The T-loop region of animal mitochondrial tRNA\textsuperscript{Ser}(AGY) is a main recognition site for homologous seryl-tRNA synthetase

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ABSTRACT

Recognition sites of bovine mitochondrial serine tRNA specific for codons AGY (tRNA\textsuperscript{Ser} (AGY)) by the cognate mitochondrial seryl-tRNA synthetase were studied using a range of tRNA\textsuperscript{Ser}(AGY) variants which were obtained by the \textit{in vitro} transcription of synthetic tRNA genes with T7 RNA polymerase. Base replacements in the anticodon and discriminator sites did not affect serine acceptance. However, deletion and/or replacement in the T-loop region completely deprived the variants of their charging activities. Point mutation experiments in this region also showed that the adenosine residue in the middle of the T-loop (position 58), which is involved in tertiary interaction between the T-loop and the truncated D-arm (de Bruijn and Klug, 1983) played a significant role in the recognition process by the synthetase.

INTRODUCTION

The question as to how aminoacyl-tRNA synthetases recognize their cognate tRNAs has been one of the substantial problems in elucidating the recognition of nucleic acid by protein. Recently, studies in this field have made rapid progress by following two approaches; \textit{in vitro} aminoacylation assay of tRNA variants synthesized from the synthetic tRNA genes by T7 RNA polymerase, and amber suppression assay \textit{in vivo} with mutated tRNA genes (1-8). These studies have revealed that most tRNAs use the anticodon as the major or decisive recognition site for the aminoacylation reaction catalyzed by aminoacyl-tRNA synthetase (9-16).

On the other hand, a number of sequence analyses of animal mitochondrial genes during this decade have revealed that the structures of most tRNAs encoded by these genes deviate from the common structure of tRNA so far elucidated (18-28). Since all mitochondrial tRNAs possess a short variable loop, a recognition mechanism through a long variable arm would not be applied in these organelles (29). It should also be pointed out that animal mitochondrial serine tRNA isoacceptors are structurally different from other mitochondrial tRNAs; serine tRNA specific for codons AGY(Y = U or C) (tRNA\textsuperscript{Ser}(AGY)) lacks the D-arm considerably (18-20), whereas the isoacceptor for codons UCN (N = A, G, C or U) (tRNA\textsuperscript{Ser}(UCN)) have a normal interaction between the D-loop and T-loop via GG-T\={C} base pairings (30).

Mitochondrial aminoacyl-tRNA synthetases are encoded on nuclear DNA and imported into mitochondria (31,32). We described that specificities of mitochondrial aminoacyl-tRNA synthetases are different from those of cytoplasm (33). It has been considered to date that a single seryl-tRNA synthetase in mitochondria recognizes both of these structurally different tRNA isoacceptors. We have shown that this enzyme also aminoacylates \textit{E. coli} serine tRNAs with normal structures (34). Thus, the question arises as to how this enzyme recognizes various serine tRNAs with different structures. To obtain information on this question, we have studied the recognition sites of tRNA\textsuperscript{Ser}(AG-Y) variants synthesized from synthetic genes by T7 RNA polymerase. Here, we describe how the T-loop region, being topologically fixed by interaction with the truncated D-arm, is mainly involved in recognition by mitochondrial seryl-tRNA synthetase.

MATERIALS AND METHODS

Various oligodeoxynucleotides for the construction of tRNA genes were synthesized with a DNA synthesizer (Applied Biosystems 391). The oligonucleotides were ligated with one another and inserted into multi-cloning sites of pUC19 by the method of Sampson and Uhlenbeck (5) with slight modification. The nucleotide sequence of each plasmid thus constructed was
checked by the dideoxy-termination method (35). Template DNA was prepared from cells cultivated on a large scale or amplified by polymerase chain reaction. The template DNA was then completely digested by BstNI and subjected to run-off transcription reaction by T7 RNA polymerase, which was purified from an overproducing strain BL21/pAR1219, kindly provided by Studier et al. (5,36). Because of the sequence near the transcription start point, the yield of transcripts of the variants of tRNA\textsuperscript{Ser}(AGY) was markedly lower than that of E. coli variants. However, we were able to obtain 5 to 20 μg of transcripts, which was sufficient to determine the aminoacylation parameters in the reactions.

