The EMBO Journal vol.15 no.21 pp.5949–5957, 1996

RNA editing changes the identity of a mitochondrial tRNA in marsupials

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In the mitochondrial genome of marsupials, the tRNA gene located at the position where in other mammals an aspartyl-tRNA is encoded carries the glycine anticodon GCC. Post-transcriptionally, an RNA editing mechanism affects the second position of the anticodon such that the aspartate anticodon GUC is created in ~50% of the mature tRNA pool. We show that the unedited version of this tRNA^{Asp}(GCC) can be specifically aminoacylated with glycine *in vitro*, while the edited version becomes aminoacylated with aspartic acid. Furthermore, we show that both forms are aminoacylated to a substantial extent *in vivo*. By replacing an amino group with a keto group, RNA editing thus changes the identity of this tRNA allowing a single gene to encode two tRNAs.

Keywords: aminoacylation/identity change/mitochondria/RNA editing/tRNA

Introduction

Since the discovery that numerous mitochondrial mRNAs in trypanosomes are post-transcriptionally altered by the insertion and deletion of uridyl residues (Benne *et al.*, 1986), sequence changes in nuclear and organellar mRNAs and tRNAs have been observed in higher plants (Covello and Gray, 1989; Gualberto *et al.*, 1989; Hiesel *et al.*, 1989; Hoch *et al.*, 1991; Wissinger *et al.*, 1991; Börner *et al.*, 1995), amoebas (Lonergan and Gray, 1993), molluscs (Yokobori and Pääbo, 1995a) and mammals (Chen *et al.*, 1987; Sommer *et al.*, 1991; Janke and Pääbo, 1993; Yokobori and Pääbo, 1995b). Regardless of whether the sequence change is achieved by insertion, deletion or replacement of one nucleotide by another this phenomenon is referred to as RNA editing.

From a functional point of view, two types of RNA editing exist. In most cases, a gene sequence gives rise to a non-functional gene product that is then 'corrected' at the RNA level (Lonergan and Gray, 1993; Börner *et al.*, 1995; Yokobori and Pääbo, 1995a,b). In contrast to this type of RNA editing, which we would like to refer to as 'correctional editing', a few cases have been described where both the genomically encoded and the edited mRNAs encode functional gene products, that differ in their properties. For example, in the mRNA for apolipoprotein B of mammals a glutamic acid codon is edited to a stop codon, resulting in the translation of a shorter version, without a receptor-binding domain (Chen *et al.*, 1987). In the liver of most mammals, only the longer form of this protein is synthesized, whereas the editing event causes the shorter form to predominate in the intestine (reviewed in Chan, 1993). Another example is the editing of several codons in mRNAs encoding subunits of glutamate receptors in the human brain which results in changes in the ion conductance of these receptors (Sommer *et al.*, 1991; Köhler *et al.*, 1993; Lomeli *et al.*, 1994). In these cases, the genomically encoded form of the RNA is functional and the RNA editing results in a change in this function. Thus, this type of RNA editing, for which we suggest the term 'transmutational editing', produces two alternative versions of a transcript from a single gene, which differ in their functional properties.

We have previously reported that in the marsupial mitochondrial genome, the gene for tRNA^{Asp} carries the anticodon 5′-GCC-3′ rather than the expected 5′-GTC-3′ (Janke and Pääbo, 1993). According to the vertebrate mitochondrial genetic code, this tRNA would recognize two out of four glycine codons (Barrell *et al.*, 1980; Anderson *et al.*, 1981). The primary sequence of this tRNA outside the anticodon is largely conserved when compared to aspartyl-tRNAs from other vertebrates. The seemingly incorrect anticodon is edited such that ~50% of transcripts carry the sequence GUC while the rest carry the sequence GCC (Janke and Pääbo, 1993; Mörl *et al.*, 1995). We have investigated the function of the unedited and edited forms of this tRNA. The data show that while the edited form functions as an aspartyl-tRNA, the unedited form serves as a glycyl-tRNA.

Results

Editing of tRNA^{Asp} in various tissues

Since no tRNA gene with a bona fide aspartic acid anticodon exists in the mitochondrial genome of the opossum (Janke *et al.*, 1994), the absence of editing of tRNA^{Asp} would probably result in an inhibition of mitochondrial translation and consequently of oxidative phosphorylation. Therefore, it is conceivable that mitochondrial function could be regulated by the editing of tRNA^{Asp}. In order to investigate this possibility, the extent of editing of tRNA^{Asp} was assayed in tissues that vary in their respiratory activities.

Total small cellular RNA was isolated from liver, skeletal muscle, heart, kidney, brain and spleen of an adult shrewlike opossum (*Monodelphis domestica*). To quantitate the relative amounts of C and U, respectively, at the second postion of the anticodon (position 35; Steinberg *et al.*, 1993) in tRNA^{Asp}, an oligonucleotide primer, complementary to the tRNA and having its 3′ end at the third nucleotide of the anticodon, was extended in the presence of [^{32}P]α-dATP or [^{32}P]α-dGTP, respectively,

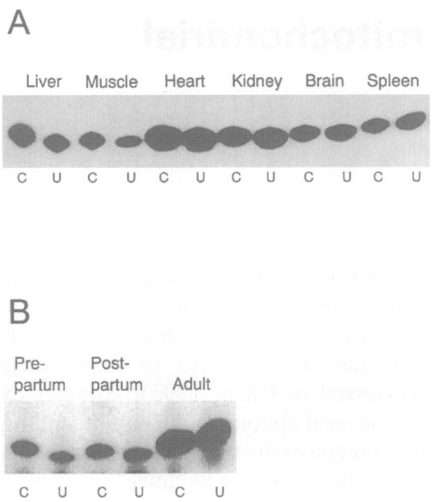

Fig. 1. Quantitation of the edited and unedited forms of tRNA^{Asp} in different tissues (A) and different developmental stages (B) of shrewlike opossum (*Monodelphis domestica*). Total small cellular RNAs were assayed by RT-minisequencing (Syvänen *et al.*, 1990) with primer MoHAC as described in Materials and methods. Identical amounts (~1 μg) of the respective RNA samples were included in the extension reactions. C, indicates lanes where the extension was done in the presence of [^{32}P]dGTP, while, U, indicates lanes where the extension was done in the presence of [^{32}P]dATP. (**A**) shows the relative abundance of tRNA^{Asp} with anticodons GCC and GUC, respectively, in liver, muscle, heart, kidney, brain and spleen. (**B**) shows the relative abundance of anticodons GCC and GUC in total extracts from embryo before birth (prepartum) after birth (postpartum) and in the liver of an adult animal. Extension products were analysed by electrophoresis in 10% polyacrylamide gels.

