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Two nucleotides next to the anticodon of cytoplasmic rat tRNA^{Asp} are likely generated by RNA editing

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ABSTRACT

The nucleotide sequences of major cytoplasmic tRNA^{Asp} from rat liver and rat ascites hepatoma comprise a U_{32} and C_{33} next to the anticodon as was confirmed by different procedures. Additionally we identified a tRNA^{Asp} with C_{32} and U_{33} in a minor proportion. We have shown earlier that the tRNA^{Asp} gene is part of a cluster of tRNA genes which is amplified at least ten times in the rat nuclear genome. Six independent isolated clones display identical sequences in the coding region of the tRNA^{Asp} gene which differ from tRNA^{Asp} in having C_{32} and T_{33}. Using a combination of single-strand conformation polymorphism (SSCP) analyses and direct sequencing of polymerase chain reaction (PCR) products we have now demonstrated that no variant allele of the tRNA^{Asp} gene with T_{32} and C_{33} exists in the rat genome. Together with the RNA sequencing data these findings strongly indicate that major rat tRNA^{Asp} is generated by post-transcriptional pyrimidine transitions at positons 32 and 33 and that the minor tRNA^{Asp} is its unedited precursor.

INTRODUCTION

The nucleotide sequences of the major tRNA^{Asp} from rat liver and a minor tRNA^{Asp} present specifically in rat ascites hepatoma were shown to comprise some interesting properties (1). The rat liver tRNA has a mannose-attached queuosine (Q^*_{34}) in the first position of the anticodon, whereas the queuosine is replaced by guanosine (G_{34}) in rat ascites hepatoma tRNA^{Asp}. The most notable feature of major and minor tRNAs^{Asp} is that they contain a cytidine in the position next to the 5'end of the anticodon (C_{33}), occupied by an uridine in all known tRNAs, with the exception of cytoplasmic initiator tRNAs from higher eukaryotes (2).

A rat DNA fragment has been cloned and sequenced which carries a cluster of genes coding for tRNA^{Leu}, tRNA^{Asp}, tRNA^{Gly} and tRNA^{Glu}. The cluster reiterates about 10 times in the haploid DNA (3, 4). Interestingly, the nucleotide sequence of the putative tRNA^{Asp} gene differs from that of rat liver tRNA^{Asp} in having C_{32} and T_{33} instead of T_{32} and C_{33}. Until now, six independent clones of this cluster have been sequenced, which all display identical sequences in the coding region of tRNA^{Asp} (4, 5). Thus, a discrepancy exists between the nucleotide sequence of the tRNA^{Asp} gene and the sequence of tRNA^{Asp} from the same organism.

Two explanations could possibly account for the observations described above: i) either a yet unknown tRNA^{Asp} gene with T_{32} and C_{33} is present within or outside the cluster in the rat genome or ii) the transcribed pre-tRNA^{Asp} is edited at the positions 32 and 33, resulting in a U to C and C to U conversion, respectively.

The well established polymerase chain reaction (PCR) to amplify DNA fragments together with the recent development of the single-strand conformation polymorphism (SSCP) analysis (6) has made possible the rapid and sensitive detection of single mutations in DNA fragments (7). Using these techniques we show here that no variant alleles of the tRNA^{Asp} gene exist in the rat genome, thus supporting the assumption that the two nucleotides U_{32} and C_{33} of mature tRNA^{Asp} are generated by RNA editing at the post-transcriptional level.

MATERIALS AND METHODS

Materials

T4 polynucleotide kinase was obtained from Boehringer, Mannheim. The AmpliTaq™ Taq DNA polymerase and the GeneAmp™ DNA Amplification kit were purchased from Perkin Elmer Cetus. The Sequenase version 2.0 (7-deaza-dGTP edition) from USB was used for sequencing reactions. [γ-^{32}P]ATP with a specific activity of 110 TBq/mmol was from Amersham.