The synthesized RNA variants were purified by 15% non-denaturing gel-electrophoresis. Mitochondrial seryl-tRNA

<table>
<thead>
<tr>
<th>tRNA or transcripts</th>
<th>Km (μM)</th>
<th>Vmax/(pmole/sec)</th>
<th>Vmax/Km(relative)</th>
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<tbody>
<tr>
<td>Native tRNA\textsuperscript{Ser}(AGY) transcripts</td>
<td>173</td>
<td>2.98x10^{-2}</td>
<td>1.15</td>
</tr>
<tr>
<td>No mutation</td>
<td>171</td>
<td>2.56x10^{-2}</td>
<td>1.0</td>
</tr>
<tr>
<td>A1</td>
<td>552</td>
<td>4.32x10^{-2}</td>
<td>0.52</td>
</tr>
<tr>
<td>A2</td>
<td>232</td>
<td>5.24x10^{-2}</td>
<td>0.72</td>
</tr>
<tr>
<td>A3</td>
<td>56</td>
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<td>1.16</td>
</tr>
<tr>
<td>D</td>
<td>1082</td>
<td>4.84x10^{-2}</td>
<td>0.30</td>
</tr>
<tr>
<td>M1</td>
<td>381</td>
<td>5.17x10^{-2}</td>
<td>0.91</td>
</tr>
<tr>
<td>M2</td>
<td>432</td>
<td>5.01x10^{-2}</td>
<td>0.77</td>
</tr>
<tr>
<td>M3</td>
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<td>5.56x10^{-2}</td>
<td>1.67</td>
</tr>
<tr>
<td>M4</td>
<td>529</td>
<td>3.88x10^{-2}</td>
<td>0.49</td>
</tr>
<tr>
<td>M5</td>
<td>675</td>
<td>4.84x10^{-2}</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Km and Vmax values were determined from a Lineweaver-Burk plot. Vmax/Km was estimated from the slope of the linear plot of initial velocity versus RNA concentration. The Vmax/Km value is relative to that of the transcript having the native sequence.
synthetase was partially purified from mitochondrial fraction prepared from bovine liver as described previously (34). Aminoacylation reaction was carried out at 37°C in 20 μl of reaction mixture consisting of 100 mM Tris-HCl (pH 8.0), 5 mM MgCl\(_2\), 2 mM ATP, 10 mM KCl, 25 μM U-[\(^{14}\)C] L-serine (6.275 TBq/mol, Amersham) and 150–630 nM transcripts (34). The reaction was started by the addition of the enzyme. The Km values for native tRNA and L-serine were 170 nM and 23 μM, respectively. The transcript having an identical sequence to tRNA\(^{Ser}\)(AGY) except for t\(^{3}A23\) had the same aminoacylation activity as native tRNA\(^{Ser}\)(AGY) (see Table 1), which indicated that the experiments conducted to determine the aminoacylation parameters using the transcripts were worthwhile.

RESULTS

Substitution of anticodon and discriminator base

Since tRNA\(^{Ser}\) isoacceptors possessing two completely different anticodon sequences, GCU and UGA, are generally considered to be aminoacylated by a single seryl-tRNA synthetase, the anticodon may not be a decisive recognition site for the enzyme (1,4). In order to check whether this is also the case in bovine mitochondrial tRNA\(^{Ser}\)(AGY), substitution in the anticodon region was first performed (Fig. 1).

As shown in Table 1, none of the replacements of, (1) the anticodon middle position (C to A, the mutant transcript was named A1), (2) the anticodon trinucleotides (GCU to AAA, named A2), and (3) the 3′-neighboring base (A to G, named A3) removed serine acceptance, indicating that the anticodon region is not involved in recognition by seryl-tRNA synthetase.

Next, the discriminator base, which is implicated as an identity element for \(E.\ coli\) tRNA\(^{Ser}\), was changed from G73 to A (the transcript was named D); however, no significant decrease in the serine acceptance was observed (Table 1). Considering that isoacceptor tRNA\(^{Ser}\)(UCN) has A at the position, the discriminator base seems not to be important for the aminoacylation process of serine tRNAs. As a combination of these mutations (A2 + D) also had no effect on aminoacylation (data not shown), it was concluded that neither the anticodon nor the discriminator base is involved in the recognition.

Amino acid acceptor stem

Normany et al. have demonstrated that a part of the acceptor stem is one of the recognition sites in \(E.\ coli\) serine tRNAs (1,4). In serine tRNAs of prokaryote and eukaryotic cytoplasm, the 5′-strand of the acceptor stem is rich in purine residues, whereas the 3′-strand of the acceptor stem is rich in pyrimidine residues (29). Most of the mitochondrial serine tRNA genes have A-T pairs in the acceptor stem, as shown in Fig. 2. These facts led us to believe that the acceptor stem is involved in the recognition sites for seryl-tRNA synthetase.

In order to examine this assumption, a stretch of A-U base pairs present in the acceptor stem of tRNA\(^{Ser}\)(AGY) was changed to a stretch of C-G pairs step by step, as shown in Fig. 3, and the resulting variant transcripts were named M1–M5 according to the number of replaced base pairs. Although almost all of these variants had values smaller than 1.0 with regard to Vmax/Km, (Table 1), the reductions observed were not very great, indicating that the acceptor stem region is not the recognition site for seryl-tRNA synthetase.