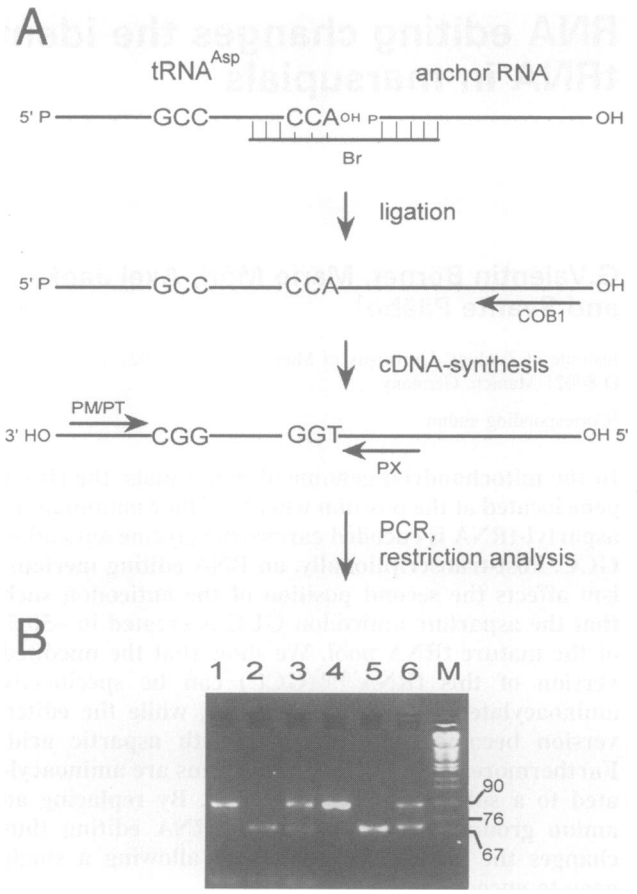

Fig. 2. Presence of CCA on tRNA^{Asp} with anticodon GCC *in vivo*. (**A**) Schematic diagram of the RNA bridging procedure. The DNA splint used for ligation (Moore and Sharp, 1992) of the tRNA^{Asp} with the *in vitro* transcript is labelled Br, the primer used for cDNA synthesis is COB1, the PCR primers are PX, which introduces an *Xcm*I site in the presence of CCA at the 3′ terminus of tRNA^{Asp}, PM, which introduces an *Mwo*I site in the presence of anticodon GCC and PT, which introduces a *Tth*111I site in the presence of anticodon GTC. (**B**) Quantitation of the fractions of tRNA^{Asp} that carry both CCA sequences and the edited (GUC), or unedited (GCC) form of the anticodon, respectively. PCR products were amplified from cDNA as described in Materials and methods and analysed on 4% agarose gels. Lane 1, uncleaved PCR products using primers PX and PT; lane 2, PCR products from lane 1 digested with *Xcm*I, diagnostic for CCA-termini; lane 3, PCR products from lane 1 digested with *Tth*111I, diagnostic for anticodon GUC; lane 4, uncleaved PCR products using primers PX and PM; lane 5, PCR products from lane 4 digested with *Xcm*I, diagnostic for CCA-termini; lane 6, PCR products from lane 4 digested with *Mwo*I, diagnostic for anticodon GCC. The length of the marker fragments is given as numbers of base pairs.

and the remaining three dideoxynucleotides. Subsequently, the extension products were analysed by polyacrylamide gel electrophoresis and autoradiography. Figure 1A shows that approximately equal amounts of tRNA^{Asp}(GCC) and tRNA^{Asp}(GUC) are present in all tissues analysed. Thus, there seems to be no correlation between the respiratory activity of different tissues and the degree of anticodon editing.

Furthermore the ratios of the two forms of tRNA^{Asp} were assayed in different developmental stages. Total small cellular RNA was extracted from embryos 14 days after fertilization (approximately half a day before birth), shortly after birth and from an adult individual. The results (Figure 1B) show that the ratios of edited versus unedited tRNA are similiar in the three developmental stages tested. Thus, there is no evidence that the extent to which the intracellular pool of tRNA^{Asp} is edited differs at different stages of marsupial developmental.

Edited and unedited tRNA^{Asp} carry CCA-termini

tRNAs carry the sequence CCA at their 3′ ends, which is required for aminoacylation and translation (reviewed in Deutscher, 1990; Samaha *et al.*, 1995). In mitochondria, this structure is not encoded in the genome but added post-transcriptionally by the ATP(CTP)-tRNA-specific nucleotidyltransferase (Rossmanith *et al.*, 1995). In order to elucidate whether the unedited form of the tRNA might become aminoacylated, it was investigated whether 3′ CCA-termini are added to the unedited transcripts. The tRNA^{Asp} was isolated from opossum liver (*Didelphis virginiana*) by hybridization to an oligonucleotide immobilized on a solid phase (Mörl *et al.*, 1994). This oligonucleotide was complementary to 29 nucleotides in the 3′ half of the tRNA and thus does not discriminate between genomic and edited forms of the tRNA. After isolation of the tRNA, an unrelated RNA molecule was ligated to the 3′ ends of tRNA^{Asp} molecules that carry CCA-termini by an 'RNA-bridging' procedure (Moore and Sharp, 1992; see Figure 2A), and a cDNA was produced by reverse transcription initiated from a primer complementary to the RNA ligated to the 3′ end of the tRNA. After PCR amplification of the cDNA, the amplification product was cloned and the inserts of seven clones with CCA sequences were sequenced. Of these, five carried the anticodon GCC whereas two carried the anticodon GTC (not shown).

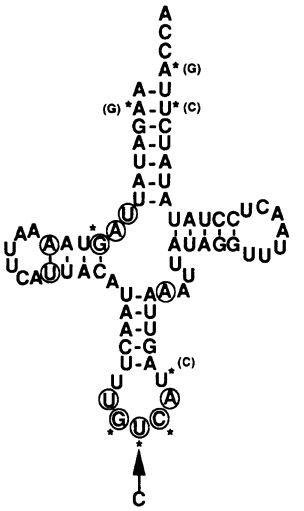

Fig. 3. Inferred secondary structure of opossum tRNA^{Asp}. The positions of identity elements for aminoacylation with aspartate in *E.coli* (Nameki *et al.*, 1992) are indicated by asterisks. Where the opossum sequence differs from *E.coli* at such positions, the *E.coli* identity elements are given within parentheses next to the secondary structure. The circled nucleotides are conserved among animal mitochondrial tRNA^{Asp}. The G at position 10, however, is present in 15 of the 22 tRNAs in opossum and is, therefore, probably not an identity element.

