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A novel cloverleaf structure found in mammalian mitochondrial tRNA^{Ser} (UCN)

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ABSTRACT

Bovine mitochondrial tRNA^{Ser}(UCN) has been thought to have two U-U mismatches at the top of the acceptor stem, as inferred from its gene sequence. However, this unusual structure has not been confirmed at the RNA level. In the course of investigating the structure and function of mitochondrial tRNAs, we have isolated the bovine liver mitochondrial tRNA^{Ser}(UCN) and determined its complete sequence including the modified nucleotides. Analysis of the 5′-terminal nucleotide and enzymatic determination of the whole sequence of tRNA^{Ser}(UCN) revealed that the tRNA started from the third nucleotide of the putative tRNA^{Ser}(UCN) gene, which had formerly been supposed. Enzymatic probing of tRNA^{Ser}(UCN) suggests that the tRNA possesses an unusual cloverleaf structure with the following characteristics. (1) There exists only one nucleotide between the acceptor stem with 7 base pairs and the D stem with 4 base pairs. (2) The anticodon stem seems to consist of 6 base pairs. Since the same type of cloverleaf structure as above could be constructed only for mitochondrial tRNA^{Ser}(UCN) genes of mammals such as human, rat and mouse, but not for those of non-mammals such as chicken and frog, this unusual secondary structure seems to be conserved only in mammalian mitochondria.

INTRODUCTION

It is well known that almost all tRNAs have the cloverleaf secondary and L-shaped tertiary structures in common (1,2), in which some invariant nucleotides, such as GG in the D loop and TΨCR in the T loop, are necessary for holding the higher-ordered structures (3,4).

On the other hand, many animal mitochondrial (mt) tRNAs are thought to possess unusual tertiary structures as inferred from their gene sequences, because they often lack the invariant nucleotiodes necessary for forming the tertiary interactions (5,6). Most mitochondrial tRNAs are known to have no tertiary interaction between the D and T loops due to lack of GG and TΨCR sequences in the D and T loops, respectively (24). Higher animal mt serine tRNAs specific for the AGY codon [tRNA^{Ser}(AGY)] are known to lack the normal D arm (7–19), whose characteristic structure has been investigated at the RNA level (20–22). Most tRNAs of nematode mitochondria are thought to lack the T arm (16,23).

As bovine mt tRNA^{Ser}(UCN) has GG and TTCG sequences in its gene, the tRNA has been thought to possess a rather common tertiary structure except for the two unusual T-T mismatches at the top of the acceptor stem. We previously reported that bovine mt tRNA^{Ser}(UCN) had the D loop/T loop interaction, as judged by the results of NucleaseS1 digestion and the cooperative melting profile, although its melting temperature (Tm) was quite low (25). The fact that bovine mt tRNA^{Ser}(UCN) can form a ternary complex with bacterial elongation factor Tu and GTP (22) suggests that the acceptor stem should take a normal double-stranded helix. For these reasons, we wondered if the U-U mismatches are in fact present in the acceptor stem of tRNA^{Ser}(UCN), as had been supposed.

To answer this question, we have isolated mt tRNA^{Ser}(UCN) from bovine liver and determined its complete nucleotide sequence. We found that the two U residues were not present at the 5′-terminus. As it is very difficult to form the normal cloverleaf structure from the determined sequence of tRNA^{Ser}(UCN), we propose here a novel, but rather unusual cloverleaf secondary structure. The structure is reasonable on the basis of enzymatic probing using double strand-specific RNaseV1 (26).

We further point out that this unusual secondary structure could hold not only for bovine but also for other mammalian tRNA^{Ser}(UCN) genes.

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MATERIALS AND METHODS

Enzymes and chemicals

RNaseT1, RNasePhyM, RNaseV1 and T4 RNA ligase were purchased from Pharmacia, NucleaseP1 from Yamasa Shoyu, RNaseU2 from Sigma and *Neurospora crassa* endonuclease from Boehringer Mannheim. RNaseCL3 and RNaseT2 were obtained from Seikagaku Kogyo and *E. coli* alkaline phosphatase from Takara Shuzo. [5'-^{32}P]pCp and [γ-^{32}P]ATP were from Amersham and [^{14}C] L-Serine from NEN Research Products. T4 polynucleotide kinase and other chemical reagents were purchased from Wako Pure Chemical Industries.

