

# Editing does not exist for mammalian selenocysteine tRNAs

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## ABSTRACT

It has been reported that selenocysteine tRNA from bovine liver is completely edited to two isoacceptor species, called tRNA<sup>[Ser]Sec<sub>NCA</sub></sup> and tRNA<sup>[Ser]Sec<sub>CmCA</sub></sup>, which differ from the gene sequence. We used direct tRNA sequencing, mobility shift analyses, primer extension, restriction enzyme digestion and single strand conformational polymorphism (SSCP) analyses of products from reverse transcription coupled with polymerase chain reaction (RT/PCR), sequencing of RT/PCR products and HPLC-coupled mass spectrometry to reproduce this result and show here that editing of these tRNAs does not occur.

## INTRODUCTION

Editing has been defined as a posttranscriptional modification process of RNA molecules which alter the DNA-coded sequence. tRNA-editing has been found in four cases: (i) the correct base pairing in the acceptor stem is generated by editing to ensure charging in several mitochondrial tRNAs from *Acanthamoeba castellanii* (1). (ii) In marsupial mitochondria the codon recognition of tRNA<sup>Asp</sup> is altered by editing from recognizing glycine codons to aspartate codons (2). (iii) In rat liver tRNA<sup>Asp</sup> two nucleotides in the anticodon loop are changed by editing (C<sub>32</sub>U<sub>33</sub> to U<sub>32</sub>C<sub>33</sub>) whereby the highly conserved U<sub>33</sub> is replaced (3). (iv) A fourth case of tRNA-editing has been described for bovine liver selenocysteine tRNA (4–6) and claimed for higher vertebrates (7). The gene sequence of bovine tRNA<sup>[Ser]Sec</sup> has been reported to be edited to two isoacceptor species, tRNA<sup>[Ser]Sec<sub>CmCA</sub></sup> and tRNA<sup>[Ser]Sec<sub>NCA</sub></sup>, which differ from the gene sequence through pyrimidine transitions at positions 21, 28 and 34 on the one hand (Fig. 1A) and 12 and 13 on the other hand (Fig. 1B). We have used restriction enzyme digestion, SSCP analyses and sequencing of RT/PCR products, direct sequencing of tRNA<sup>[Ser]Sec</sup> from bovine liver and combined HPLC/mass spectrometric analyses as well as primer extension of tRNA from human placenta in order to substantiate the edited products. Our results indicate that editing in mammalian tRNA<sup>[Ser]Sec</sup> does not exist.

## MATERIALS AND METHODS

### RNA preparation and purification of tRNA

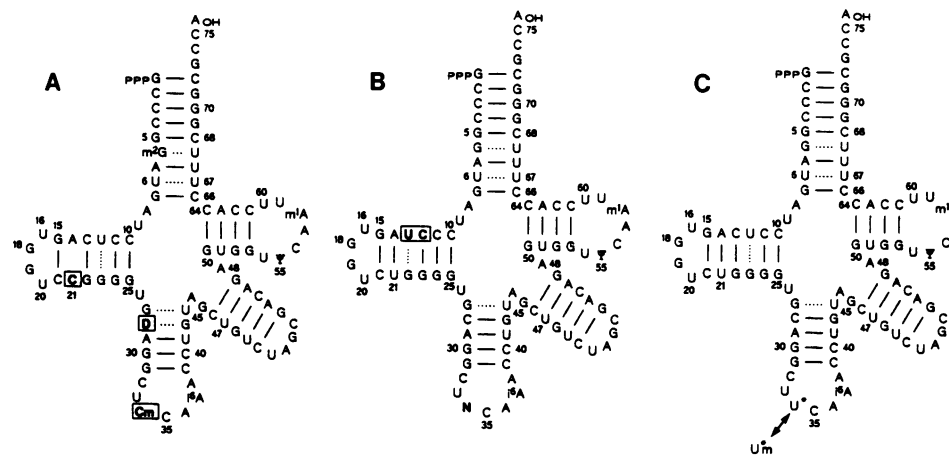
Total RNA was isolated from fresh bovine liver according to the methods of Roe (8) from animals which had an optimal selenium supply in their diet. High molecular weight RNA was removed by high salt (3M Na-acetate, pH 4.5) and the soluble low molecular weight RNAs were further purified by DEAE-cellulose chromatography (9). The resulting tRNA fraction was deacylated (30 min, 37°C, 0.1 M Tris-HCl, pH 9.0) and fractionated on BD-cellulose with a linear gradient from 0.35 to 1 M NaCl and then with 2 M NaCl and 0 to 15% ethanol. Fractions containing selenocysteine tRNA were pooled and further fractionated by preparative polyacrylamide gel electrophoresis, first under non-denaturing conditions at pH 8.3 (10% PAA/10% glycerol), followed by a denaturing 12.5% PAA/8 M urea gel at pH 8.3. tRNA bands in the gels were visualized by staining with Toluidine blue O (Merck) and recovered from gel slices by elution (10) and EtOH precipitation. Selenocysteine tRNAs in column fractions and from extracted gel slices were identified by dot-blot hybridization with a 5'-labelled oligonucleotide complementary to the 3' end of tRNA<sup>[Ser]Sec</sup> (5'-CGCCCGAAAGGTGGAATTGAACC3'). Quantitations of dot-blots were performed with a Molecular Dynamics Phosphor Imager 425. Purity of tRNAs was documented by mobility shift analyses after 5'-labelling.

