assessed by purification and nuclease treatment of the mitochondrial fraction. Three tRNA genes were tested in this system: (i) a mutated version of the trypanosomal tRNA^{Tyr}; (ii) a cytosolic tRNA^{His} of yeast; and (iii) a human cytosolic tRNA^{Lys}. The tRNAs were expressed in their own genomic context, or containing various lengths of the 5'-flanking sequence of the trypanosomal tRNA^{Tyr} gene. In all cases efficient import of each of the tRNAs was observed. We independently confirmed the mitochondrial import of the yeast tRNA^{His}, since *in organello* [α -³²P]ATP-labelling of the 3'-end of the tRNA was inhibited by carboxyatractyloside, a highly specific inhibitor of the mitochondrial adenine nucleotide translocator. Import of heterologous tRNAs in their own genomic contexts supports the conclusion that no specific targeting signals are necessary to import tRNAs into mitochondria of *T. brucei*, but rather that the tRNA structure itself is sufficient to specify import.

Keywords: mitochondria/mitochondrial biogenesis/RNA import/tRNA/trypanosomes

Introduction

Mitochondria contain their own genome and are able to transcribe and translate the genetic information of their DNA. There are, however, only about a dozen proteins encoded and synthesized in that compartment. In contrast, mitochondria do carry genes for most of their RNAs. However, there is an increasing number of evolutionarily divergent organisms which carry only nuclear genes for some of their mitochondrial RNAs, suggesting that RNA import might be a universal process in eukaryotes (Dietrich *et al.*, 1992; Schneider, 1994).

Most evidence for mitochondrial RNA import concerns the tRNAs. Import of tRNAs has been implicated in the mitochondrial biogenesis of yeast, plants and protozoa. Much of what is known about the process is based on indirect data. More recently, however, two more direct approaches have been used in a number of organisms.

First, by exploiting DNA transformation technology, it was possible to set up *in vivo* import systems in potatoes (Small *et al.*, 1992), trypanosomatids (Chen *et al.*, 1994; Schneider *et al.*, 1994a) and mouse cardiomyocytes (Li *et al.*, 1994) and demonstrate directly mitochondrial RNA import in those species. Second, *in vitro* studies were set up to measure tRNA import into mitochondria from yeast (Tarassov and Entelis, 1992) and *Leishmania* (Mahapatra *et al.*, 1994). Not much is yet known about the signals and the mechanism of RNA import. Targeting is highly specific in yeast, where only one of two cytosolic tRNAs^{Lys} is imported (Tarassov and Entelis, 1992). Less specificity is found in trypanosomatids, where almost all tRNAs are imported (Simpson *et al.*, 1989; Hancock and Hajduk, 1990).

The parasitic protozoan *Trypanosoma brucei* provides an excellent system to study RNA import. The whole set of mitochondrial tRNAs, which includes more than 30 species, needs to be imported. Two-dimensional polyacrylamide gel analysis showed that only few tRNAs are specific for the cytosol or mitochondria, whereas the majority of tRNAs are found in both compartments (Hancock and Hajduk, 1990; Mottram *et al.*, 1991). *In vivo*, import of a mutationally tagged tRNA suggested that the same gene may code for both cytosolic and mitochondrial isotypes of those tRNAs (Schneider *et al.*, 1994a). This was confirmed by direct enzymatic RNA sequencing showing that mitochondrial and cytosolic isotypes of three different tRNAs share the same sequence (Schneider *et al.*, 1994b). Two features distinguish the mitochondrial and cytosolic forms of these tRNAs. First, a unique nucleotide modification at the penultimate position before the anticodon seems to be a general feature of tRNAs imported into mitochondria. This modification does not provide a signal for, but rather is a consequence of import, as a mutated variant of a tRNA which cannot be modified any more can still be imported into mitochondria (Schneider *et al.*, 1994b). Second, high molecular weight forms of tRNAs were detected exclusively in the mitochondrial fraction (Hancock *et al.*, 1992). High molecular weight forms are highly unusual for tRNAs, as they are normally transcribed with short 5'- and 3'-extensions only. These molecules were converted to mature form by treatment with *Escherichia coli* RNase P or mitochondrial extract of trypanosomes. The exact structure and sequence of the high molecular weight forms are not known, but primer extension analysis indicates that they are in part due to 5'-extensions of the tRNA. Preliminary Southern analysis demonstrated that the 5'-extensions are coded for by the nuclear genome (Hancock *et al.*, 1992). It has therefore been suggested that the tRNAs are imported as long precursor forms which, after import into the matrix, are processed to mature-sized tRNAs.

The aim of this study was to define the features of

a tRNA required for its import into mitochondria of trypanosomes. We have previously established an *in vivo* import system where import of a mutated variant of the tRNA^{Tyr} could be demonstrated directly (Schneider *et al.*, 1994a). In this study we developed an episomal *in vivo* tRNA expression system to define some of the extra- and intragenic sequence requirements necessary for import of a tRNA into mitochondria.

Results

Episomal expression of various tRNAs

In vivo import of the endogenous mutationally tagged tRNA^{Tyr} has been demonstrated previously. The gene for that tRNA had been integrated into the nuclear genome by homologous recombination (Schneider *et al.*, 1994a). Since one aim of this study was to test whether 5'- and 3'-flanking sequences of defined lengths are involved in import, genomic integration would not be optimal, as influences of sequences upstream or downstream of the transfected gene cannot be excluded.