Hybridization procedures

The dot hybridization assay method was performed as reported previously (25). An oligodeoxyribonucleotide (17mer), 5'-ATCATAACCTCTATGTC-3', complementary to the D arm region of the bovine mt tRNA^{Ser}(UCN) gene (see Fig. 1), was synthesized on an Applied Biosystems 391 DNA synthesizer, and labeled at the 5'-end with [γ-^{32}P]ATP using T4 polynucleotide kinase. This labeled oligodeoxynucleotide was used as the hybridization probe. The dot hybridization was performed at 41°C.

Preparation procedures of mt tRNA^{Ser}(UCN)

Total mt tRNA (tRNA^{Mix}) was extracted from mitoplast of bovine liver as described previously (25). Isolation of mt tRNA^{Ser}(UCN) from mt tRNA^{Mix} was carried out by the procedures described previously (25), using successive column chromatographies of BD-cellulose and RPC-5, and gel electrophoresis.

RNA sequencing

Nucleotide sequence of tRNA^{Ser}(UCN) was determined by the post-labeling method of Kuchino *et al* (27), and Donis-Keller's method (28) using RNase's T1, U2, PhyM and CL3 (29,30). Modified nucleotides were identified by two-dimensional thin layer chromatography using two solvent systems. Solvent System 1, isobutylic acid / 25% ammonia / water (66 : 1 : 33, by vol.) for the first dimension and isopropyl alcohol / 36% HCl / water (70 :15 : 15, by vol.) for the second dimension. Solvent System 2, isobutylic acid / 25% ammonia / water (66 : 1 : 33, by vol.) for the first dimension and 100ml of 0.1M sodium phosphate (pH 6.8), 60g of ammonium sulfate and 2ml of n-propyl alcohol for the second dimension (27).

Enzymatic probing

Mt tRNA^{Ser}(UCN) labeled with [^{32}P] at the 5'-end or at the 3'-end with [5'-^{32}P]pCp (10,000cpm each) containing 83 pmol carrier tRNA was digested with 0.01unit or 0.002unit of RNaseT2, essentially according to the method of Vary *et al* (31). The RNaseV1 digestion was carried out as described in the literature (26), using 0.07unit or 0.014unit of RNaseV1. Both of these digestions with RNases T2 and V1 were performed at 37°C for 10min. in 50mM Tris-HCl (pH 7.5) and 10mM MgCl2. Alkaline digestion was performed at 90°C for 9min. in 50mM sodium carbonate (pH 9.0) and 1mM EDTA. The digestion with *Neurospora crassa* endonuclease was performed at 55°C for 10min. in 2.5mg/ml endonuclease, 20mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol and 8M urea. The digested samples were immediately loaded on 15% polyacrylamide gel with 7M urea and 10% glycerol.

RESULTS

Isolation of bovine mt tRNA^{Ser}(UCN)

Mt tRNA^{Ser}(UCN) was isolated from bovine liver by the same procedure as reported previously (25). During purification tRNA^{Ser}(UCN) was detected by hybridization as well as aminoacylation assay. The hybridization probe, shown in Fig. 1, was synthesized by taking hybridization efficiency into

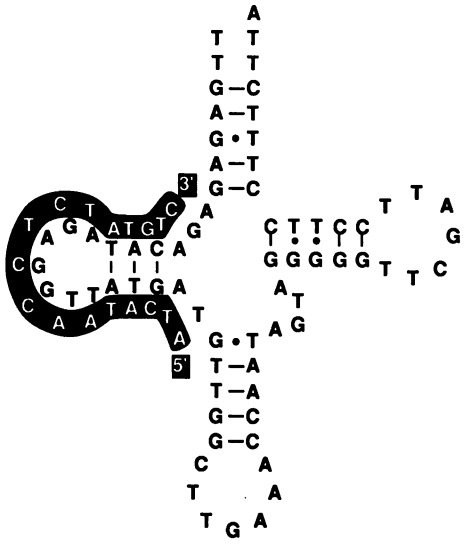

Figure 1. The putative cloverleaf structure of the tRNA^{Ser}(UCN) gene of bovine mitochondria proposed by Anderson *et al* (8). The oligodeoxyribonucleotide used for hybridization, which is complementary to the D arm region of tRNA^{Ser}(UCN), is shown by a black background.