### RT/PCR and analysis by SSCP

20 ng tRNA from the EtOH fraction of the BD-cellulose chromatography or 200 ng of DEAE-cellulose purified tRNA were reverse transcribed with Mn<sup>2+</sup> and *Tth*-polymerase (final concentrations 10 mM Tris-HCl pH 8.3, 90 mM KCl, 1 mM MnCl<sub>2</sub>, 200 μM dNTPs, 0.75 μM primer Sec2 or Sec3 or Sec4 and 1.25 U *Tth*-polymerase from Biozym, Hameln, in a final volume of 5 μl) by 15 min incubation at 70°C (11). After adding amplification buffer (10 mM Tris-HCl pH 8.3, 100 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.75 mM EGTA, 200 μM dNTPs, 0.625 μM primer Sec 1, 0.437 μM primer Sec2 or Sec3 or Sec4 in 50% glycerol in a volume of 20 μl) the generated cDNA was amplified immediately after prior denaturation (2 min at 94°C) in 35 cycles (30 s at 94°C, 1 min at 54°C, 30 s at 72°C) with a Perkin Elmer

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**Figure 1.** Secondary structures of tRNA<sup>[Ser]Sec</sup> from bovine liver. Sequences for edited tRNAs postulated in earlier reports (5, 6) are shown in A ('CmCA') and B ('NCA'). (C) Non-edited bovine tRNA<sup>[Ser]Sec</sup> as revealed from mobility shift (Fig. 3) and sequence analyses (Fig. 4). U<sub>20</sub> is partially modified to D. U\* in the anticodon is mcm<sup>5</sup>U and the ribose-methylated mcm<sup>5</sup>Um, respectively, as revealed by HPLC-coupled mass spectrometry. Numbering of nucleotides and secondary structures are according to Sturchler *et al.* (26).

Cetus Thermocycler 480. For reverse transcription three different primers, complementary to several parts of the 3' half of the tRNA<sup>[Ser]Sec</sup> and containing a nonbinding 5' end with an *EcoRI* or *BamHI* restriction site (underlined) were used: Sec2 (5'GCGAATTCGCCCCGAAAGGTG<sup>3'</sup>), Sec3 (5'AGCGGATCCGACAGCTACAGTTTG<sup>3'</sup>) and Sec4 (5'AGCGGATCCTACAGGTTTG<sup>G/A</sup>AGCC<sup>3'</sup>). The primer Sec1 (5'GCGAATTCGCCCCGGATGATCC<sup>3'</sup>), which is identical to the 5' end of the tRNA and contains an *EcoRI* site at its 5' end, was added for amplification. For SSCP analyses one of the two primers for amplification was weakly (5%) labelled with [<sup>32</sup>P]-phosphate at the 5' end. After denaturation the RT/PCR samples were loaded on a 10% PAA/10% glycerol gel and electrophoresis was performed at 4°C, 20 W with effective cooling, as described by Orita *et al.* (12). The amplification products were visualized by autoradiography and the bands of single-stranded DNA were excised, the DNA eluted, amplified by PCR with one labelled primer and sequenced by chemical cleavage (13).