We therefore decided to express the tRNAs episomally using the pTbo plasmid (Metzenberg and Agabian, 1994). pTbo contains a PARP (procyclic acidic repetitive protein)/procyclin promoter/hygromycin resistance gene cassette followed by a minicircle sequence of 1 kb in length (Figure 1A). This sequence is responsible for extrachromosomal maintenance and replication of the plasmid. The different tRNA gene fragments were inserted upstream of the PARP/procyclin promoter using the unique *KpnI* and *BgIII* sites. One mutated trypanosomal and two different heterologous tRNA genes were expressed in various genomic backgrounds with the aim, (i) to test if a tRNA needs to be of trypanosomal origin in order to be imported into mitochondria and (ii) to investigate the role of 5'- and 3'-flanking regions in the targeting of the tRNAs. Of particular interest, hereby, are the mitochondria-specific 5'-extensions (Hancock *et al.*, 1992). Two constructs contained a mutated version of the endogenous intron-containing tRNA^{Tyr} gene. The mutations present in this tRNA led to the accumulation of the unspliced precursor as observed previously (Schneider *et al.*, 1993). In the first construct the mutated tRNA^{Tyr} was expressed in the context of 300 bp upstream and 23 bp downstream flanking regions (Figure 1B, pT-Y300). In the second construct most of the upstream sequence was deleted, leaving only 25 bp (Figure 1B, pT-Y25). In another set of constructs the mutated tRNA^{Tyr} gene, including its 3'-flanking region, was replaced by a cytosolic tRNA^{His} gene from *Saccharomyces cerevisiae* flanked by its own 3'-region. That gene was either fused to 300 bp (Figure 1B, pY-H300) or to 25 bp (Figure 1B, pY-H25) of the tRNA^{Tyr} gene 5'-flanking sequence, or it was expressed in its own genomic context (Figure 1B, pY-H). Finally, a human tRNA^{Lys} was expressed, again in its own genomic context (Figure 1B, pH-K).

All three tRNAs could be expressed in transgenic trypanosomes as illustrated in the Northern blots shown in Figure 2A. The cell lines transformed with the trypanosomal tRNA^{Tyr} gene and two of those transfected with the yeast tRNA^{His} gene efficiently expressed the corresponding tRNAs, whereas the yeast tRNA^{His} in its own genomic context was expressed at comparably low levels only.

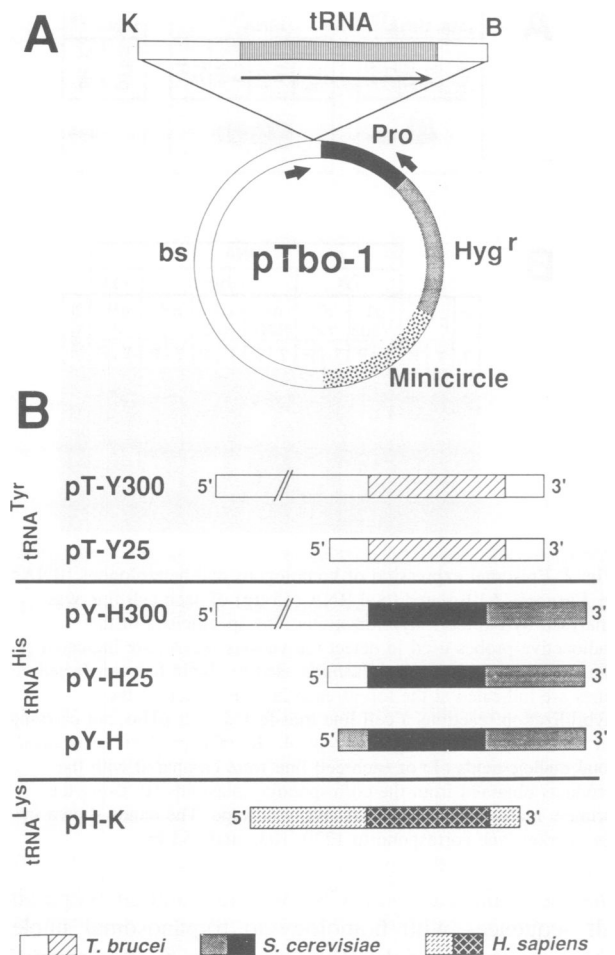


Fig. 1. Plasmids used to transfect *T.brucei*. (A) All constructs are based on pTbo-1 which is episomally maintained in trypanosomes. The fragments were inserted into the unique *KpnI* (K) and *BgIII* (B) sites. Direction of transcription is marked by the long arrow. The two primers used to amplify the insert containing region are indicated by the small solid arrows. Pro, PARP/procyclin promoter; Hyg^r, hygromycin resistance gene; bs, pBluescript. (B) Six different inserts were cloned into pTbo-1. pT-Y300 and pT-Y25 contain a mutated version of the trypanosomal (T) tRNA^{Tyr} (Y) gene flanked by either a 300 or a 25 nucleotide upstream region. pY-H300, pY-H25 and pY-H contain the yeast (Y) tRNA^{His} (H) gene flanked by 300 and 25 nt of the trypanosomal tRNA^{Tyr} 5'-region or by its own. pH-K contains the human (H) tRNA^{Lys} (K) gene in its own genomic context.

Both the tRNA^{Tyr} and the tRNA^{His} were detected using labelled oligonucleotide probes, whereas the human tRNA^{Lys} could be detected only by a random hexamer-labelled DNA fragment complementary to the entire length of the tRNA. This probe exhibits a much higher specific radioactivity than the labelled oligonucleotides, indicating that the tRNA^{Lys} is expressed only at very low levels. Trypanosomes transformed with pTbo, not containing an insert, served as a control for a drug-resistant cell line not expressing episomally encoded tRNAs.

All the transfected cell lines were tested for the presence of the original plasmid used for transformation (Figure 2B). Two primers were synthesized, one hybridizing to the pBluescript sequence found 40 bp upstream of the *KpnI* site, the other complementary to the 3'-end of the PARP/procyclin promoter of pTbo (Figure 1A, solid arrows). These primers allowed amplification by poly-

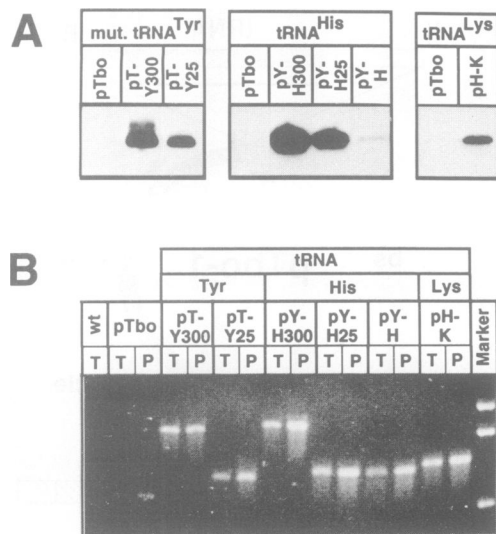


Fig. 2. Episomal expression of homologous and heterologous tRNAs in *T. brucei*. (A) Isolated total RNA (15 µg) of each cell line was analysed by Northern hybridizations. The specificities of the radioactive probes used to detect the various tRNAs are indicated at the top of each panel. The plasmids used to obtain the transfected cell lines are indicated at the top of each lane. For each of the hybridization reactions a cell line transfected with pTbo, not carrying an insert, served as a negative control. (B) PCR products of isolated total nucleic acids (T) of each cell line were compared with the products obtained from the corresponding plasmids (P) using the primers indicated in Figure 1A. Wt, wild-type. The bands shown in the marker lane correspond to 1230, 1033 and 653 bp.

merase chain reaction (PCR) of a fragment encompassing all sequences with homology to trypanosomal nuclear DNA consisting of the PARP/procyclin promoter and the inserts containing the tRNA genes. In all cell lines the fragment amplified from isolated total nucleic acids showed the expected length and was identical to that amplified from the original plasmid DNA (Figure 2B).