Sequencing and labelling of polydeoxyribonucleotides

Oligonucleotide primers Asp1 (5'-TCCTCGTTAGTATAG-TGGTG-3') and Asp2 (5'-CTCCCCGTCGGGGAATCGAA-3'), identical and complementary, respectively, to the 5' and 3' ends of the non-coding, tRNA-like strand of the tRNA^{Asp} gene (Fig. 1A) were synthesized on the Applied Biosystems Model 380 B DNA Synthesizer and purified on a 12.5% polyacrylamide/7M urea gel. The primers (100 pmoles each) were end-labelled with [γ-^{32}P]ATP (33 pmoles) by T4 polynucleotide kinase (10 units). This mixture (14 μl) was incubated for 45 min at 37°C, heated for 10 min at 68°C and

stored at −20°C. The efficiency of ^{32}P-transfer to the oligonucleotide was monitored by Polygram CEL PEI cellulose chromatography in 4M ammonium acetate. Under the conditions described above, more than 90% of the radioactivity was transferred to the primers which were therefore not further purified.

DNA amplification by the polymerase chain reaction (PCR)

The amplification reactions were performed according to Hayashi *et al.* (8) using three different sets of primers (see legend to figure 2) in a 10 μl final volume containing 0.1 μg rat liver DNA (Donryu strain), 10 pmol of each primer, 2 nmol each of the four deoxyribonucleotides and 0.25 units of Taq DNA polymerase. The mixture was subjected to 30 cycles of PCR in a Perkin-Elmer Cetus Thermocycler. Each cycle consisted of 94°C, 55°C and 72°C for 0.5, 0.5 and 1 min, respectively.

Single-strand conformation polymorphism (SSCP) analysis

The PCR products were analysed by the SSCP method essentially as described by Orita *et al.* (6, 7). A portion of the reaction mixture (1 μl out of 10 μl) was withdrawn and mixed with 50 μl of either F-dye (10 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol in 96% formamide) or S-dye (10 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol, 0.1% SDS, 10% glycerol). The F-dye samples were heated at 80°C for 3 min, the S-dye samples (4 μl/lane) were applied without prior heating to a 6% non-denaturing polyacrylamide gel (20×40×0.04 cm), containing 10% glycerol. Electrophoresis was performed with 0.5×TBE (Tris-Borate-EDTA buffer, pH 8.0) at 1000 Volt for about 3.5 h until bromphenol blue had migrated 25 cm. The desired DNA fragments were isolated from the dried gel by cutting with a razor-blade the area of the gel corresponding to the appropriate region on the autoradiogram. The pieces were placed in 100 μl of water and kept for 60 min at room temperature with occasional shaking. The eluted material was then transferred to another tube.

Asymmetrical polymerase chain reaction (asPCR)

The eluted DNA from the PCR-SSCP gel was subjected to another PCR in a mixture containing an unequal molar ratio (1:10) of the primers (9). The amplification reaction was performed in a 40 μl final volume, containing 10 μl solution of the eluted DNA, 0.1 μM primer Asp1, 1 μM primer Asp2 (or vice versa) and 1 unit Taq DNA polymerase. The mixture was subjected to 55 cycles consisting of 94°C, 55°C and 72°C for 0.5, 0.5 and 1 min, respectively. The amplification mixture was extracted once with 50 μl chloroform and diluted with 2 ml of water. Desalting and removal of dNTPs was achieved by centrifugating the sample in a Centricon 30 micro-concentrator. About 50 μl of DNA solution were retained.

Direct sequencing of PCR products

Sequence determination of DNA fragments from the asPCR was according to Gyllensten and Erlich (10) using ^{32}P-labelled oligonucleotides as sequencing primers (11). For the annealing reaction, 1 μl 5′-labelled primer prepared as described above, 2 μl 5×sequenase buffer and 7 μl DNA template (out of 50 μl purified asPCR solution) were incubated for 10 min at 65°C and then slowly cooled down. After addition of 1 μl 0.1 M DTT and 2 μl H_2O, the template-primer mixture was divided into four samples of 2.5 μl to which 2.5 μl of the corresponding termination mix and 1 μl diluted sequenase were added. For the dilution of sequenase, 1 μl sequenase (13 units/μl), 5 μl dilution buffer and 2 μl Mn buffer were mixed together. Incubation was for 5 min at 37°C, followed by the addition of 4 μl stop solution.