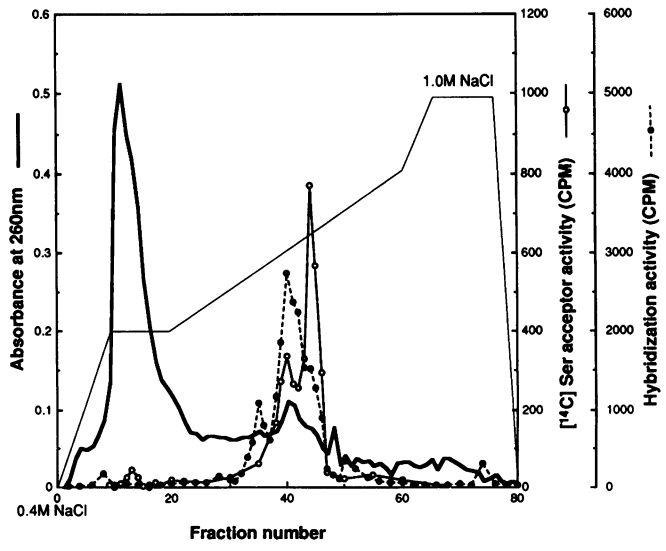

Figure 2. RPC-5 column chromatography of partially-purified bovine liver mt tRNA. 10 A260 units of tRNA^{Ser}(UCN)-rich fraction on BD cellulose column chromatography was applied onto a RPC-5 column (5mm×10 cm). A linear gradient of NaCl from 0.4 M to 1.0 M was used in elution with a buffer containing 10 mM Tris-HCl (pH 7.5) and 10 mM Mg(OAc)2. The thick solid line indicates the absorbance monitored at 260 nm, the line with open circles ^{14}C serine acceptor activity, the dashed line with closed circles hybridization activity measured by the Cherenkov method, and the light line the gradient profile of the NaCl concentration. 0.28 A260 unit of tRNA^{Ser}(UCN) was recovered from the fractions between numbers 43 and 46.

consideration (Kumazawa *et al.*, in preparation), and corresponds to a tRNA region different from that used before (25).

Fig.2 shows the elution profile of $tRNA^{Ser}$(UCN) by RPC-5 column chromatography. Fractions having high [^{14}C]Ser acceptor activity as well as showing hybridization activity with the [^{32}P]-labeled DNA probe were collected. $tRNA^{Ser}$(UCN) with the longest chain of $tRNA^{Ser}$'s was finally purified by 15% polyacrylamide gel electrophoresis with 7M urea and 10% glycerol.

Primary sequence of mt $tRNA^{Ser}$(UCN)

The 5'-terminal nucleotide of $tRNA^{Ser}$(UCN) purified above was determined to be guanosine-5'-phosphate, as shown in Fig. 3a, by conventional procedures (27). The nucleotide sequencing by Donis-Keller's method (28) indicates that the terminal G is the third base of the putative $tRNA^{Ser}$(UCN) gene (Fig. 3b), which had been proposed by Anderson *et al* (8).

The complete nucleotide sequence containing modified nucleotides was determined as shown in Fig. 3c, by the post-labeling method of Kuchino *et al* (27), by which most of the modified nucleotides, m^1A, Ψ, , m^3C and D could be identified and positioned. The only nucleotide which could not be identified is ms^2i^6A or i^6A at the position 3'-adjacent to the anticodon, because it migrates similarly on the two different chromatograms used in this study.

As it is very difficult to construct the normal cloverleaf structure from the deduced primary sequence, we propose here a novel, but rather unusual secondary structure for $tRNA^{Ser}$(UCN) as shown in Fig. 3c. The base numbering does not conform to the literature (29), but is given in order.

Enzymatic probing using RNaseV1 and RNaseT2

To see if the novel secondary structure proposed here is in fact the case, enzymatic probing was performed using double strand-specific RNaseV1 (26) and base non-specific RNaseT2.