#### Molecular cloning, restriction enzyme analysis and sequencing of PCR-clones

Amplification products were treated with proteinase K (Boehringer) to remove *Tth*-polymerase (14) followed by a subsequent restriction with *EcoRI* or *EcoRI/BamHI* to generate sticky ends and then cloned into pUC19. Positive clones were analysed by digestion with *DdeI* and *AvaII*, respectively. Several clones were sequenced by the chain termination method using Klenow polymerase.

Genes for the two types of 'edited' tRNA<sup>[Ser]Sec</sup> were produced by site-specific mutagenesis of the cloned wild-type gene according to Kunkel (15) and then cloned into pUC19.

#### Primer-extension

Primer-extension using DEAE-cellulose purified tRNA from human placenta was performed exactly according to Schmutzler and Gross (16). The primer used was complementary to the anticodon stem, D stem and loop, from G<sub>15</sub>, to C<sub>32</sub> (5'GCC-TGCACCCAGACCAC<sup>3'</sup>).

#### Direct sequencing of RNA

RNA sequence analyses were performed as described by Stanley and Vassilenko (17) or by mobility shift analyses according to Silberklang *et al.* (18).

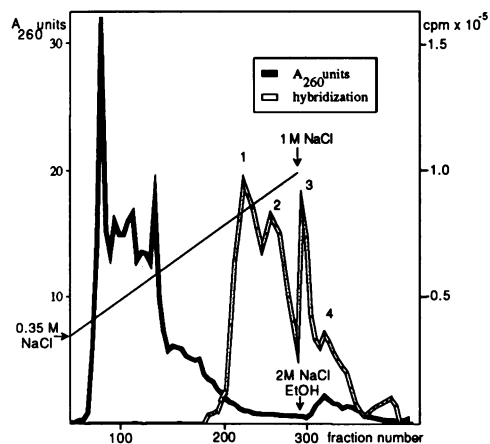
#### Identification of modified nucleotides

Modified nucleotides from sequence analyses according to Stanley and Vassilenko (17) were identified by cellulose thin layer chromatography. Identification of 5-methylcarboxymethyluridine (mcm<sup>5</sup>U) and 5-methylcarboxymethyl-2'-O-methyluridine (mcm<sup>5</sup>Um) was performed by directly combined high performance liquid chromatography/mass spectrometry (HPLC/MS) according to the method of Pomerantz and McCloskey (19, 20). Gel-purified selenocysteine tRNAs were digested successively with nuclease P1 and snake venom phosphodiesterase and dephosphorylated with bacterial alkaline phosphatase (19). The hydrolysates were separated by reversed-phase chromatography on a Sulpeco LC18S column; nucleosides were detected by measurement of UV-absorbance and from mass spectra recorded every 1.6 s (20). Authentic mcm<sup>5</sup>U was used as standard; its elution time is about 1.4 min before adenosine, mcm<sup>5</sup>Um is known to elute about 3.7 min after adenosine (21).

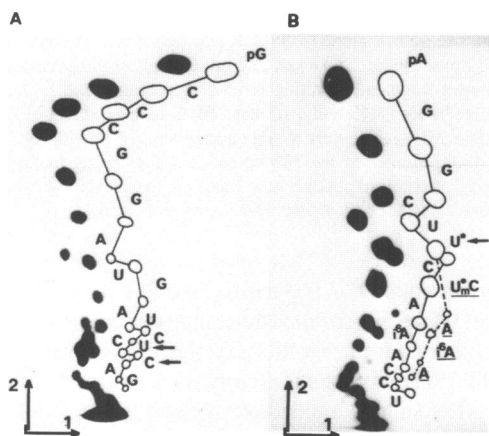
## RESULTS

#### BD-cellulose chromatography of bovine liver tRNA and purification of tRNA<sup>[Ser]Sec</sup>

The majority of the selenocysteine tRNA from bovine liver elutes in two peaks from the BD-cellulose column (Fig. 2) at 0.8 M NaCl before the EtOH gradient, quite in contrast to earlier reports (5). A third and a minor fourth peak appeared in the EtOH-fraction. Fractions of peak 1 and peak 2 were separated in two main fractions, 3 and 4 were collected in one fraction. The tRNAs from each main fraction were further purified by two successive polyacrylamide gel electrophoreses. Selenocysteine tRNA has been found in three neighbouring bands in the non-denaturing gel. Separation of these RNAs on the denaturing gel resulted in only one single band. tRNAs eluted from these gel slices were examined by sequence analyses according to Stanley and