RESULTS

Major cytoplasmic tRNA^{Asp} contains two nucleotides which differ from the corresponding tRNA^{Asp} gene sequence

Cytoplasmic tRNA^{Asp} was first isolated from rat liver and its sequence was determined by post-labelling procedures (1). This tRNA is shown in Fig. 1B. A minor tRNA^{Asp} which contains guanosine instead of queuosine in the first position of the

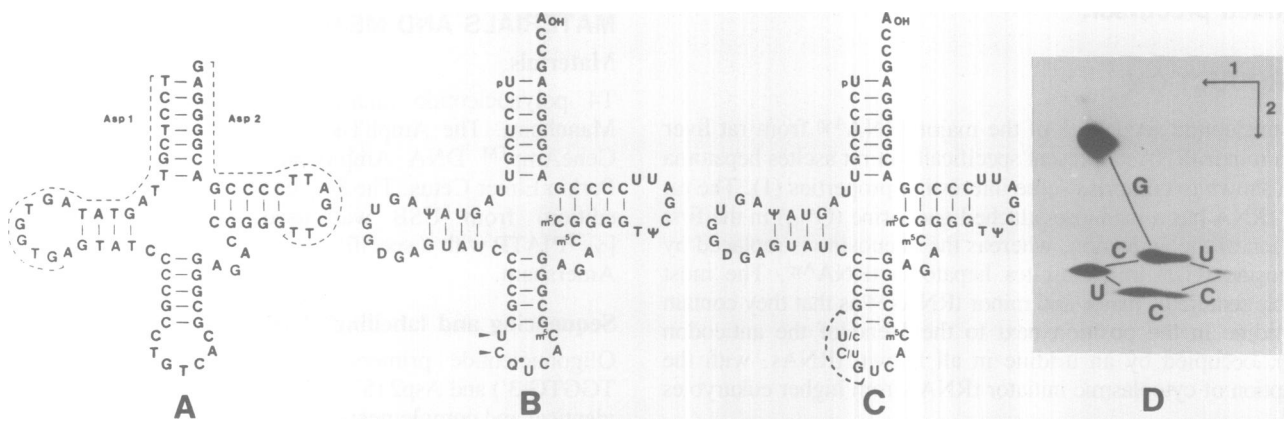

Figure 1. Secondary structures of the rat liver tRNA^{Asp} gene (A), tRNA^{Asp} from rat liver (B) and tRNA^{Asp} from rat ascites hepatoma (C). The regions to which the two primers (Asp1 and Asp2) hybridize to the tRNA^{Asp} gene are indicated by dotted lines. Asp1 has the nucleotide sequence corresponding to that of the tRNA, while Asp2 is complementary to the tRNA sequence. The arrows in B point to the two nucleotides which differ in the tRNA as compared to the tRNA gene sequence. Q* is mannose-attached queuosine (1). The dotted line in C indicates the RNase T1 fragment whose composition was analysed by mobility shift analysis as shown in D. This tetranucleotide was isolated from a fingerprint of 5′-[^{32}P]-labelled RNase T1 fragments (spot 3, ref. 12) of tRNA^{Asp}. Electrophoresis in the first dimension was on cellulose acetate strips in pyridine/acetate buffer (pH 3.5) at 4000 V and in the second dimension was on DEAE-cellulose paper in 7% formic acid at 750 V.

anticodon was detected in rat ascites hepatoma cells by analysis using *Escherichia coli* guanosine insertion enzyme (12). Otherwise the sequence of this $tRNA^{Asp}$ appeared to be identical to that of rat liver tRNA (1). Because of the unusual occurrence of a cytidine at position 33 in both tRNA species, we re-evaluated the nucleotide sequence of minor $tRNA^{Asp}$ from rat ascites hepatoma in the questionable region, using a combination of RNase T1 fingerprint and mobility shift analyses.

The putative oligonucleotide (pCUCG), comprising nucleotides 31 to 34 of mature tRNA (Fig. 1C), was eluted from the DEAE-cellulose paper. The composition of this material was determined by limited nuclease P1 digestion and subsequent mobility shift sequence analysis as seen in Fig. 1D. The mobility shift pattern clearly reveals that the major sequence of the tetranucleotide is CUCG and that a minor sequence CCUG can also be read, indicating that major rat $tRNA^{Asp}$ carries indeed an uridine at position 32 and a cytidine at position 33. Moreover, it demonstrates that a $tRNA^{Asp}$ species with a C_{32} and U_{33}, presumably the precursor of the major species, is present in minor amounts in rat ascites hepatoma cells. However, there is no direct evidence for such a precursor-product relationship.