Fig.4(a and b) shows the autoradiograms of 5'- and 3'-labeled $tRNA^{Ser}$(UCN)'s digested with various RNase's. The most susceptible sites towards RNaseV1 digestion were A10, U11, U63, U65 and U67 and the susceptible sites next to them were G3, G5−C9, A12, A19, U20, Ψ23−Ψ26 and C66. This result

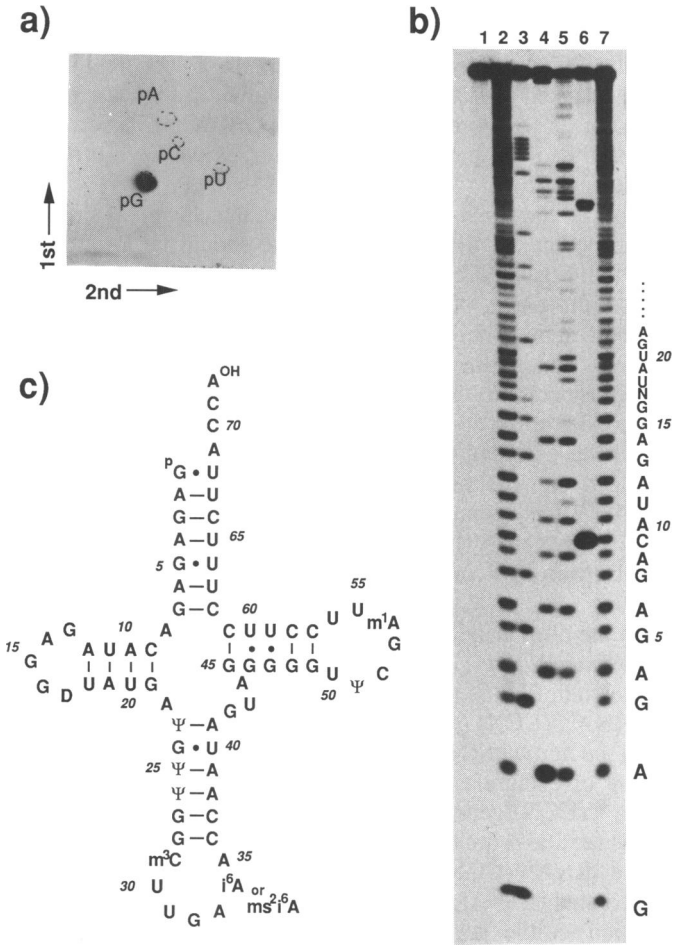

Figure 3. Analyses of the 5'-terminal nucleotide and a whole sequence of $tRNA^{Ser}$(UCN). a) Two dimensional thin layer chromatogram of the 5'-terminal nucleotide of $tRNA^{Ser}$(UCN), which was developed using System 1. The conditions for chromatography are described in Materials and methods. b) Enzymatic sequencing by the Donis-Keller method; lane 1, intact tRNA; lanes 2 and 7, alkaline digestion; lane 3, digestion with RNaseT1; lane 4, digestion with RNaseU2; lane 5, digestion with RNasePhyM; lane 6, digestion with RNaseCL3. c) The complete nucleotide sequence determined by the the post-labeling method of Kuchino *et al*. The base numbering is given in order.