Unspliced mutant tRNA^{Tyr} is imported into mitochondria

To test whether the tRNAs expressed in the different cell lines are imported into mitochondria, cytosolic and mitochondrial fractions of the transformants were prepared. The fractionation procedure involves initial hypotonic lysis of the trypanosomes, which disrupts the outer membrane of the mitochondria and converts them to mitoplasts (Brady *et al.*, 1974; Harris *et al.*, 1990; Schneider *et al.*, 1994a). RNA extracted from cytosolic and mitoplast fractions was analysed by Northern hybridizations using radioactive probes specific for the different tRNAs. As a control we used a cell line which had been transfected with pTbo not containing any insert. In order to see if a specific tRNA is enriched in mitoplasts it was essential to assess the cytosolic cross-contamination in the mitochondrial fraction of each cell line. Although the best markers for that would be cytosol-specific tRNAs, they have not yet been cloned in *T. brucei*. Previously, we have used 5S rRNA as a cytosolic marker; however, a recent report suggests that a small fraction of that RNA may be imported into mitochondria in several species (Yoshinari *et al.*, 1994). We therefore decided to use the 7SL RNA of the signal recognition particle as a cytosolic marker. This marker may overestimate the cross-contamination,

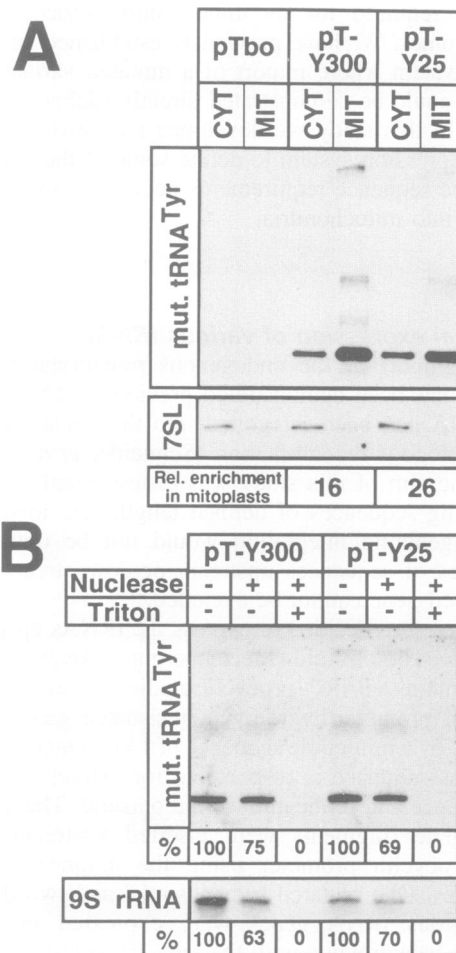


Fig. 3. Mutated trypanosomal tRNA^{Tyr} expressed in different genomic contexts is imported into mitochondria. (A) A Northern blot containing 0.8 µg of cytosolic (CYT) and 4 µg of mitoplast (MIT) RNA from cells transfected with either pTbo, pT-Y300 or pT-Y25 was hybridized with an oligonucleotide (see Materials and methods) specific for unspliced mutant tRNA^{Tyr} (upper panel) and subsequently re-probed with an oligonucleotide recognizing cytosolic 7SL RNA (lower panel). The relative enrichment of the tRNA^{Tyr} in mitoplasts was determined by comparing the ratios of the tRNA^{Tyr} and 7SL RNA signals in both the cytosolic and the mitoplast fractions. (B) Mitoplasts originating from the tRNA^{Tyr}-expressing transformants were either left untreated or were incubated with micrococcal nuclease (Nuclease) in the presence or absence of 1% Triton X-100 (Triton) and their respective RNAs were separated on a polyacrylamide gel. The corresponding Northern blot was probed for unspliced mutant tRNA^{Tyr} (upper panel) and subsequently re-probed for mitochondrial 9S rRNA (lower panel). The percentages of nuclease-resistant molecules, when compared with untreated samples, are indicated at the bottom of each panel.

as 7SL RNA is expected to be partly associated with the endoplasmic reticulum, a known contaminant of the mitochondrial preparations. By quantitatively comparing the ratios of the tRNA and 7SL RNA signals in both the cytosolic and the mitoplast fractions it was possible to obtain a factor indicating the relative enrichment of the tRNA in mitoplasts. Any value >1.0 means that more of the tRNA is found in mitoplasts than expected for a cytosolic contaminant.

Figure 3A shows the cell fractionation results of the two cell lines expressing the mutated tRNA^{Tyr}. In both transformants the tRNA is found in the cytosol and in mitoplasts. The cell line T-Y300 confirms the result

obtained before by genomic integration (Schneider *et al.*, 1994a), namely that unspliced tRNA^{Tyr} can be imported into mitochondria. Results obtained with T-Y25 indicate that most of the 5'-flanking sequence is dispensable for import of the mutated tRNA^{Tyr}. Figure 3B shows that 69–75% of the tRNAs are localized within the matrix as they were found to be resistant to micrococcal nuclease treatment, unless the mitochondrial inner membrane was disrupted with detergent. A similar percentage of the mitochondrial 9S rRNA was also resistant (Figure 3B, lower panel). We therefore presume that 25–37% signal reduction was due to loss of mitochondrial membrane integrity during the incubation.

Mitoplast-specific high molecular weight forms of the tRNA are also detected. They appear to be independent of most of the 5'-flanking sequence of the tRNA^{Tyr} gene, as they appear identical in the T-Y300 and the T-Y25 cell line. In addition, they are resistant to nuclease treatment and are therefore localized within mitoplasts.