Single-strand conformation polymorphism (SSCP) analysis of $tRNA^{Asp}$ genes in rat liver DNA

A technique has recently been presented which allows the rapid detection of base changes including single nucleotide substitutions in sequences of genomic DNA (7). The target sequence is amplified by PCR using labelled primers, followed by denaturation and electrophoresis for SSCP analysis. The procedure is based on the fact that in nondenaturing polyacrylamide gels, the electrophoretic mobility of single-stranded nucleic acids depends on their size and their sequence (6).

The synthetic primers used to amplify DNA fragments from rat liver DNA in the PCR were complementary to the ends of the coding region of the $tRNA^{Asp}$ gene as illustrated in Fig. 1A, thus permitting the detection of any $tRNA^{Asp}$ gene which would comprise a different sequence in the anticodon-loop region. The PCR products were analysed on a 6% non-denaturing polyacrylamide gel containing 10% glycerol. Fig. 2 shows the analysis of PCR products using a mixture of primers in which either Asp1, Asp2 or both primers were labelled. The major single-stranded DNA fragments are species 3 and 8, which very likely comprise the coding and non-coding strand of the $tRNA^{Asp}$ gene. They do not display a pronounced difference in their migration behaviour. The double-stranded DNA products of 72 bp length (species 1 and 5) migrate more slowly than the ssDNA fragments. Minor traces of these products—due to renaturation or incomplete denaturation—are also seen in lanes which show the analysis of ssDNAs. The two minor species 2 and 4 and species 6 and 7 were consistently detected in gel electrophoretic analyses using either labelled Asp1 or Asp2, respectively (Fig. 2a, b). All fragments, including the ds DNAs, were eluted from the gel and amplified again by asymmetric PCR.

Direct sequencing of PCR-amplified $tRNA^{Asp}$ genes

The direct sequencing by the dideoxy chain termination method using unequal molar amounts of the two amplification primers has been established by Gyllensten and Erlich (10). In contrast to the method described by these authors, the actual amount of template DNA in the amplification mixture is unknown if this DNA derives from a non-denaturing gel as described above. Routinely we used 10 out of 100 µl elution extract which proved to be optimal at the end.

DNA species 1 to 8 (Fig. 2) were subjected to asPCR. Either primer Asp1 or primer Asp2 were present in a reduced molar amount in parallel amplification mixtures. This procedure results in the production of an excess of ssDNA of a chosen strand. For direct sequencing, aliquots of 7 µl out of 50 µl purified amplification mixture were used as ssDNA template, and the labelled sequencing primers were the same as used in the PCR.

A number of variations were introduced in the sequencing protocol which finally resulted in clear and reproducible sequencing ladders as seen in Fig. 3. The best resolution up to nucleotide 72 was obtained by using a 8% polyacrylamide/7M urea gel. By adding Mn^{2+} to the normal sequencing reaction (in the form of a buffered $MnCl_2$ solution which was added to the sequenase dilution mixture) the nucleotides close to the primer could be identified. For instance, the G at position 22, the second nucleotide in the elongated primer Asp1 (Fig. 1A) was clearly visible (Fig. 3). No labelling mix was used in the sequencing reaction, instead the template-primer mixture (together with sequenase) was directly added to the corresponding termination mix, in which the ratio of deoxyribonucleotides to dideoxyribonucleotides was 10:1. This also improved the reading of sequences close to the primer.

The eight DNA species (including dsDNA fragments) eluted from the gel shown in Fig. 2 comprised a DNA sequence identical to that in Fig. 1A as was verified by sequencing complementary and non-complementary strands from each species. Examples of the corresponding sequencing ladders are shown in Fig. 3. At some positions apparent sequence variations were observed which could, however, not be confirmed in the complementary strand. For example, species 4 in Fig. 3 displays an A residue at position 41 which is a G in mature $tRNA^{Asp}$