To quantitate the numbers of edited and unedited tRNA^{Asp} molecules with CCA-termini, the primers used for the PCR were designed such that an *Xcm*I site was created if a CCA sequence was present at the 3′ end of the tRNA, and an *Mwo*I site was created if the template carried the anticodon GCC. When the PCR products were restricted with *Xcm*I, more than 90% of the PCR products are cleaved (Figure 2B), indicating that the majority of the molecules selected for by the bridging procedure carry CCA-termini at their 3′ ends as expected. When the same PCR product was restricted with *Mwo*I, approximately half of the molecules were cleaved indicating the presence of the genomic anticodon GCC. In an analogous experiment, a primer which introduces a *Tth*111I site if the template carried the anticodon GUC was used. In this case, approximately a third of the molecules were cleaved (Figure 2B). Thus, both the edited and unedited forms of the tRNA^{Asp} carry 3′ CCA-termini and could therefore potentially be charged with an amino acid.

In vitro aminoacylation of the edited form of tRNA^{Asp}

The primary sequences of animal mitochondrial tRNAs are weakly conserved compared to their prokaryotic and eukaryotic counterparts (Kumazawa and Nishida, 1993; Steinberg *et al.*, 1993). In particular, not all identity elements that are essential for recognition of tRNA^{Asp} by its cognate aminoacyl-tRNA sythetase in other systems are found in the mitochondrial tRNA^{Asp}. Thus, of the eight elements that are essential for aminoaylation of tRNA^{Asp} in *Escherichia coli* (Nameki *et al.*, 1992), only four are present in the opossum mitochondrial tRNA^{Asp} (Figure 3). Since extrapolations from prokaryotic systems may therefore be difficult, we investigated whether the edited form of the tRNA becomes specifically aminoacylated with aspartate. To that end, transcripts carrying a 3′ CCA-terminus and a U at position 35 were incubated with a mitochondrial extract from marsupial liver in the presence of [^{3}H]aspartic acid. Figure 4A shows that the edited version of the tRNA is efficiently aminoacylated with aspartic acid. In order to investigate the effect of the editing event on aminoacylation, an identical tRNA with the genomically encoded anticodon GCC was incubated under the same conditions. This unedited tRNA^{Asp} could not be aminoacylated with aspartate. When a 20-fold molar excess of the remaining 19 amino acids was added under conditions where the tRNA was limiting for the reaction, no reduction of the aminoacylation of the tRNA^{Asp}(GUC) with aspartic acid was detected (not shown). Thus, while the edited version of the tRNA^{Asp} is recognized efficiently and specifically by the mitochondrial aspartyl-tRNA synthetase, the unedited version tRNA$^{'Asp'}$(GCC) is not.

Aminoacylation with glycine of the unedited form of tRNA$^{'Asp'}$

In order to investigate whether the unedited form of the tRNA^{Asp} can be aminoacylated with glycine, *in vitro* synthesized tRNAs with anticodons GCC and GUC were incubated with a mitochondrial extract in the presence of radioactively labelled glycine. Figure 4B shows that the tRNA with the anticodon GCC can be aminoacylated with glycine while the edited tRNA can not. When a 20-fold molar excess of the other 19 amino acids was included in the experiments, no reduction of the level of aminoacylation with glycine was observed (not shown). Thus, the presence of a C at position 35 allows aminoacylation with glycine and blocks charging with aspartic acid. By contrast, the U allows charging with aspartic acid while preventing charging with glycine.

Glycine aminoacylation of tRNA^{Asp} synthesized in vivo

Some modifications of tRNAs can serve as negative identity elements that prevent interaction with non-cognate tRNA synthetases (Muramatsu *et al.*, 1988; Perret *et al.*, 1990). In order to elucidate whether the aminoacylation of the unedited form of the marsupial tRNA^{Asp} with glycine might be prevented by modifications present in the tRNA synthesized *in vivo*, tRNA^{Asp} was isolated from marsupial liver by hybridization to an immobilized oligonucleotide that is specific for tRNA^{Asp} and does not discriminate between genomic and edited forms (Mörl *et al.*, 1995). When this tRNA preparation was incubated with a mitochondrial extract, it could be charged with glycine (Figure 5A) as well as with aspartic acid (Figure 5B). Neither reaction was affected by the addition of a 20-fold molar excess of the 18 remaining amino acids (not shown). Moreover, it was observed that ~10 times smaller amounts of the unedited tRNA isolated from liver was needed to detect aminoacylation with glycine than of the *in vitro* transcript. Thus, the unedited form of tRNA^{Asp} is a better substrate for aminoacylation with glycine when synthesized *in vivo* rather than *in vitro*, a fact that can probably be explained by the presence of modifications and/or a more stable tertiary structure in the tRNA isolated from the tissue.