Heterologous tRNAs are imported into mitochondria independently of their genomic context

A similar analysis was undertaken with the cell lines expressing the yeast tRNA^{His}. Only the transformants Y-H300 and Y-H25 (Figure 1B) could be analysed by Northern hybridization; for the cell line Y-H the signal was too low. Cell fractionation revealed that the yeast tRNA^{His} in both transformants (Y-H300 and Y-H25) was enriched in the mitoplast fraction (Figure 4A). Figure 4B shows that the tRNAs do not just co-fractionate with but are localized within mitoplasts, since they exhibit the same sensitivity to nuclease as mitochondrial 9S rRNA.

In order to analyse the transformant Y-H, expressing the tRNA^{His} in its own genomic context, primer extension analysis was performed. The primer chosen hybridized to an internal region of the yeast tRNA^{His} and therefore can be extended by reverse transcriptase to the 5'-end of mature tRNA^{His} resulting in a cDNA 16 nt longer than the original oligonucleotide (Figure 5). As expected, no extension products are seen in the transformant Tbo since it does not express any tRNA^{His}. In the three other cell lines, however, extension products corresponding to mature tRNA^{His} are observed in both the cytosolic and the mitoplast fractions. To determine the relative enrichment of the tRNAs in mitoplasts we quantified the extension products and assessed the cytosolic contamination of the various mitoplast preparations by Northern hybridization. For cell lines Y-H300 and Y-H25 those numbers agree well with the values obtained by Northern analysis (Figure 4A) and also show that the tRNA^{His} expressed in its own genomic context (Y-H) is imported efficiently into mitochondria. This is surprising, since in pY-H neither the tRNA gene nor any of its flanking sequences are of trypanosomal origin. Another unexpected feature is the detection of mitoplast-specific 5'-extension products, reminiscent of the high molecular weight forms of the mutant tRNA^{Tyr} (Figure 3), which are extended further than just to the mature end of the tRNA. These products are detected in the mitoplast fractions of all three cell lines but are most prominently seen in Y-H25. Whereas they appear to be identical in the transformants Y-H25

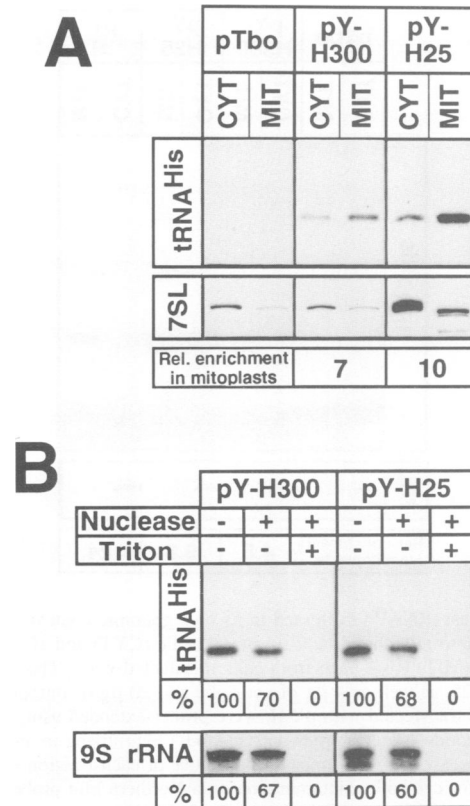


Fig. 4. Yeast cytosolic tRNA^{His}, expressed in different genomic contexts, is imported into mitochondria. (A) A Northern blot containing 0.8 µg of cytosolic (CYT) and 4 µg of mitoplast (MIT) RNA from cells transfected with either pTbo, pY-H300 or pY-H25 was hybridized with an oligonucleotide (see Materials and methods) specific for yeast tRNA^{His} (upper panels) and subsequently re-probed with an oligonucleotide recognizing cytosolic 7SL RNA (lower panels). The relative enrichment of the tRNA^{His} in mitoplasts was determined by comparing the ratios of the tRNA^{His} and 7SL RNA signals in both the cytosolic and the mitoplast fractions. (B) Mitoplasts originating from the tRNA^{His}-expressing transformants were either left untreated or were incubated with micrococcal nuclease (Nuclease) in the presence or absence of 1% Triton X-100 (Triton) and their respective RNAs were separated on a polyacrylamide gel. The corresponding Northern blot was probed for yeast tRNA^{His} (upper panel) and subsequently re-probed for mitochondrial 9S rRNA (lower panel). The percentages of nuclease-resistant molecules, when compared with untreated samples, are indicated at the bottom of each panel.

and Y-H (Figure 5, indicated by ■) they have different lengths in Y-H300 (Figure 5, indicated by *). We do not know the exact nature of those extensions; however, they are clearly specific for mitoplasts and apparently do not depend on a specific sequence upstream of the gene.

Yeast cytosolic tRNA^{His}, when expressed in trypanosomes, could be imported into mitochondria, even though it was expressed in its own genomic context. In order to see if any tRNA transcribed in trypanosomes would be imported, another heterologous tRNA was tested. We decided to use the human tRNA^{Lys} since in evolutionary terms, humans are widely separated from both trypanosomes and yeast. Like the yeast tRNA^{His}, the human tRNA^{Lys}, expressed in its own genomic context, is imported into mitochondria (Figure 6).

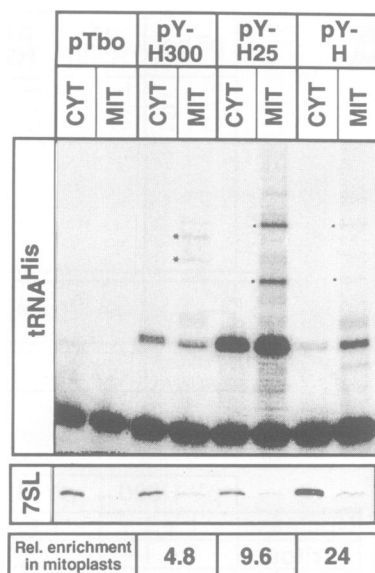


Fig. 5. Yeast tRNA^{His} expressed in its own genomic context is imported into mitochondria. 2 µg of cytosolic (CYT) and 10 µg of mitoplast (MIT) RNA each from cells transfected with pTbo, pY-H300 and pY-H25, as well as 4 µg of cytosolic and 20 µg of mitoplast RNA from cells transfected with pY-H, were primer-extended using an oligonucleotide (see Materials and methods) hybridizing to an internal region of yeast tRNA^{His} (upper panel). 10% of each fraction was analysed for cytosolic contamination on a Northern blot probed with an oligonucleotide recognizing cytosolic 7SL RNA (lower panel). The relative enrichment of the tRNA^{His} in mitoplasts was determined by comparing the ratios of the main tRNA^{His} primer extension product and 7SL RNA signals on Northern blots in both the cytosolic and the mitoplast fractions. High molecular weight primer extension products unique to Y-H300 are indicated by *, whereas those shared by Y-H25 and Y-H are marked by ■.