**Figure 2.** Elution profile of bovine liver tRNAs from BD-cellulose. 6400  $A_{260}$  units of DEAE-cellulose purified and deacylated total tRNA were eluted by a NaCl gradient as indicated, followed by an EtOH gradient from 0 to 15% in 2M NaCl. Selenocysteine tRNA-containing fractions were identified by dot-blot hybridization with a 5' labelled oligonucleotide complementary to the 3' end of the tRNA<sup>[Ser]<sup>Sec</sup> (5'CGCCGAAAGGTGGAATTGAACC3') and collected as indicated. Quantitations of dot-blot were performed as indicated in Materials and Methods.</sup>

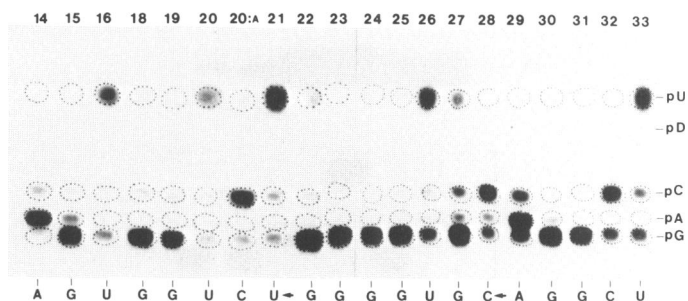


**Figure 3.** Autoradiograms of mobility shift analyses. First dimension: high voltage electrophoresis at pH 3.5 on cellulose acetate; second dimension: homochromatography in a 5 mM KOH 'homomix' on DEAE-cellulose thin layer plates at 65°C. (A) Mobility shift from the 5' end of tRNA<sup>[Ser]<sup>Sec</sup> from peak 3 + 4 of the EtOH fraction of BD-cellulose chromatography, representing the sequence from G<sub>1</sub> to G<sub>15</sub>. (B) Mobility shift of a 5' labelled tRNA<sup>[Ser]<sup>Sec</sup> fragment purified from peak 1 of BD-cellulose chromatography, showing the sequence from A<sub>29</sub> to U<sub>41</sub>. The U\* indicates mcm<sup>5</sup>U at position 34 of the anticodon. The sequence of a minor tRNA<sup>[Ser]<sup>Sec</sup> containing the ribose-methylated mcm<sup>5</sup>Um in position 34 of the anticodon can also be read (underlined). Numbering is according to Sturchler *et al.* (26). Arrows indicate putative editing sites. For interpretation of mobility shift analyses see Fig. 5 of Silberklang *et al.* (18).</sup></sup></sup>

Vassilenko (17), mobility shift analyses (18), and nucleoside modifications were detected by HPLC/MS analyses after total digestion and dephosphorylation.

**Direct sequencing and identification of modifications**

Mobility shift analyses of tRNA<sup>[Ser]<sup>Sec</sup> show that they are essentially free of contaminating tRNAs and that positions 12</sup>

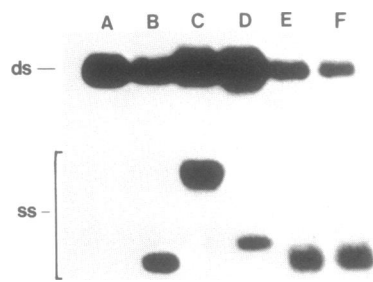


**Figure 4.** Thin layer chromatograms of nucleotides 14 to 33 from a Stanley-Vassilenko analysis of gel-purified tRNA from the EtOH fraction of the BD-cellulose chromatography. The solvent for cellulose thin layer chromatography is isopropanol, 32% HCl and H<sub>2</sub>O (700:173:127, by vol.). Positions of unlabelled pN markers are indicated by dotted circles. Nucleotides are identified on the bottom, postulated editing sites are marked by arrows. Numbering is according to Sturchler *et al.* (26). Contaminating nucleotides in positions 27 to 29, 32 and 33 derive from cross contamination of neighbouring bands. The modified uridine(s) in position 34 are not shown since they appeared to be labile during thin layer chromatography as pointed out by Kuntzel *et al.* (27).