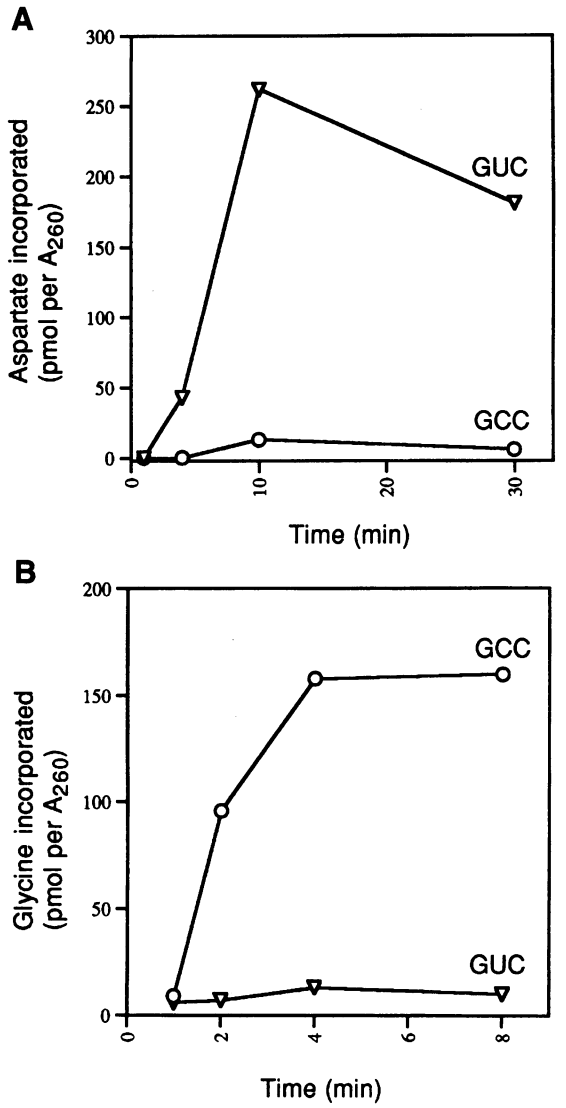

Fig. 4. Time course of aminoacylation of *in vitro* transcripts with aspartate (A) and glycine (B). (**A**) Aminoacylation of *in vitro* transcripts of $tRNA^{Asp}$ with anticodons GUC and GCC, respectively, in the presence of [^{3}H]aspartate and marsupial mitochondrial extract. (**B**) Aminoacylation of *in vitro* transcripts of $tRNA^{Asp}$ with anticodons GUC and GCC, respectively, in the presence of [^{3}H]glycine and marsupial mitochondrial extract.

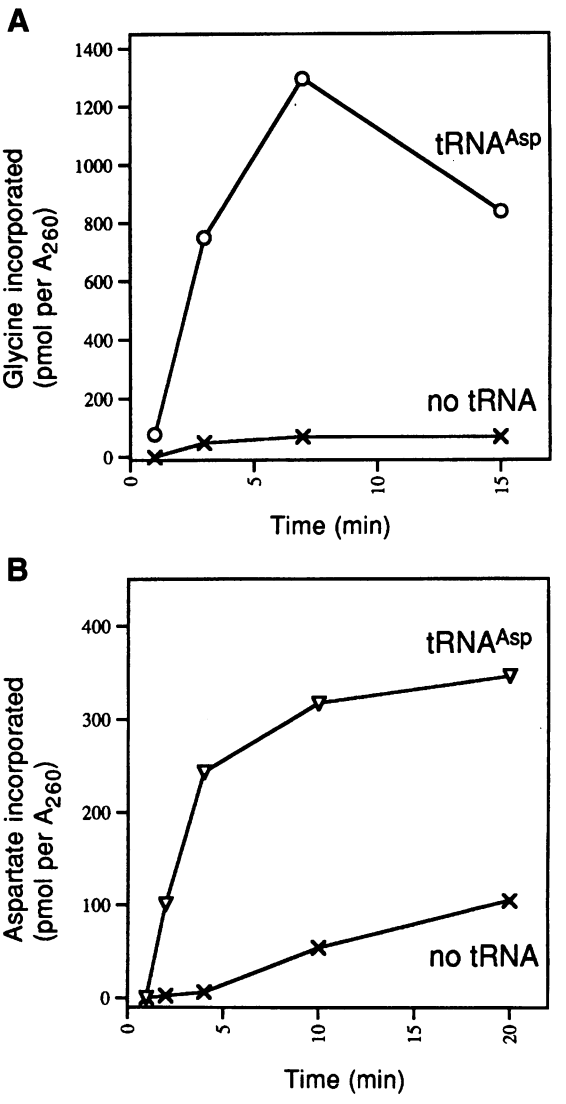

Fig. 5. Time course of *in vitro* aminoacylation of $tRNA^{Asp}$ isolated from opossum liver with glycine (A) and aspartate (B). (**A**) The relative abundance of C versus U in the total $tRNA^{Asp}$ isolate was determined by RT-minisequencing and the intensity of the bands compared to reactions with known amounts of *in vitro* transcripts. The total concentration of $tRNA^{Asp}$ in this *in vitro* aminoacylation was 43 nM, 25 nM of which carried the unedited base C. Approximately 80% of the input $tRNA^{'Asp'}$(GCC) becomes aminoacylated with glycine. When an *in vitro* transcript with anticodon GCC was added at 25 nM, no aminoacylation with glycine could be detected (not shown). (**B**) The concentration of $tRNA^{Asp}$ in this experiment was 102 nM, 58 nM of which carried the edited base U in the anticodon. Approximately 20% of the input $tRNA^{Asp}$(GUC) becomes aminoacylated with aspartate. The increase in the reaction with no (external) tRNA is presumably due to a high level of endogenous tRNA in this extract preparation.

Two mitochondrial tRNAs for glycine in marsupials

In the mitochondrial genome of marsupials, a gene for $tRNA^{Gly}$ with the expected anticodon TCC exists at the location where other mammalian mitochondrial genomes also carry such a gene (Janke *et al.*, 1994). Although this tRNA gene is similar to other mitochondrial tRNAs in lacking most elements responsible for glycine identity in *E.coli* (McClain *et al.*, 1991), its inferred secondary structure seems fully compatible with it being a functional tRNA (Figure 6A). In agreement with this, aminoacylation experiments with an *in vitro* transcript of this tRNA showed that it can be aminoacylated with glycine (not shown).

To investigate whether the the two tRNAs with glycine identity are present in comparable amounts *in vivo*, we assayed for these two tRNAs in total small cellular RNA. To this end, two primers specific for the $tRNA^{'Asp'}$(GCC) and $tRNA^{Gly}$(UCC) that exhibit similiar sensitivity when tested with the respective T7 transcript (not shown) were used in an RT-minisequencing assay. Figure 6B shows that both primers yielded comparably strong signals, indicating that $tRNA^{'Asp'}$(GCC) and $tRNA^{Gly}$(UCC) are present in marsupial mitochondria at comparable concentrations. Thus, the $tRNA^{Gly}$(UCC) and the $tRNA^{'Asp'}$(GCC) both seem to be functional and will compete for the glycyl-tRNA synthetase *in vivo*.