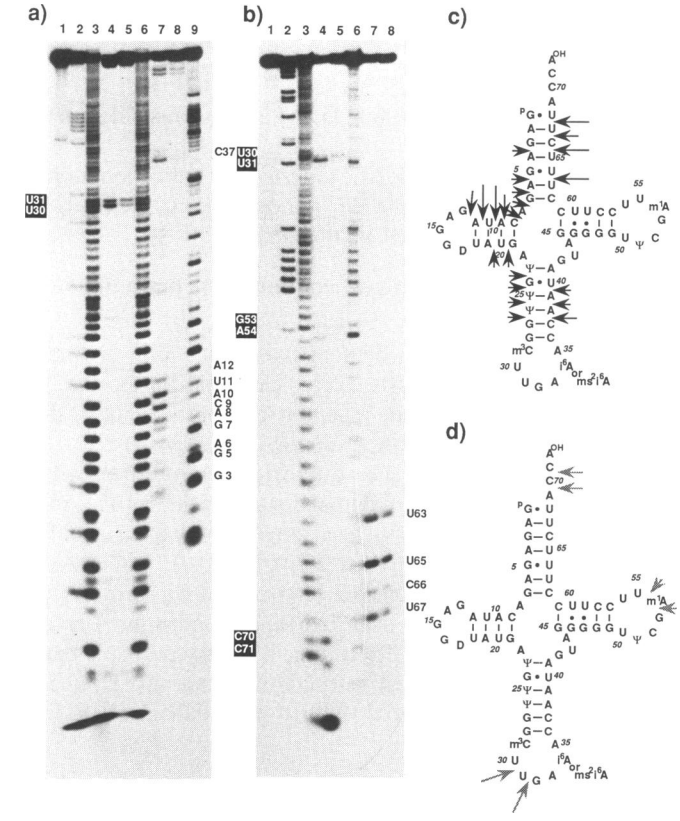

Figure 4. Enzymatic probing using RNaseV1 and RNaseT2. a) Limited digestion patterns of 5'-labeled $tRNA^{Ser}$(UCN); lane 1, intact tRNA; lane 2, digestion with RNaseT1; lanes 3 and 6, alkaline digestion; lane 4, digestion with 0.01 unit of RNaseT2; lane5, RNaseT2 0.002 unit; lane 7, digestion with 0.07 unit of RNaseV1; lane 8, RNaseV1 0.014 unit; lane 9, digestion with *Neurospora crassa* endonuclease. b) Limited digestion patterns of 3'-labeled $tRNA^{Ser}$(UCN); lane 1, intact tRNA; lane 2, digestion with RNaseT1; lane 3, alkaline digestion; lane 4, RNaseT2 0.01 unit; lane 5 RNaseT2 0.002 unit; lane 6 digestion with *Neurospora crassa* endonuclease; lane 7, RNaseV1 0.07 unit; lane 8, RNaseV1 0.014 unit. c) Digestion pattern with RNaseV1 on the cloverleaf structure of $tRNA^{Ser}$(UCN). Arrows indicate the cleavage points and the length of the arrow is proportional to the susceptibility towards RNaseV1 digestion. d) Digestion pattern with RNaseT2. Dark arrows indicate the cleavage points.

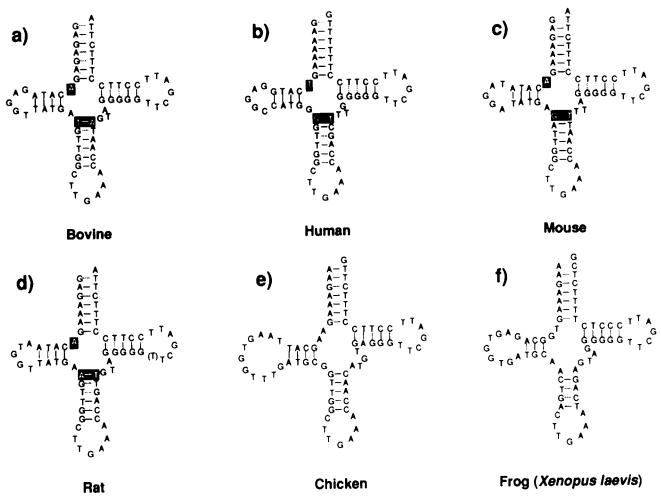

Figure 5. Comparison of the secondary structures of various vertebrate mt tRNA^{Ser}(UCN) genes (7,8,9,13,18,19,33). The cloverleaf structures of mammalian mt tRNA^{Ser}(UCN) genes are arranged as described in RESULTS. A black background shows a nucleotide characteristic to the cloverleaf structure proposed in this work. (T) in the T loop of the rat tRNA^{Ser}(UCN)gene in Fig. 5d shows the nucleotide lacking in the report of Gadaleta *et al* (18).

strongly supports the acceptor, D and anticodon stem structures shown in Fig. 4c.

On the other hand, RNaseT2 digested the anticodon loop and CCA end considerably, and the T loop to a lesser extent. This also supports the proposed secondary structure.

Novel cloverleaf structures common to mammalian mt tRNA^{Ser}(UCN) genes

This work has clearly demonstrated that the bovine mt tRNA^{Ser}(UCN) gene starts from G3, and lacks the U-U mismatches at the top of the acceptor stem of the putative tRNA gene which have so far been supposed to exist.