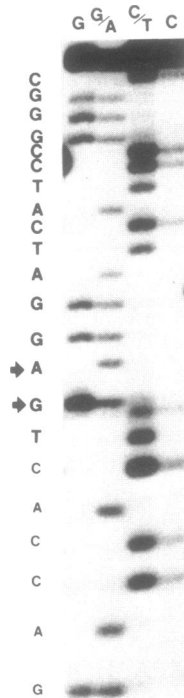
**Table 1.** Distribution of mcm<sup>5</sup>U and mcm<sup>5</sup>Um in bovine selenocysteine tRNAs from BD-cellulose chromatography (Fig. 2), determined by HPLC/MS

Peak	mcm <sup>5</sup> U	mcm <sup>5</sup> Um
1	++++	trace
2	+	+++
3 + 4	trace	++++

and 13 are unedited (Fig. 3A). Direct sequencing according to Stanley and Vassilenko (17) of the gel-purified tRNA<sup>[Ser]<sup>Sec</sup> of all fractions resulted in one sequence which is unedited at positions 21 and 28 (Fig. 4), in contrast to earlier reports (4-6). We found several base modifications. Position 34 contains two modified U derivatives, one of them (from peak 3 + 4 from BD-cellulose chromatography, Fig. 2) is 2'-O-methylated. The mobility shift pattern in Fig. 3B shows the sequence of a tRNA fragment (including the anticodon) from peak 1 of selenocysteine tRNA of the BD-cellulose chromatography (Fig. 2). There is only a weak ribose methylation present since the corresponding gap can only be recognized within the mobility shift pattern of a very minor species (Fig. 3B). i<sup>6</sup>A, U, Ψ and m<sup>1</sup>A have been found in positions 37, 54, 55 and 58, respectively (Fig. 1C). The modified uridines in position 34 are 5-methylcarboxymethyl-uridine (mcm<sup>5</sup>U) and 5-methylcarboxymethyl-2'-O-methyl-uridine (mcm<sup>5</sup>Um). This has been shown through total digestion of tRNA and separation of the dephosphorylated nucleosides on HPLC followed by mass spectrometry. Retention times and the appearance of peaks at m/z of 185 (protonated base) and 317 (protonated molecule) for mcm<sup>5</sup>U and 185 (protonated base) and 331 (protonated molecule) for mcm<sup>5</sup>Um clearly indicate that selenocysteine tRNA contains these modified nucleosides (21) (data not shown). U<sub>34</sub> is the only possible position for this modification in bovine selenocysteine tRNA, because all other uridines except those specified above (Fig. 1) are unmodified as evidenced by thin layer chromatography. The distribution of 2'-O-methylated and unmethylated mcm<sup>5</sup>U in selenocysteine tRNA from different fractions of BD-cellulose chromatography is shown in Table 1. HPLC/MS analyses also revealed that there is no Cm present in any of the fractions of the BD-cellulose</sup>



**Figure 5.** SSCP analyses of PCR and RT/PCR products from amplification with primer Sec1 and Sec3, which terminates in the anticodon of tRNA<sup>[Ser]Sec</sup> (see Materials and Methods). Lanes A to D show the SSCP analyses of the PCR-products of the wild type gene, not denatured (A) and denatured (B), of the 'CmC-A' clone (C) and of the 'NCA' clone (D), both denatured. Lanes E and F display RT/PCR products from bovine liver tRNA of the EtOH fraction from BD-cellulose chromatography and from DEAE-cellulose purification only, respectively. Double (ds) and single stranded (ss) products are indicated.

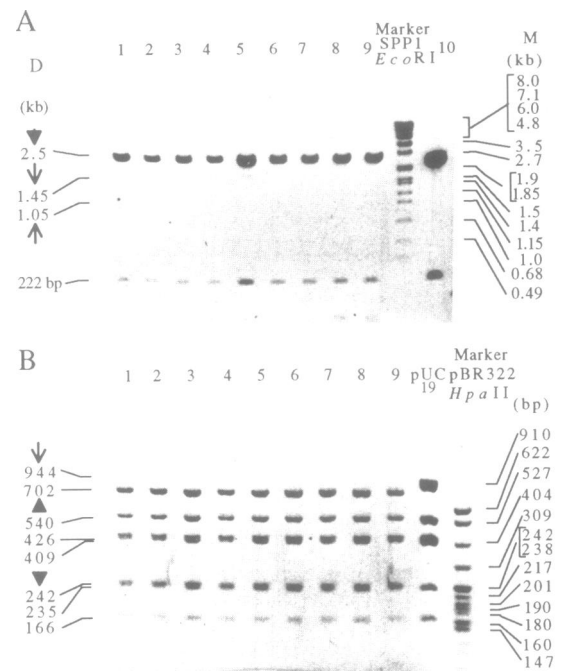


**Figure 6.** Sequence analysis by chemical cleavage (13) of a primer extension product from DEAE-cellulose purified tRNA from human placenta. The primer sequence is indicated by smaller letters. The possible editing sites are indicated by arrows.