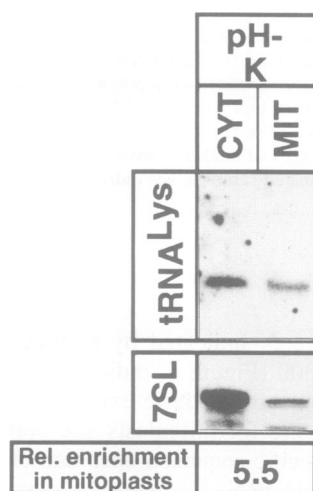


Fig. 6. Human cytosolic tRNA^{Lys} expressed in its own genomic context is imported into mitochondria. A Northern blot containing 3 µg of cytosolic (CYT) and 15 µg of mitoplast (MIT) RNA from cells transfected with pH-K was hybridized with a random primed DNA fragment (see Materials and methods) specific for human tRNA^{Lys} (upper panel) and subsequently re-probed with an oligonucleotide recognizing cytosolic 7SL RNA (lower panel). The relative enrichment of the tRNA^{Lys} in mitoplasts was determined by comparing the ratios of the tRNA^{Lys} and 7SL RNA signals in both the cytosolic and the mitoplast fractions.

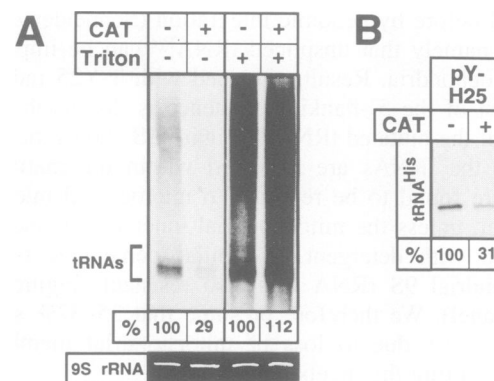


Fig. 7. Carboxyatractylide (CAT)-sensitive labelling of tRNA^{His}. (A) Isolated mitoplasts from wild-type cells were labelled with [α -³²P]ATP in the presence or absence of CAT (see Materials and methods) and their respective RNAs were separated on a polyacrylamide gel. When indicated, the labelling was performed in the presence of Triton X-100. The percentage of labelling of the tRNA region (bracket) of the drug-treated samples, as normalized to untreated samples, is indicated at the bottom of the panels. The lower panel shows the ethidium bromide-stained mitochondrial 9S rRNA of the top part of the gels shown in the upper panel. (B) Isolated mitoplasts from cells transformed with pY-H25 were labelled as described above in the presence or absence of CAT, and their respective RNAs were subjected to affinity isolation using a 5'-biotinylated oligonucleotide and avidin agarose (see Materials and methods). The radioactive tRNA^{His} eluted off the avidin agarose from the samples, labelled in the absence or in the presence of CAT, is shown. The percentage of labelling of the tRNA^{His} of the drug-treated samples, as normalized to untreated samples, is indicated at the bottom of the panel.

Carboxyatractylide-sensitive labelling proves tRNA^{His} is localized within the matrix

The use of CAT (carboxyatractylide), a highly efficient and specific inhibitor of the adenine nucleotide translocator of the inner mitochondrial membrane, offers a tool to assess the location of a tRNA independently of the quality of the cell fractionation. Trypanosomal tRNAs, like all other eukaryotic tRNAs, have a post-transcriptionally added CCA sequence at their 3'-end. It has been shown that after arresting transcription, mitochondrial tRNAs can be labelled *in organello* with [α -³²P]CTP due to an endogenous nucleotidyl transferase activity (Hancock *et al.*, 1992).

Transcription arrest is achieved by a 30 min incubation during which endogenous nucleotide pools become depleted. We have modified this procedure by using a mixture of unlabelled CTP and [α -³²P]ATP. Figure 7A shows the labelling pattern obtained with this technique. The tRNA region is labelled most prominently; however, there is also a smear visible which most likely is due to polyadenylation of mitochondrial mRNAs. In the presence of CAT the [α -³²P]ATP labelling is reduced by 71% when compared with the control in the absence of the drug, as the only access of ATP to the matrix is via the adenine nucleotide translocator. Identical amounts of mitoplasts were used in the presence or absence of CAT as indicated by the same amount of mitochondrial 9S rRNAs being found in both lanes (Figure 7A, bottom panel). As expected for an inhibitor of the adenine nucleotide translocator, reduction of labelling depends on an intact inner membrane and is not seen in the presence of detergent (Figure 7A, right panel). A duplicate experiment to that shown for

pT-Y300

..cggcacatataacaagtctccccgactactcgccgagtcagCCTTCTGTAGCTCAATTGGTAGAGCATGTGACTCTAAATCATGCGGGTATCACAGGGTCGCTGGTTCGATTCGGCCGGAAGGAActtttttcaagccac..
ooo

pT-Y25

..tgggtaccgctacgtctccccgactactcgccgagtcagCCTTCTGTAGCTCAATTGGTAGAGCATGTGACTCTAAATCATGCGGGTATCACAGGGTCGCTGGTTCGATTCGGCCGGAAGGAActtttttcaagccac..
ooo

pY-H300

..cggcacatataacaagtctccccgactactcgccgagtcagGCCATCTTAGTATAGTGGTTAGTACATCGTTGTGGCCGATGAAACCCCTGGTTCGATTCAGGAGATGGCActtttttttaactg..
ooo

pY-H25

..tgggtaccgctacgtctccccgactactcgccgagtcagGCCATCTTAGTATAGTGGTTAGTACATCGTTGTGGCCGATGAAACCCCTGGTTCGATTCAGGAGATGGCActtttttttaactg..
ooo

pY-H

..aagggaacaaaagctgggtaccgttctccattgaaaagtcGCCATCTTAGTATAGTGGTTAGTACATCGTTGTGGCCGATGAAACCCCTGGTTCGATTCAGGAGATGGCActtttttttaactg..
ooo

pH-K

..agaacctcttataagcaaaaagtaagctctcgtgaagaGCCCGATAGCTCAGTCGGTAGAGCATCAGACTTTTAATCTGAGGGTCCAGGGTTCAGTCCCTGTTGGGGCGcgtgttttgcctt..
ooo