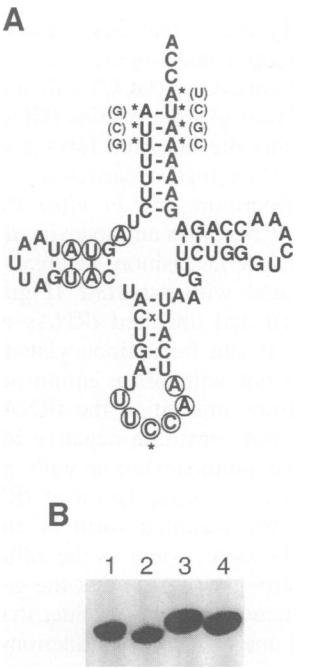

Fig. 6. Inferred secondary structure of the mitochondrial $tRNA^{Gly}$ of opossum and its abundance compared to $tRNA^{'Asp'}$(GCC). (**A**) The positions that serve as identity elements in *E.coli* (McClain *et al.*, 1991) are indicated by an asterisk. At positions where the base in opossum deviates from the identity element in *E.coli*, the latter is given next to the secondary structure. Circled positions are conserved in the $tRNA^{Gly}$ genes from animal mitochondria. (**B**) The RT-minisequencing was performed on total small RNA from opossum liver in the presence of [^{32}P]dGTP with the primers DidHAC (lane 1) and MoGlyHAC (lane 2), respectively. In parallel, identical amounts of T7 *in vitro* transcripts of $tRNA^{'Asp'}$(GCC) (lane 3) and $tRNA^{Gly}$(UCC) (lane 4) were assayed.

Aminoacylation state in vivo

The identity of a tRNA is determined by the competition of 19 non-cognate and one cognate aminoacyl-tRNA synthetases (Sherman *et al.*, 1992). Under *in vitro* conditions, deviations from the relative *in vivo* concentrations of tRNAs and synthetases can result in misacylation. It is therefore conceivable that the $tRNA^{Gly}$(UCC), but not the $tRNA^{'Asp'}$(GCC), would be aminoacylated *in vivo*.

To investigate whether the unedited form of $tRNA^{Asp}$ is indeed aminoacylated *in vivo*, we designed the OXOCIRC assay that takes advantage of the fact that aminoacylation protects the 3′ ends of tRNAs against oxidation by $NaIO_4$ (Andrulis and Arfin, 1979). In this assay (Figure 7), tRNAs isolated under non-deacylating conditions are incubated with $NaIO_4$. This results in the oxidation of the 2′ and 3′ hydroxylgroups of non-acylated tRNAs. Subsequently, aminoacylated tRNAs are deacylated, circularized by ligation (Pan *et al.*, 1991; Yokobori and Pääbo, 1995a) and separated by gel electrophoresis from the oxidized tRNAs, which remain linear since they lack 3′ OH-groups that could take part in the ligation reaction.

In an initial experiment, the amount of $NaIO_4$ required for complete oxidation of non-aminoacylated RNAs was determined. Small RNAs were isolated from opossum heart under acidic conditions. A fraction was deacylated by incubation at pH 9.0 and incubated with an excess of $NaIO_4$. Subsequently, the state of the 3′ ends was assayed by deacylation and 3′ labelling. Figure 8A shows that of the total small RNAs, a substantial number resisted $NaIO_4$

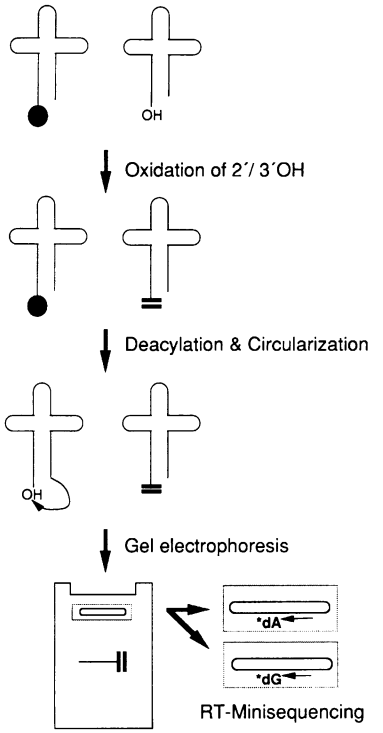

Fig. 7. Schematic representation of the OXOCIRC assay for determination of the state of aminoacylation *in vivo*.

oxidation and were therefore aminoacylated tRNAs, while this proportion was decreased at least 6-fold by deacylation prior to oxidation.

To analyse the extent of aminoacylation of the edited and unedited forms of $tRNA^{Asp}$, small RNAs were isolated from opossum heart and analysed as described. To analyse total tRNAs regardless of their aminoacylation as well as to control for possible modifications introduced by the oxidation, an aliquot of the RNA was not oxidized prior to deacylation, but instead after deacylation and circularization. Subsequently, circularized forms of the two tRNA aliquots were separated from linear tRNAs by electrophoresis in polyacrylamide gels, eluted and assayed by RT-minisequencing. Figure 8B shows the relative amounts of the non-edited and edited forms of $tRNA^{Asp}$ in the total tRNA fraction, and in the aminoacylated fraction. The genomic version is equally abundant among the aminoacylated tRNAs as in the total tRNA pool. Since oxidation reduces the amount of non-aminoacylated tRNAs at least 6-fold (Figure 8A), these data show that the unedited $tRNA^{Asp}$ gets aminoacylated *in vivo* to the same extent as the edited version.

When the RT-minisequencing assay was performed with the same RNA fractions with a primer specific for $tRNA^{Gly}$, similar amounts were detected in both the total and the aminoacylated fractions (Figure 8C). Thus, not only the unedited $tRNA^{'Asp'}$(GCC) but also the $tRNA^{Gly}$(UCC) is aminoacylated *in vivo*. Consequently, two tRNAs with anticodons for glycine are functional in marsupials.

Discussion

It has been shown previously that the $tRNA^{Asp}$ is encoded in the mitochondrial genome of marsupials with the