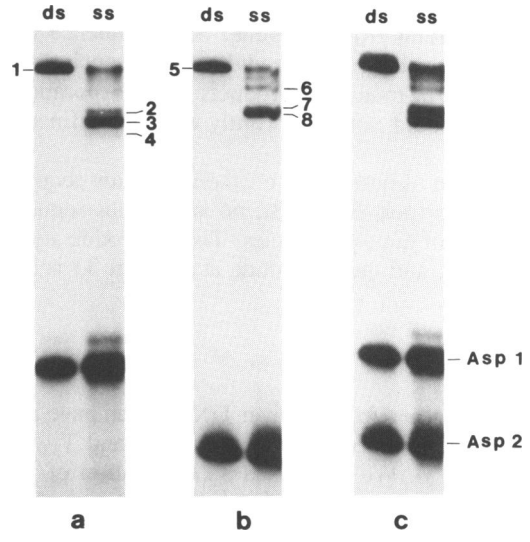

Figure 2. PCR-SSCP analysis of the $tRNA^{Asp}$ gene from rat liver DNA. Genomic DNA from rat liver (Donryu strain) was amplified by PCR in the presence of labelled primer Asp1 and unlabelled primer Asp2 (a); labelled primer Asp2 and unlabelled primer Asp1 (b); labelled primers Asp1 and Asp2 (c). Aliquots of the amplification products were denatured in F-dye to generate single-stranded DNAs (ssDNA) or not denatured (dsDNA) and applied to a 6% non-denaturing polyacrylamide gel containing 10% glycerol. The DNA fragments, numbered 1 to 8, were cut out from the dried gel and amplified by asPCR.

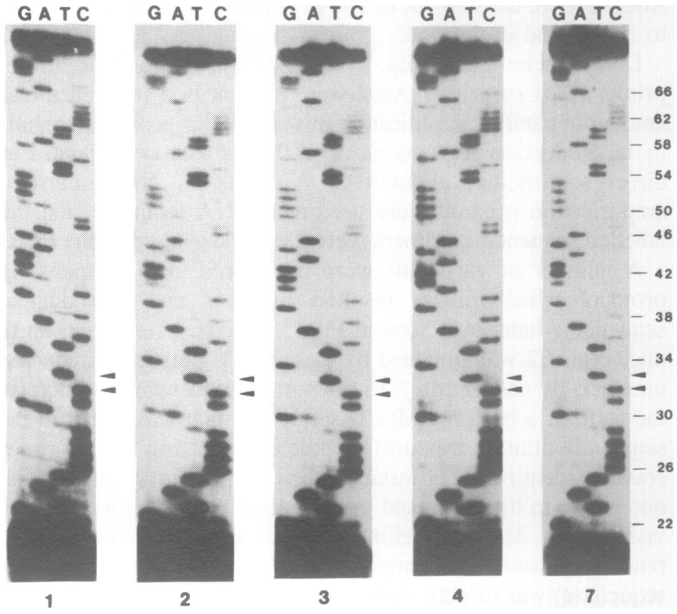

Figure 3. Direct sequencing of ssDNAs generated by asPCR. DNA fragments 1 to 8 (Fig. 2) were amplified in a PCR reaction in which primers Asp1 and Asp2 were present at concentrations of 1 and 10 μM, respectively. For the sequencing reaction, labelled primer Asp1 was used. Samples of 2 μl were loaded on a 8% polyacrylamide/7M urea gel (20×40×0.04 cm). The numbers to the right indicate the nucleotide position in mature $tRNA^{Asp}$, the arrows point to the two positions which differ in $tRNA^{Asp}$ and its gene (Fig. 1). The numbers below the sequencing ladders indicate the DNA fragments eluted from the gel shown in Fig. 2.

(Fig. 1B) and is also a G in species 3 and 7. The species 1 and 2 comprise both G and A at this position in the sequencing ladder. On the opposite strand, position 41 is occupied by a C in all species including 1, 2 and 4 (not shown), implying that species 4 is not a variant $tRNA^{Asp}$ gene. Such sequence aberrations appear to be mistakes introduced during sequencing since in the direct analysis of total PCR products misincorporations by Taq polymerase should not significantly create a defined sequence population.

In the region of question, i.e., the nucleotide sequence of the anticodon loop (pos. 32 to 38), no ambiguous sequences were visible in any of the eight species. The nucleotide at position 32 is always a C, and the nucleotide at position 33 is always a T (Fig. 3).

DISCUSSION

The $tRNA^{Asp}$ genes from rat liver DNA which have been cloned and sequenced until now all contain a C_{32} and T_{33} next to the anticodon (4, 5). We have used a combination of polymerase chain reaction (PCR), single-strand conformation polymorphism (SSCP) and direct sequencing of PCR products in order to detect a variant allele of the $tRNA^{Asp}$ gene in rat liver DNA whose sequence would correspond to that of major rat liver $tRNA^{Asp}$. This tRNA carries a U_{32} and C_{33} (1).