Fig. 8. Sequence of the tRNA genes and their corresponding flanking sequences in the different constructs. A sequence segment consisting of the tRNA coding region (upper case) and an additional 40 nt upstream and 15 nt downstream flanking sequence (lower case) is shown for the inserts present in each of the plasmids used in the transfection experiments. Consensus RNA polymerase III box A and box B promoter elements present within the tRNA coding region are underlined. 5'-flanking vector sequences are shown in lower case and are underlined. The triplet encoding the anticodon is indicated by the small circles.

mitochondrial tRNAs from wild-type trypanosomes (Figure 7A) was performed using mitoplasts isolated from a transformant expressing yeast cytosolic tRNA^{His} (Y-H25). [α -³²P]ATP-labelled RNAs were isolated from untreated and CAT-treated mitoplasts and radioactive tRNA^{His} was affinity-selected with avidin agarose using a complementary biotinylated oligonucleotide. As for total tRNAs, the labelling of the tRNA^{His} was reduced by 69% in the presence of CAT, proving its location in the matrix space of mitochondria (Figure 7B). The labelling is not completely abolished in the presence of CAT. This is most likely due to leakage of mitoplasts during incubation. Indeed, the percentage of inhibition correlates well with the percentage of nuclease-resistant imported tRNAs (Figures 3B and 4B). The CAT experiment proves that the tRNA^{His} expressed in Y-H25 is located within the matrix space of mitochondria. It is therefore concluded that cell fractionation and nuclease treatment of intact mitoplasts are valid criteria to assess the mitochondrial location of a tRNA.

Discussion

The aim of the present study was to identify the features which render a tRNA competent for import into mitochondria of *T.brucei*. The problem was approached using an *in vivo* import system based on episomal expression of different tRNAs in various genomic contexts. Our experiments showed that in trypanosomes tRNAs from various organisms are imported into mitochondria independently of their genomic context.

A yeast tRNA was imported into mitochondria of *T.brucei*, irrespective of whether its gene was expressed fused to various lengths of 5'-flanking sequences of the trypanosomal tRNA^{Tyr} gene, or in its own genomic context. In addition, it could be demonstrated that the human tRNA^{Lys} transcribed from a cloned fragment of the human genome was imported as well. Neither of the sequences upstream of the three different tRNA genes shared significant homology (Figure 8). The only common motif is a tetranucleotide TCTC which is found at different positions in each of the three sequences. Those results show that the upstream sequence of the trypanosomal tRNA^{Tyr} gene

does not specify any targeting information. The same is true for the downstream region of the gene. 3'-Flanking sequences of tRNA genes of three different organisms were all compatible with mitochondrial tRNA import. Unlike the upstream flanking regions, those sequences necessarily shared some homology (Figure 8), as an oligo(T) stretch compromises an universal stop signal for RNA polymerase III.

If the targeting information is not found in the 5'- or 3'-flanking region of the tRNA^{Tyr} gene, then it must be located within the tRNA itself. What feature of the structure might allow the tRNA to be imported? One of the tRNAs used as substrates in our *in vivo* import system is a mutated version of the endogenous tRNA^{Tyr}. As a result of the mutations present in that tRNA, it accumulates in its unspliced form (Schneider *et al.*, 1993) and its anticodon loop structure is therefore greatly altered. Nonetheless, efficient import of that molecule was observed (Schneider *et al.*, 1994a). Thus, the targeting does not depend on an intact anticodon loop structure. Two heterologous substrates, a tRNA^{His} of yeast and a tRNA^{Lys} of human, were also shown to be import-competent. The yeast cytosolic tRNA^{His} is not imported into yeast mitochondria and is therefore not expected to contain an import signal. It is not known how homologous yeast tRNA^{His} is to the endogenous tRNA^{His} of trypanosomes, since that tRNA has not yet been cloned. However, no cross-reaction to trypanosomal tRNAs was observed by Northern blots when using two non-overlapping oligonucleotide probes specific for the yeast tRNA. Human cytosolic tRNA^{Lys} (Roy *et al.*, 1982) shows 66% identity to the tRNA^{Lys} of *T.brucei* (Mottram *et al.*, 1991) and is therefore not more closely related to the trypanosomal tRNA^{Lys} than to the tRNA^{Lys} of most other eukaryotes. Mitochondrial tRNA import is absent in mammals. The human tRNA^{Lys} should therefore not contain a specific targeting signal. Thus, it appears as if the tRNA structure itself, which is the only common denominator of the three tested tRNAs, includes the determinant for mitochondrial import. These results are in sharp contrast to the highly specific mitochondrial tRNA import in yeast where the targeting process discriminates even between two iso-

acceptors of the very same tRNA (Tarassov and Entelis, 1992).

Our findings indicate that any tRNA expressed in *T.brucei* is in part imported into mitochondria. However, it is clear that there are exceptions to this rule as a few cytosol-specific tRNAs do appear to exist in trypanosomes (Hancock and Hajduk, 1990). Also, the genetic codes of the cytosol and mitochondria are not identical, indicating that the tRNA populations in both compartments cannot totally overlap. The structure of these cytosol-specific tRNAs might specify retention in the cytosol, or be missing a crucial structural feature that prevents their import into mitochondria. Unfortunately, as yet no cytosol-specific tRNA which could be tested in our system has been cloned in *T.brucei*; hence, it is not known how specific cytosolic location of a tRNA is achieved.