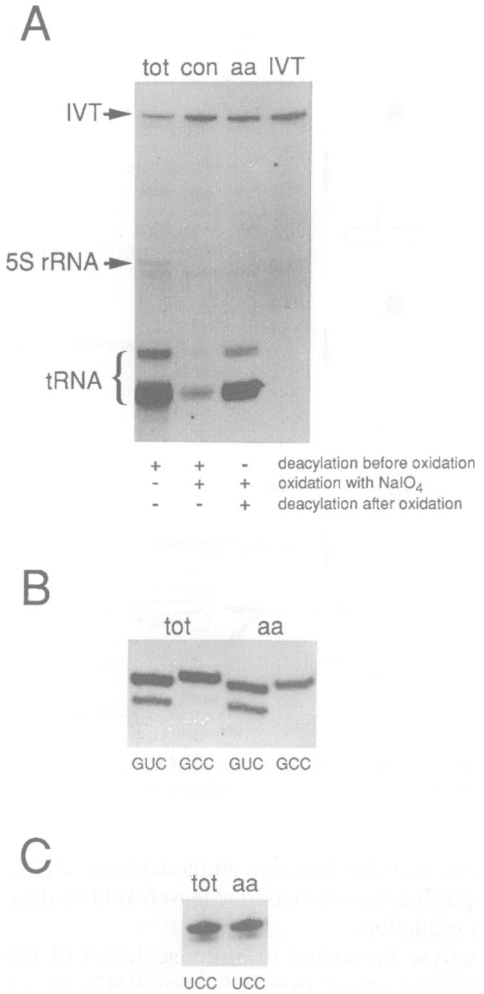

Fig. 8. Aminoacylation of the unedited tRNA$^{'Asp'}$(GCC) and tRNA^{Gly}(UCC) *in vivo*. (**A**) 3′ end-labelling of total small RNAs from opossum heart after deacylation (tot), after deacylation and subsequent oxidation (con) and after oxidation without previous deacylation (aa). To compare the efficiency of the pCp end-labelling in the reactions, identical amounts of an unrelated *in vitro* transcript (IVT) with a length of 302 nt were added to all labelling reactions. 5S rRNA indicates the position of a cellular RNA species (presumably 5S rRNA), which is not aminoacylated *in vivo*. The reactions were analysed in a 12.5% polyacrylamide gel. (**B**) Quantification of edited (GUC) and unedited (GCC) forms of tRNA^{Asp} in total tRNA (tot) and aminoacylated tRNAs (aa) by RT-minisequencing. The edited version (GUC) is represented by two bands, where the upper band represents the expected stop two nucleotides after the primer, while the shorter product is caused by the queuine base at position 34 which is mainly present in the edited version (Mörl *et al.*, 1995) and in its oxidized form blocks reverse transcription. When RT-minisequencing is performed with an *in vitro* transcript carrying anticodon GUC, only the upper band is visible (G.V.B., unpublished data). Extension products were analysed by electrophoresis in 20% polyacrylamide gels. (**C**) Quantification of mitochondrial tRNA^{Gly} with anticodon UCC in total (tot) and aminoacylated tRNA (aa). Equal amounts of the two RNA fractions were used in the circularization reaction, the subsequent gel purification of circular molecules and the RT-minisequencing. The weaker, lower band is present also when the extension is done with an *in vitro* transcript of tRNA^{Gly} and is thus not caused by a base modification.

anticodon GCC, instead of the expected GTC, and that the aspartate anticodon GUC is created post-transcriptionally by an RNA editing mechanism. At steady-state, the amounts of the edited and unedited forms is approximately equal (Janke and Pääbo, 1993; Mörl *et al.*, 1995) and it is shown here (Figure 1) that this is the case in different tissues and developmental stages.

The unedited anticodon (GCC) will potentially recognize two out of four glycine codons (GGC and GGU). A first indication that the unedited form may be functional comes from the fact that it carries a 3′ CCA-terminus (Figure 2). Furthermore, an *in vitro* transcript of the unedited tRNA^{Asp} becomes aminoacylated with glycine in an efficient and specific fashion, whereas the edited form gets aminoacylated with aspartate (Figure 4). When a mixture of edited and unedited tRNAs is isolated from marsupial liver, it can be aminoacylated with aspartate and glycine but not with other amino acids (Figure 5). Thus, modifications present in the tRNA *in vivo* (Mörl *et al.*, 1995) do not constitute negative identity elements that preclude the aminoacylation with glycine. Finally, when the extent of aminoacylation of tRNA^{Asp} is determined *in vivo*, the unedited form is aminoacylated to approximately the same extent as the edited form (Figure 8). These data strongly suggest that the gene for tRNA^{Asp} in marsupials encodes a gene product that performs two functions. In its unedited form it functions as a tRNA for glycine whose anticodon will recognize two glycine codons (GGC and GGU). The edited form of the tRNA^{Asp}, on the other hand, functions in the translation of aspartate codon (GAC and GGC) in a fashion similar to other vertebrate mitochondrial systems. The RNA editing of tRNA^{Asp} thus changes one functional form of the tRNA into another and joins two other mammalian cases, apolipoprotein B and a familiy of glutamate-gated ion channels, as examples of transmutational RNA editing, the type of RNA editing which changes one functional form of the transcript into another.

It is of interest that two *in vitro* transcripts, that differ only in having a C or a U residue at position 35, become specifically aminoacylated with glycine and aspartate, respectively. While the presence of the amino-group at the second position of the anticodon allows the interaction with the glycyl-tRNA synthetase, and prevents interaction with the aspartyl-tRNA synthetase, the presence of the keto-group reverses the situation. The presence of an amino- or a keto-group, therefore, constitutes a major identity element for aminoacylation with glycine or aspartate, respectively. Furthermore, the identity switch induced by the editing at position 35 must be almost complete *in vivo*, since misacylation would result in mistranslation of mitochondrially encoded proteins and would presumably not be compatible with a functional oxidative phosphorylation. In this context, it is interesting that the U at position 35 of yeast cytoplasmic tRNA^{Asp} has been shown to interact with several amino acid residues in the aspartyl-tRNA synthetase (Cavarelli *et al.*, 1993). While these amino acid residues are conserved in several prokaryotic aspartyl synthetases, as well as the yeast mitochondrial synthetase (Delarue *et al.*, 1994), it is not known whether they occur in the aspartyl-tRNA synthetase of animal mitochondria.

The only other case where a single naturally occurring modification changes both the codon specificity and the identity of a tRNA comes from aminoacylation studies of a minor tRNA^{Ile} in *E.coli*, which is encoded in the genome with the methionine anticodon CAT (Muramatsu *et al.*, 1988). The unmodified version of this tRNA is aminoacyl-

ated with methionine *in vitro*, while after modification of the first anticodon position to a lysidine residue, a base with the pairing capacities of U, the tRNA becomes aminoacylated with isoleucine. However, nothing is known about the *in vivo* abundance or a possible physiological function of the non-modified version of this tRNA (Muramatsu *et al.*, 1988).