chromatography (peak 1, 2, 3 + 4, respectively). At an elution time of 14.3 min there is no signal in UV-absorbance or mass, as would be required for a sample containing any Cm (20). HPLC/MS analyses also showed the presence of i<sup>6</sup>A, Ψ and m<sup>1</sup>A in the same tRNA<sup>[Ser]Sec</sup> digests, in accordance with the thin layer chromatographic results.

**RT/PCR-amplification and analysis by SSCP**

Fig. 5 shows the SSCP analysis of PCR amplification products derived from the wild type selenocysteine tRNA gene, from the two synthetic 'edited' species, and from RT/PCR-amplification



**Figure 7.** Restriction enzyme analysis of cloned RT/PCR amplification products with primers Sec1/Sec3 from total bovine liver tRNA. (A) *Avall* digests (separated on a 1% agarose gel) of cloned RT/PCR products from unedited tRNA<sup>[Ser]Sec</sup> result in two fragments of 2.5 kb and 222 bp. Editing would generate a cleavage site in the larger fragment, resulting in its disappearance and in the generation of two smaller bands (1.45 and 1.05 kb). (B) Edited products in *Ddel* digests (separated on a 2% agarose gel) would cause a shift of the 702 bp band to 944 bp and the disappearance of the 242 bp band. Disappearing bands are marked by arrowheads, positions of possible new bands are indicated by arrows. Fragment lengths of markers (M) and digests (D) are as indicated.

products from total DEAE-purified bovine liver tRNA and from the total EtOH fraction of the BD-cellulose chromatography (Fig. 2). The electrophoretic mobility of the single-stranded, tRNA-derived RT/PCR products is clearly the same as that of the wild type gene. The single-stranded products of the two 'edited' genes have different mobilities and were not detected in lanes E and F (Fig. 5), even after overexposure. The primer Sec3 used for these amplifications ends at position 35 in the anticodon and hence avoids termination of reverse transcription presumably caused by i<sup>6</sup>A<sub>37</sub>. The ratio of possible edited and unedited products should therefore not be affected by this modification. To rule out the possibility that the U<sub>34</sub> modification also impairs reverse transcription we used also another primer, Sec4, which covers the anticodon loop and ends at position 30 of the tRNA in the anticodon stem. Sec4 carries either G or A in the position complementary to the first nucleotide in the anticodon in order to amplify both postulated isoacceptor species. The SSCP experiment revealed again that edited tRNA<sup>[Ser]Sec</sup> cannot be detected in bovine liver tRNA (not shown). Furthermore, sequencing of the amplification products of these bands showed only the unedited sequence. Corresponding sequence analyses of the synthetic 'edited' products (lanes C and D, Fig. 5) gave the expected results (not shown).

**Primer extension and sequencing**

Primer extension of tRNA from human placenta purified by DEAE-cellulose chromatography and sequencing of the product

by chemical cleavage (13) using a primer complementary to positions 15 to 32 of the selenocysteine tRNA showed no editing at positions 12 and 13 (Fig. 6). Due to the method of chemical cleavage, a G residue appears in two lanes, one in the G lane and one in the purine lane. An A residue appears only in the purine lane. If there would be a mixture of A and G at this position of the sequence, a minor A residue would not be detected. Although a U at position 13 cannot be ruled out, its existence at this position, however, is not very likely, because the complementary G residue mentioned above represents the strongest signal in the entire lane. In the case of a mixture of two species we would expect a weaker, but not a stronger signal (Fig. 6). Position 12 is clearly an 'A' and thus we conclude that both positions are unedited.