The results presented here suggest that no such $tRNA^{Asp}$ gene exists in rat liver DNA supported by the following observations: i) We have amplified the $tRNA^{Asp}$ genes from genomic rat DNA by using oligodeoxynucleotide primers hybridizing to the ends of the tRNA coding sequence (Fig. 1A). Consequently, any $tRNA^{Asp}$ gene with the same ends but with different sequences in the region between the D-stem and T-stem, including the anticodon loop, should have been amplified. ii) We used the sensitive technique of separating ssDNAs by electrophoresis in non-denaturing gels (PCR-SSCP) in order to identify amplified $tRNA^{Asp}$ genes which differ in their nucleotide composition from the known one. However, none of the DNA fragments revealed an altered nucleotide sequence (Figs. 2 and 3). The different mobilities of some minor ssDNA species seen in Fig. 2 were obviously caused by a variant conformation of the major ssDNA species rather than by a different nucleotide composition. This behaviour is not completely unexpected for rat $tRNA^{Asp}$ which contains long stretches of G:C-pairs in three out of four arms (Fig. 1), leading to altered conformations after denaturation and renaturation. iii) All DNA fragments eluted from the non-denaturing gel (Fig. 2) were sequenced directly upon asymmetric PCR. One virtue of direct sequence analysis versus cloning of PCR products in M13 or pUC19 DNA is the simultaneous detection of wild-type and variant sequences in a given gene (10). This approach did not indicate the presence of traces of a variant sequence in the region around the anticodon of the $tRNA^{Asp}$ gene (Fig. 3).

The nucleotide sequence of rat $tRNA^{Asp}$ had originally been determined by limited formamide hydrolysis according to Stanley and Vassilenko (13) and subsequent two-dimensional thin-layer chromatography of 5'end-labelled nucleotides (1). We evaluated the sequence of a RNase T1 fragment (comprising nucleotides 31 to 34 of rat $tRNA^{Asp}$) by mobility shift analysis and confirmed that major $tRNA^{Asp}$ contains a U_{32} and C_{33} (Fig. 1C, D). Moreover, we identified a small amount of $tRNA^{Asp}$ with C_{32} and U_{33}, presumably the precursor of the major species (Fig. 1C, D). Taken together with the DNA sequencing data, these findings strongly indicate that $tRNA^{Asp}$ from rat liver and from rat ascites hepatoma is generated by editing of a pre-$tRNA^{Asp}$ with C_{32} and U_{33} to a $tRNA^{Asp}$ with U_{32} and C_{33}.

RNA editing is a recently described form of RNA processing which leads to post-transcriptional alterations of coding sequences. Since the initial report of RNA editing in the mitochondria of Trypanosoma (14), several examples of RNA editing in other RNA species, including human intestinal apolipoprotein B mRNA (15), plant mitochondrial (16–18) and chloroplast mRNA (19) have been described. In the case of mitochondrial mRNA editing displayed by a number of protozoa, U residues are posttranscriptionally inserted or deleted at multiple sites (14, 20), whereas in plant mitochondrial mRNAs C residues are converted to U (18, 21) and some U residues are exchanged to C (21, 22).

Another example of RNA editing has recently been claimed to occur in bovine liver. Only one locus was detected in bovine DNA which hybridized to a human selenocysteine tRNA gene probe (23). The bovine tRNA gene was sequenced and it was found that it differed in two and three positions, respectively, from the two known bovine selenocysteine tRNA isoacceptors (24). As a consequence of these and our findings, tRNA sequences should not necessarily be revised on the basis of a differing gene sequence.

RNA editing in plant mitochondria alters codons in the mRNA that specify amino acids which are conserved in the homologous proteins of other species (18, 21). Editing of mitochondrial mRNA has also been observed in silent codon positions and more recently it was shown that it creates translational stops in open reading frames coding for functional proteins (25).

The significance of the editing events in bovine selenocysteine tRNA and rat $tRNA^{Asp}$, respectively, is unclear. Most pyrimidine transitions include nucleotides which are not involved in maintaining the secondary or tertiary structure of the tRNA and might be regarded as 'silent' events. One exception, however, is the exchange of U_{33} to C_{33} in rat $tRNA^{Asp}$. The uridine at position 33 is highly conserved in all elongator tRNAs and is thought to play a key role in establishing the sharp turn of the polynucleotide chain in the anticodon region (26). Thus it remains open whether rat $tRNA^{Asp}$ is fully active in the translational process.

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