According to the genetic code in vertebrate mitochondria (Barrell *et al.*, 1980; Anderson *et al.*, 1981), the unedited version of $tRNA^{Asp}$(GCC) could recognize two (GGY) out of four glycine codons. At the same time, the $tRNA^{Gly}$(UCC) found in all vertebrates is thought to recognize all four glycine codons (GGN), which all occur in the marsupial mitochondrial genome at frequencies similar to those in other mammals (Janke *et al.*, 1994). In view of the fact that the mammalian mitochondrial translation machinery uses only 22 tRNAs and that the $tRNA^{Gly}$, with the anticodon UCC, can be efficiently aminoacylated with glycine and thus seems to be fully functional, it is hard to understand why there should be two tRNAs recognizing an overlapping set of glycine codons in marsupials. A possible explanation has been suggested elsewhere (Börner and Pääbo, 1996). Further work will be needed to elucidate the functional roles of the $tRNA^{Gly}$(UCC) and $tRNA^{Asp}$(GCC) in mitochondrial translation.

Materials and methods

Oligonucleotides

DidHAC: 5′-AAACCTATAATTTAACTATG-3′, 20mer; MoHAC: 5′-GCTTGTAATTTAACTATG-3′, 18mer; MoGlyAC: 5′-CCCAGAACTT-AATGATTG-3′, 18mer; tAspPu: 5′-B-TAAGATATATAGGAGTTAAA-CCTATAATA-3′, 29mer; tAspBr: 5′-CCTTTTGTTCCCTGGTAAGA-TATATA-3′, 26mer; COB1: 5′-TCTGTCCATAAACACAAC-3′, 18mer; PX: 5′-ACACAACAATAACCATTTG-3′, 19mer; PM: 5′-AGTAAAAT-TCATTAGCTAAC-3′, 20mer; PT: 5′-GTAAAATTCATTACATGACT-3′, 20mer.

RT mini-sequencing

For annealing, 10 pmol of a gel purified oligonucleotide that recognizes the sequence immediately downstream of the second position of the anticodon (DidHAC for detection of $tRNA^{Asp}$ in *Didelphis*, MoHAC for $tRNA^{Asp}$ in *Monodelphis* and MoGlyAC for $tRNA^{Gly}$ in both *Monodelphis* and *Didelphis*) were heated with 0.1–1μg of the respective total small cellular RNAs in 44 mM Tris–HCl, pH 8.3, 40 mM KCl, 8 mM $MgCl_2$, 6 mM DTT at 92°C for 2 min and put on ice. Either 10 μCi [^{32}P]α-dATP (3000 Ci/mmol, Amersham) and 20 μM each of ddGTP, ddCTP, ddTTP (for detection of the edited base) or 10 μCi [^{32}P]α-dGTP (3000 Ci/mmol, Amersham) and 20 μM each of ddATP, ddCTP, ddTTP (for detection of the non-edited base) were subsequently added together with 10 U of AMV reverse transcriptase (Pharmacia, Sweden) and incubated at 42°C (Syvänen *et al.*, 1990). In all experiments, amounts of GCC versus GUC were determined with radionucleotides of identical specific activity. The resulting extension products were analysed by PAGE. Controls with the same amounts of total cellular tRNA from *E.coli* showed that the assays are specific for marsupial $tRNA^{Asp}$ and $tRNA^{Gly}$ (not shown).

Isolation of total small RNA for quantitation of RNA editing

Total RNA for quantitation of editing was prepared by the guanidine thiocyanate method (Chomczynski and Sacchi, 1987) from frozen tissue from *Monodelphis domestica*. High molecular weight RNA was precipitated by the addition of NaCl to a final concentration of 1 M at 4°C. After centrifugation, the tRNA fraction in the supernatant was ethanol precipitated, redissolved in water and analysed by electrophoresis on a 10% polyacrylamide gel containing 7 M urea.

Isolation of mitochondrial $tRNA^{Asp}$

The isolation of mitochondrial $tRNA^{Asp}$ from frozen opossum liver was as described (Mörl *et al.*, 1995) using the biotinylated primer tAspPu.

RT–PCR assay for 3′ CCA detection

An *in vitro* transcript was synthesized from a plasmid carrying an exon sequence from the mitochondrial *COB* gene of *Saccharomyces cerevisiae*. Ligation of the isolated $tRNA^{Asp}$ with this *in vitro* transcript was essentially as described by Moore and Sharp (1992). Prior to ligation, the *in vitro* transcript was dephosphorylated and rephosphorylated according to standard procedures. The 26mer DNA splint (tAspBr) was designed to base pair with 14 nucleotides at the 3′ end of $tRNA^{Asp}$ including the CCA-end and 12 nucleotides at the 5′ end of the *in vitro* transcript. This procedure results in preferential ligation of the anchor-RNA to $tRNA^{Asp}$ with CCA-terminus when a mixture of T7 transcripts of $tRNA^{Asp}$ with and without CCA terminus is present (data not shown). cDNA was synthesized using a primer (COB1) complementary to the *in vitro* transcript and AMV reverse transcriptase (Pharmacia, Sweden). The cDNA was amplified by PCR (Saiki *et al.*, 1988) using primers PX, which is complementary to the *in vitro* transcript and introduces an *Xcm*I site in the presence of CCA at the 3′ end of the tRNA, and PM, which represents a sequence from the $tRNA^{Asp}$ 5′ of the anticodon and introduces an *Mwo*I site in the presence of anticodon GCC. PT was used instead of PM to introduce a *Tth*111I site in the presence of anticodon GTC. PCR was performed in 50 μl containing 0.4 μM of the respective primers, 0.25 mM deoxynucleotides, 1× reaction buffer and 1 μl of the cDNA reaction. An initial denaturation step at 92°C for 5 min was followed by 10 cycles consisting of 1 min at 92°C, 1 min at 43°C and 30 s at 72°C. For the following 30 cycles the annealing temperature was raised to 48°C. The PCR products were subsequently digested with the relevant restriction enzymes and analysed on a 4% agarose gel (Metaphor, FMC, USA).

Preparation of template DNA and in vitro transcription

The templates coding for $tRNA^{Asp}$ with anticodons GCC and GTC, and $tRNA^{Gly}$ with anticodon UUC, including the T7 promotor and a *Bst*N1 recognition site, were generated by PCR from cloned regions of mitochondrial DNA, or cloned RT–PCR products (Janke and Pääbo, 1993) using appropriate synthetic oligonucleotides and cloned into pIC20-H (Marsh *et al.*, 1984). The sequence of all constructs was confirmed by dideoxy sequencing (Sanger *et al.*, 1977). Preparation of templates and *in vitro* transcription were performed as described (Wyatt *et al.*, 1991). For $tRNA^{Gly}$, the first nucleotide, which is genomically encoded as A, had to be replaced by G to enhance transcription. Also, the Mg^{2+} concentration had to be increased from 28 to 42 mM. Following transcription, the tRNA transcripts were purified by PAGE.