Fig. 5 shows that the cloverleaf structures of mt tRNA^{Ser}(UCN) genes of mammals, such as human (7), mouse (9) and rat (18, 33) could be rearranged so as to make similar structures to that of bovine mt tRNA^{Ser}(UCN), while those of non-mammals, such as chicken (19) and *Xenopus laevis* (13), were not able to undergo such rearrangement in the secondary structure of their tRNAs. Therefore, it is very probable that the novel, unusual cloverleaf structure found in bovine mt tRNA^{Ser}(UCN) is conserved only in mammalian mt tRNAs.

DISCUSSION

There have been only a few reports concerning structural or functional studies of mt tRNAs at the RNA level, in which mt tRNA^{Ser}(AGY) has mainly been studied due to ease of purification on account of its short chain length (20,21). There have been only two reports for tRNA^{Ser}(UCN) studied at the RNA level. One is the report that bovine mt tRNA^{Ser}(UCN) can form a ternary complex with bacterial EF-Tu (22) and GTP, and the other is our previous study indicating that the tRNA forms D loop/T loop interaction (25).

In the present work, we determined the primary sequence of bovine mt tRNA^{Ser}(UCN) containing modified nucleosides and found that guanosine was located at the 5′-terminus, which corresponded to the third nucleoside of the putative gene (Fig. 1). This result shows that mt tRNA^{Ser}(UCN) does not have the two U-U mismatches which have been thought to exist at the top of the acceptor stem, and its gene starts two nucleotides ahead. The RNaseV1 digestion pattern shows that the acceptor stem has 7 base pairs containing two G-U pairs, so that the stem is more stable than has been supposed.

Gebhardt-Singh and Sprinzl have pointed out that bovine mt tRNA^{Ser}(UCN) can form a ternary complex with bacterial EF-Tu and GTP (22), while footprinting studies have shown that bacterial EF-Tu interacts with the aminoacyl stem and T stem of tRNA (34,35). These findings support the validity of the new acceptor stem structure of mt tRNA^{Ser}(UCN) which we have proposed here. From RNaseV1 digestion patterns as shown in Fig. 4(a and b), it is most probable that the D stem is formed as shown in Fig.4c, so that only one nucleotide exists between the acceptor stem and the D stem.

For the present, we may not have sufficient evidence to affirm that the anticodon stem has 6 base pairs, because we have only the result with RNaseV1 digestion. If Y23-A41 does not exist at the top of the acceptor stem, there will be two bases between the D stem and the anticodon stem, which also results in an unusual cloverleaf structure different from those of most tRNAs.

RNaseT2 preferentially attacked the U turn in the anticodonloop and the CCA end, and slightly cleaved the T loop region, but never attacked any other regions, as shown in Fig. 4(a and b). Thus, it is supposed that mt tRNA^{Ser}(UCN) has a very compactly folded higher-order structure as we have proposed previously (25).

From all the results mentioned above, the cloverleaf structure of the bovine mt tRNA^{Ser}(UCN) gene should be arranged as shown in Fig. 5a. Cantatore *et al.* have already pointed out that mouse mt tRNA^{Ser}(UCN) would have a more reasonable acceptor stem if its gene started one nucleotide ahead (36). However, taking the homology with bovine mt tRNA^{Ser}(UCN) into consideration, one nucleotide at the 3′-end, as well as two nucleotides at the 5′-end of the mouse mt tRNA^{Ser}(UCN) gene should be excluded so that the cloverleaf structure would be arranged in a similar manner to that of the bovine mt tRNA^{Ser}(UCN) gene. Similar rearrangement could be carried out for mt tRNA^{Ser}(UCN) genes of human (7) and rat (18,33), and is probably common to mammalian mt tRNA^{Ser}(UCN) genes.

On the other hand, mt tRNA^{Ser}(UCN) genes of chicken (19) and *Xenopus laevis* (13) could have normal cloverleaf structures, as shown in Fig. 5e and f respectively. Especially, the *Xenopus* mt tRNA^{Ser}(UCN) gene has the invariant nucleotide U8 found in all the normal tRNAs. These observations suggest that in the course of evolution, the cloverleaf structure of vertebrate mt tRNA^{Ser}(UCN) genes acquired unusual features.

The next tasks are to determine the three-dimensional structure of the tRNA^{Ser}(UCN) having the unusual cloverleaf structure and to study the effects of this unusual tRNA structure on its function in the aminoacylation and also in the translation processes.

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