Two working models have been proposed for the translocation of tRNAs across the mitochondrial membranes. The tRNA could either be naked during import, or it could be complexed with proteins (Nagley, 1989). In the first case it may use its own import system, whereas in the second case it may be co-imported through the protein import channel. *In vitro* import of tRNA into yeast mitochondria requires an intact protein translocation machinery and exhibits the same energy requirements as protein import (Tarassov *et al.*, 1994). Those results provide strong evidence for the co-import model in this organism. In other systems, however, no experimental data for either of the two models have been presented. If the co-import model is indeed correct, precursors of mitochondrial aminoacyl-tRNA synthetases would be prime candidates for carrier proteins (Suyama and Hamada, 1978) as they all are imported from the cytosol. The compartment-specific localization of tRNAs in *T.brucei* could in this case be explained by specific localization of their cognate synthetases. The fact that mutated unspliced tRNA^{Tyr} was imported efficiently in our system but could not be aminoacylated by its cognate synthetase (Schneider *et al.*, 1994a), seems to contradict this model. This result makes direct coupling of import and charging unlikely, but it cannot be excluded that the intron-containing tRNA^{Tyr} is still able to interact with its synthetase without being aminoacylated.

High molecular weight forms of tRNAs are found exclusively in the mitochondrial fraction of *T.brucei* (Hancock *et al.*, 1992). We have shown here that mitochondria-specific high molecular weight forms of the tRNAs are detected independently of the genomic context of the tRNA gene. Using Northern blots we were able to detect the same set of high molecular weight forms of the tRNA^{Tyr} transcribed from the gene containing 300 or 25 bp of 5'-flanking region. Surprisingly, similar forms were also detected by primer extension of the mitochondrial isotype of heterologous tRNA^{His}, independent of whether the trypanosomal tRNA^{Tyr} or its own genomic 5'-flanking sequence was fused to its gene. The common features of all the high molecular weight forms detected in the different cell lines are their exclusive presence in the mitochondrial fraction, that they are detected for homologous and heterologous tRNAs and, that they are not due to a specific 5'-flanking sequence of the gene. The high molecular weight forms were efficiently detected on Northern blots for the tRNA^{Tyr} only. It was therefore

decided to test if these extensions originate from the sequence found upstream of the tRNA^{Tyr} gene. However, Northern hybridization using an oligonucleotide probe complementary to the immediate 5'-flanking region of the tRNA^{Tyr} gene failed to detect any of the high molecular weight bands (not shown). These results suggest that, at least for the tRNA^{Tyr}, the high molecular forms might not be the primary transcripts of the tRNA genes, but rather that the 5'-extension is acquired after transcription by some novel form of trans-splicing or a ligation reaction. In order to elucidate how that process might work, and to investigate any role it might play in import, it is essential to determine the exact structure of the high molecular weight molecules.

In conclusion, our *in vivo* data provide strong evidence that the requirements for import of a tRNA into mitochondria of *T.brucei* are not stringent: the tRNA structure itself is a sufficient determinant for import. Even the classical anticodon loop structure within a tRNA is not essential for import, as dramatic alterations within that region are tolerated. The fact that no specific import signal is required and that heterologous tRNAs are imported may allow us in the future to use the *in vivo* import as a tool to manipulate mitochondrial gene expression by import of RNAs designed to interfere with intramitochondrial processes.

Materials and methods

Transfection of cells

Procyclic *T.brucei*, stock 427, were grown in SDM-79 medium supplemented with 10% fetal bovine serum. Electroporation and transfection of the cells were performed as described (Zomerijk *et al.*, 1990; Hehl *et al.*, 1994) using 15 µg of the corresponding plasmid DNA isolated from *E.coli* (strain UT580) using a plasmid purification kit (Qiagen, Germany). After electroporation, cells were cultured for 24 h without selection, before adding hygromycin B (Calbiochem, California) to 75 µg/ml. After 10–15 days of drug selection, resistant cells started to grow. Resistant cell lines were split 1/20 at least 5–10 times before expression of the modified tRNAs was analysed. The presence of the plasmid pTbo-1, containing the inserts of the correct sizes, in the transformants was verified by amplifying a DNA fragment by PCR which includes the *KpnI*-*BglII* insert (see below) and the PARP/procyclin promoter region. The primers used for such experiments hybridized to the pBluescript II KS (Stratagene) sequence, nucleotides 629–612 (5'-GGCCAGTG-AGCGCGCT-3') present in pTbo-1, and the 3'-region of the PARP/procyclin promoter (5'-CCCAAGCTTCGAGGGGAGCTGTGA-3').

Recombinant plasmids

pTbo-1 (generously provided by Drs Stan Metzberg and Nina Agabian), which due to the minicircle sequence is maintained and replicated as an episome in *T.brucei* (Metzberg and Agabian, 1994), was used as a vector for the transfection experiments. The plasmid was modified by inserting a synthetic sequence, containing a unique *BglII* site, immediately downstream of the unique *KpnI* site located 5' of the PARP/procyclin promoter.

The tRNA genes containing inserts of the constructs were amplified by PCR using specific primers carrying 5'-flanking *KpnI* and *BglII* sites, respectively, allowing forced cloning into the modified pTbo. The tRNA^{Tyr} gene-containing inserts in pT-Y300 and pT-Y25 were obtained by PCR amplification using pHyg-Sup (Schneider *et al.*, 1993) as a template. Amplifying a fragment from yeast genomic DNA yielded the yeast tRNA^{His} gene insert in pY-H. The primers for that PCR reaction were designed using the published sequence of pYB12 (del Rey *et al.*, 1983) containing a cytosolic yeast tRNA^{His} gene. The other two tRNA^{His} genes containing inserts of pY-300 and pY-25 were obtained by fusion of different PCR products using either pT-Y300 or pY-H as templates. Finally, the insert of pH-K was amplified from human genomic DNA. Specific primers for that PCR were designed according to sequence information of pAT 153 (Roy *et al.*, 1982) encoding a human cytosolic

tRNA^{Lys}. All tRNA genes were transcribed in the *KpnI/BglII* direction with regard to the insert.

Isolation of mitoplasts and RNA

Mitoplast and cytosolic fraction were purified, using published procedures (Harris *et al.*, 1990). For each preparation 4 l of each of the transformed cell lines (1.5×10^7 – 2.5×10^7 cells/ml), grown in SDM-79 containing 5% fetal bovine serum and 75 µg/ml hygromycin B were used.

RNA of total cells, cytosolic or mitochondrial fractions was purified by the acid guanidinium isothiocyanate method as described by Chomczynski and Sacchi (1987). Isolated RNA fractions were routinely treated with RQ-DNase (Promega) (1–5 units/100 µg of nucleic acids) for 20 min at 37°C in a buffer containing 70 mM Tris–HCl, pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol and 40 U of human placental RNase inhibitor, RNAGuard (Pharmacia).