#### RT/PCR, molecular cloning and restriction enzyme analysis

In order to provide more evidence for the presence or absence of edited tRNAs and to rule out any minor edited species, we amplified DEAE-cellulose purified tRNA from bovine liver and also the EtOH fraction of the BD-cellulose chromatography (Fig. 2) via RT/PCR with three different primer pairs (see Materials and Methods) and cloned the products in pUC19. From each amplification we examined 24 clones for editing by restriction enzyme analyses. Editing to the postulated tRNA<sup>[Ser]<sup>Sec</sup><sub>NCA</sub> would destroy a *DdeI*-site in the tRNA-gene at position 12/13 (Fig. 1) and editing to the postulated tRNA<sup>[Ser]<sup>Sec</sup><sub>CmCA</sub> would generate an *AvaII*-site at position 21. Clones carrying a copy of the 'NCA' species should therefore be restriction endonuclease resistant at these positions, whereas clones of the 'CmCA' species should be cleaved by both restriction enzymes. However, the analyses of 84 clones again revealed only clones derived from unedited tRNAs with a *DdeI* and no *AvaII* site (Fig. 7).</sup></sup>

#### DISCUSSION

Editing of selenocysteine tRNAs has been described for bovine liver tRNA<sup>[Ser]<sup>Sec</sup> (4–6). Since we were interested in studying the editing mechanism, we first tried to reproduce the previous results. We have used direct tRNA sequencing, RT/PCR with SSCP-separation and sequencing, RT/PCR with restriction enzyme analyses and combined HPLC/mass spectrometry in order to elucidate the ratio of edited to unedited tRNA<sup>[Ser]<sup>Sec</sup>. tRNA from human placenta was also examined using the sensitive method of primer extension. Neither method yielded any indication for the presence of edited tRNA<sup>[Ser]<sup>Sec</sup>. Moreover, our nucleotide analysis according to Stanley and Vassilenko and our analyses of nucleosides of tRNA<sup>[Ser]<sup>Sec</sup> from all selenocysteine tRNA fractions of the BD-cellulose chromatography (Fig. 2) by HPLC/MS revealed that there are two modified uridines in the first position of the anticodon rather than Cm (Figs. 1A and 3B). This result explains the finding that selenocysteine tRNA recognizes UGA but cannot read UGG tryptophan codons, as expected in case of a CmCA anticodon (5). The chromatographic behaviour on HPLC and the MS analyses show that these modified uridines are mcm<sup>5</sup>U and mcm<sup>5</sup>Um, respectively, as published for rat liver selenocysteine tRNA (21). In BD-cellulose chromatography the mcm<sup>5</sup>U-containing tRNA<sup>[Ser]<sup>Sec</sup>-species elutes first, followed by the tRNA<sup>[Ser]<sup>Sec</sup> with mcm<sup>5</sup>Um in the first position of the anticodon, independent of the ethanol gradient (Fig. 2 and Table 1). This notion is also confirmed by the mobility shift analysis (Fig. 3B).</sup></sup></sup></sup></sup></sup>

Our results indicate that the finding of Kato *et al.* (22), who found unedited tRNA<sup>[Ser]<sup>Sec</sup> in HeLa cells (presumably with a mcm<sup>5</sup>s<sup>2</sup>U in position 34), is not a unique exception. Recently Diamond *et al.* (21) reported the sequence of rat selenocysteine tRNA, which is also unedited. They described, for the first time in vertebrate tRNA, mcm<sup>5</sup>U and for the first time in any tRNA, mcm<sup>5</sup>Um in the first position of the anticodon as found here in bovine liver tRNA<sup>[Ser]<sup>Sec</sup>. Accordingly, we could not find an edited NCA species in human placenta (Fig. 6) as previous postulated for human selenocysteine tRNA of HL60 cells (23). We therefore conclude that there is no evidence for editing in mammalian selenocysteine tRNAs. All earlier chromatographic data providing evidence for two different ('edited') species in eukaryotes (23–25) may be caused by the partial 2'-O-methylation of mcm<sup>5</sup>U in the first position of the anticodon. Ribose methylations are often partial and thus a mixture of methylated and unmethylated tRNA exists. Recently, Diamond *et al.* (21) have shown that this methylation is selenium dependent in several rat tissues. Whether differences in mcm<sup>5</sup>U ribose methylation are important for the recognition of selenocysteine-specific UGA codons or for selenocysteine synthesis from seryl-tRNA<sup>[Ser]<sup>Sec</sup> is not clear (21).</sup></sup></sup>

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