Preparation of mitochondrial S100 extracts

Liver tissue of freshly killed animals was homogenized with a motor driven Wheaton homogenizer. Mitochondria were prepared by differential centrifugation according to Lansman *et al.* (1981) and frozen in a buffer containing 210 mM mannitol, 70 mM sucrose, 50 mM Tris–HCl, pH 7.5 and 10 mM EDTA. For lysis, the mitochondria were pelleted and disolved in 1 M KCl, 20 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT. Lysis was by sonication with three bursts of a Branson cell disruptor (output control 4, 30% duty cycle) for 30 s. After centrifugation for 30 min at 20 000 *g* the lysate was dialysed against 10% glycerol, 50 mM Tris–HCl pH 8.0, 15 mM $MgCl_2$, 0.1 mM PMSF, 1 mM DTT and subsequently centrifuged at 100 000 *g* for 1 h. Protein concentration was determined by the Bio-Rad assay. Lysates were stored as aliquots at –80°C.

In vitro aminoacylation

All *in vitro* synthesized tRNAs were heated for 5 min to 68°C in water and allowed to cool slowly to room temperature. Aminoacylation was performed at 37°C in a reaction mixture containing 55 mM Tris–HCl, pH 7.7, 12.5 mM $MgCl_2$, 15 mM KCl, 1 mM DTT, 5% glycerol, 0.05 μg/μl BSA, 5 mM ATP and 2 μg/μl proteins of mitochondrial extracts. The concentration of amino acids was either 20 μM for aspartate, including 5 μM L-[2,3-^{3}H]aspartic acid (21 Ci/mmol), or 6 μM for [^{3}H]glycine (19.2 Ci/mmol). Only in the aminoacylation of $tRNA^{Asp}$ isolated from opossum liver, the concentration of aspartate was 6 μM. The concentration of tRNA was 1 μM except for experiments with tRNA isolated from liver tissue, where concentrations are given in the respective figure legends. Where indicated, a 20-fold molar excess of cold amino acids was included. Aliquots (6 μl) of the reaction mixtures were transferred onto pieces of Whatman 3MM paper which were submitted to 5% trichloroacetic acid. Amino acids not bound to tRNA were removed by one wash in 5% trichloroacetic acid and one wash in 80% ethanol. Radiolabelled aminoacyl-tRNA was measured by liquid scintillation counting.

Isolation of small RNA under acidic conditions

Fresh or freshly frozen liver was homogenized with an ultra-turrax at maximal speed in 50 mM sodium acetate, pH 5.0, 1% SDS, 10 mM $MgCl_2$, extracted with 1 volume of phenol saturated with the extraction buffer and twice with 1 volume of chloroform. To the supernatant, 0.2 M potassium acetate (pH 5.0) was added followed by isopropanol precipitation. The RNA was applied to a DEAE–cellulose column (DE 52, Whatman), and after washing with 50 mM sodium acetate, pH 5.0, 10 mM $MgCl_2$, 100 mM NaCl, tRNAs were eluted with the same buffer containing 900 mM NaCl. Aliquots were precipitated with ethanol and stored as a dried pellet at –20°C.

In vivo aminoacylation assay (OXOCIRC assay)

Oxidation with $NaIO_4$. To determine the amount of $NaIO_4$ necessary for quantitative oxidation of non-acylated 3′ ends, an RNA sample was deacylated in 0.1 M Tris–HCl, pH 9.0 for 1 h at 37°C followed by ethanol precipitation. Aliquots were subsequently oxidized with different concentrations of $NaIO_4$ (Sigma, USA). Non-oxidized and oxidized RNA preparations were subsequently radioactively labelled by incubation with T4-RNA ligase and [^{32}P]pCp (England and Uhlenbeck, 1978) and analysed by electrophoresis and autoradiography. Only RNAs that have not been oxidized can be labelled by this procedure. The oxidation with an ~1000-fold excess of $NaIO_4$ over tRNAs reduced the amount of tRNAs with intact 3′ end by a factor of at least six. The amount of a small cellular RNA species which probably represents 5S rRNA was even further reduced by a factor of 20, indicating that oxidation was complete whereas deacylation in the control tRNA fraction was probably not complete. After adjusting the oxidation conditions, pellets of small cellular RNAs isolated under acidic conditions were disolved in modification buffer at 1.5 μg/μl and treated essentially as described (Andrulis and Arfin, 1979). One volume of 65 mM $NaIO_4$ disolved in modification buffer was added, which equals an ~1000-fold molar excess of $NaIO_4$ over 3′ ends of RNA. After 20 min in the dark, the reaction was stopped by the addition of an equimolar amount of glucose. Nucleic acids were recovered by three subsequent precipitations with isopropanol, followed by extraction with acidic phenol and ethanol precipitation. Subsequently, the tRNAs were deacylated as above. In parallel, an aliquot was treated identically except that $NaIO_4$ was added only after circularization with T4-RNA ligase.

Circularization of tRNAs. Circularization of tRNAs was achieved by incubation of total small cellular RNAs as described by Pan *et al.* (1991) except that the reaction mixture was heated to 70°C and slowly cooled to 37°C before addition of ATP, DMSO and T4-RNA ligase (New England Biolabs, USA). Approximately 15 μg circularized tRNAs were subsequently loaded on a 10% polyacrylamide gel and coelectrophoresed in parallel with a 5′ labelled, cicularized *in vitro* transcript, until linear and circularized forms were completely separated. Circularized molecules were eluted from the gel overnight at 4°C, phenol extracted, precipitated and analysed by RT-minisequencing.

Acknowledgements

We are indebted to H.Zischler, H.Poinar, S.Ribeiro, M.Sprinzl, W.K. Thomas, T.Pan, M.Dörner, S.Yokobori, C.Baron, A.Böck, H.J.Gross, W.Studier, T.Ueda, U.Zeller, W.Schaffner, U.Gyllensten, W.Schartau and K.Reimer for help in many ways. Financial support came from the DFG (Mo 634/1-1, Pa 452/2-1), Genzentrum München and the Fonds der Deutschen Chemischen Industrie.

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Received on March 26, 1996; revised on July 16, 1996