Northern analysis

DNase-treated RNAs were separated electrophoretically on 8 M urea–10% polyacrylamide gels. Electrophoretic transfer and Northern analysis were performed as described previously (Schneider *et al.*, 1993). All oligonucleotide probes were 5'-labelled using polynucleotide kinase and hybridizations were performed at high stringency, corresponding in all cases to 55°C hybridization temperature. The sequences of the oligonucleotides were as follows: 5'-CCC GCATGATTTAGAGTC-3' for the probe complementary to nucleotides 29–46 of unspliced mutant tRNA^{Tyr} expressed in T-Y300 and T-Y25 and 5'-GTGGTGCCATCTCC-TAGAATCGAA-3' hybridizing to nucleotides 53–72 of the tRNA^{His} and its CCA-tail in cell lines Y-H300 and Y-H25. Human tRNA^{Lys} was detected using the PCR fragment containing the whole insert and the PARP/procyclin region of pH-K (Figure 2B, H-K) as probe. The fragment was labelled by [α -³²P]dATP using a random primed DNA labelling kit (Boehringer Mannheim Biochemicals, Germany). In this case hybridization was performed at 65°C.

Cytosolic contamination was assessed using the oligonucleotide 5'-GAACCCCGCTGTGTTCAAG-3' hybridizing to nucleotides 56–75 of 7SL RNA (Mottram *et al.*, 1991; Béja *et al.*, 1993).

Micrococcal nuclease treatment

Nuclease treatment of isolated mitoplasts was carried out as described by Schneider *et al.* (1994a), except that 5000 U of micrococcal nuclease were added to each sample. Mitochondrial 9S rRNA served as a compartment-specific marker and was detected using the oligonucleotide probe 5'-AGGAGATAGGACTTGCCCT-3'.

Primer extension analysis

Oligonucleotides for primer extension were gel-purified before labelling. ~0.3 pmol of the phosphorylated primer, equivalent to $\sim 5 \times 10^5$ c.p.m., was used in each reaction. The oligonucleotide 5'-CACAACG-ATGTGTACTAACC-3', hybridizing to the nucleotides 17–36 of the yeast tRNA^{His}, was used as a primer. 1–20 µg of DNase-treated cytosolic or mitochondrial RNA was hybridized with the labelled primer, in 4 µl of 62.5 mM Tris–HCl, pH 9.0, containing 125 mM KCl, by incubation for 2 min at 90°C and subsequent quenching on ice for 30–60 s. After annealing, 6 µl of 42 mM Tris–HCl, pH 9.0, containing 13 mM MgCl₂, 8 mM dithiothreitol, 1 mM dNTPs and 5 U of AMV reverse transcriptase (Boehringer Mannheim) was added, and the sample was incubated for 30 min at 48°C. The reactions were subsequently treated with 5 µg of RNase A for 10 min at 48°C and, after addition of 10 µg glycogen (Boehringer Mannheim), precipitated with 2.5 volumes of ethanol. Primer extension products were analysed on 8 M urea–10% polyacrylamide gels.

Carboxyatractyloside-sensitive 3'-CCA-labelling of tRNAs

Freshly isolated mitoplasts (2 – 5×10^9 cell equivalents) were re-isolated and re-suspended in 200 µl of 5 mM HEPES, pH 7.6, 3 mM potassium phosphate, pH 7.7, 125 mM sucrose, 10 mM magnesium acetate, 5 mM 2-mercaptoethanol, 1 mM EDTA, pH 8, 1 mg/ml bovine serum albumin (fatty acid-free) and incubated for 30 min at 27°C to deplete the endogenous nucleotide pools. The mitoplasts were then divided into two equal samples. To one tube 2.5 µl of 5 µg/µl CAT (generously provided by Dr Gottfried Schatz), dissolved in 50% ethanol, was added, whereas the other sample was mock-treated with 50% ethanol. Both samples were incubated for 10 min at 30°C. After adding CTP to 0.5 mM, labelling was started by adding 20 µCi of [α -³²P]ATP (3000 Ci/mmol) and the reaction was incubated for 5 min at 30°C. Labelled mitoplasts were quenched on ice and re-isolated at 4°C before the RNA was extracted. As a control, the labelling reactions, with and without CAT, were performed in the presence of 1% (v/v) Triton X-100. In this case

no re-isolation step was possible and RNA was prepared directly. All samples were analysed on 8 M urea–10% polyacrylamide gels.

Affinity isolation of labelled tRNA^{His}

The labelling procedure described above was performed using 5×10^{10} cell equivalents of freshly isolated mitoplasts originating from the transformant Y-H25. All the volumes and the amount of CAT were scaled up by a factor of five. RNA, isolated from the CAT-treated and the mock-treated samples, was subjected to affinity isolation using the 5'-biotinylated oligonucleotide 5'-ATATATGGTGCCATCTCCTAGA-ATCGAACCAGGGTTTC-3' and avidin agarose (Pierce, IL). In order to reduce background, 100 µl of avidin agarose beads (1:1 slurry) were blocked in 500 µl of blocking buffer consisting of 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM Na₂PO₄, pH 7.7, 1 mM EDTA) containing 5× Denhardt's solution [0.1% (w/v) each Ficoll, polyvinylpyrrolidone, bovine serum albumin] and 0.5% (v/v) of Nonidet P-40 for 20 min at 37°C. This blocking procedure was repeated twice. Isolated [α -³²P]ATP-labelled RNA fractions were incubated for 60 min at 45°C in 250 µl each of blocking buffer containing 50 pmol of the biotinylated oligonucleotide. Next, 50% of the pre-adsorbed beads were added to each reaction and the incubation was continued at the same temperature for 45 min. Finally, the beads were washed three times using 1 ml of 2× SSPE containing 0.5% (v/v) Nonidet P 40 before the affinity-selected tRNAs were eluted by boiling of the beads in 10 mM Tris–HCl, pH 8.0, containing 1 mM EDTA. After addition of 10 µg of glycogen the eluates were ethanol-precipitated and analysed on 8 M urea–10% polyacrylamide gels.

Quantification

The radioactive signals obtained on the Northern blots, in the primer extensions and in the labelling experiments were quantified using a PhosphorImager (Molecular Dynamics).

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