Deletion of the T arm

Next, we synthesized mini-helices consisting of the acceptor and anticodon stems as well as a micro-helix consisting of the acceptor stem and the anticodon loop (37), as shown in Fig. 4, to examine the contribution of the T-arm and the anticodon stem to recognition. None of these variants showed any aminoacylation activity at all (data not shown), suggesting that the T-arm is involved in the recognition sites, or that disruption of the tertiary interaction between the T loop and the truncated D-arm (38) leads to a loss of affinity with seryl-tRNA synthetase. To examine these possibilities further, the following tRNA variants lacking the tertiary interaction were constructed and their charging activities analyzed.

Fig. 3. The acceptor stem of bovine mitochondrial tRNA\(^{Ser}\)(AGY) in which a stretch of A-U base-pairs was replaced by a stretch of C-G pairs step by step. The resulting variants were named M1 (single replacement), M2 (double), M3 (triple), M4 (four) and M5 (five replacements), respectively. The kinetic parameters of the variants are shown in Table 1.

Fig. 4. Two mini-helices (left and center) made by deleting the T arm of bovine mitochondrial tRNA\(^{Ser}\)(AGY) and a micro-helix (right) made by joining the acceptor stem to the anticodon loop. These variants showed no serine acceptor activity, although 20 pmole of each transcript was subjected to one assay mixture, as described in Materials and Methods. Their apparent relative Vmax/Km values were estimated to be less than 0.01 in comparison to that of the transcript without mutation.
Replacement of D-arm and T-loop

de Bruijn and Klug have proposed a tertiary structural model of tRNA\textsuperscript{Ser(AGY)} from human and bovine mitochondria based on chemical probing experiments (38), in which they assumed tertiary interaction is bound by several hydrogen bondings between the T loop and the truncated D loop, as shown by the dotted lines in Fig. 5.

In order to examine the involvement of tertiary interaction in recognition by seryl-tRNA synthetase, the UAU sequence in the truncated D arm and the AAUA sequence in the T loop were replaced by GGG and CCCC sequences, respectively (the former mutant was named Q1 and the latter Q2, as shown in Fig. 5). It is clear that although individual mutations lead to disruption of T loop/D loop interaction, a combination of both mutations (named Q3) is expected to restore the interaction. The results, however, were all negative; not only the Q1 and Q2 mutants but also the Q3 mutant showed no aminoacylation activity (data not shown). This observation strongly suggests that the D loop/T loop interaction is in some way related to recognition, but it may not be the recognition site itself. Rather, it serves to arrange the recognition site(s) in the proper position(s) necessary for synthetase recognition.

Replacement of the nucleotides involved in tertiary interaction

In order to examine this tertiary interaction in more detail, point mutations were introduced into the regions where the tertiary interactions take place. According to the model proposed by de Bruijn and Klug (38) nine nucleotide residues, U8, A9 and U10 in the truncated D loop, U46 in the variable loop, and U54, A57, A58, U59 and A60 in the T loop are involved in tertiary interaction. All of these nucleotides were replaced by others, as indicated in Fig. 6.

As shown in Table 2, mutations at U54 and/or A58 interacting with each other in the T loop led to a complete loss of serine acceptance in the variants (R4 means U54 changed to A54, R5 is A58 changed to U58 and R6 is a double mutation of R4 and R5). Thus, the interaction between these two nucleotides is important for recognition, which suggests that both U54 and A58 are involved in recognition sites. In particular, A58 is highly conserved in animal mitochondrial serine tRNAs, as shown in Fig. 2, so A58 may be a recognition site of serine tRNAs in most animal mitochondria.

Table 2. Kinetic parameters of tRNA variants in which nucleotides involved in the tertiary interaction were substituted (see Fig. 6).

<table>
<thead>
<tr>
<th>Transcripts</th>
<th>Km (µM)</th>
<th>Vmax(pmoles/sec)</th>
<th>Vmax/Km (relative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No mutation</td>
<td>171</td>
<td>2.56×10\textsuperscript{-2}</td>
<td>1.0</td>
</tr>
<tr>
<td>R1</td>
<td>184</td>
<td>1.59×10\textsuperscript{-2}</td>
<td>0.58</td>
</tr>
<tr>
<td>R2</td>
<td>61</td>
<td>5.73×10\textsuperscript{-3}</td>
<td>0.62</td>
</tr>
<tr>
<td>R3</td>
<td>52</td>
<td>4.31×10\textsuperscript{-2}</td>
<td>5.56</td>
</tr>
<tr>
<td>R4</td>
<td>ND</td>
<td>ND</td>
<td>&lt;0.01\textsuperscript{a}</td>
</tr>
<tr>
<td>R5</td>
<td>ND</td>
<td>ND</td>
<td>&lt;0.01\textsuperscript{a}</td>
</tr>
<tr>
<td>R6</td>
<td>ND</td>
<td>ND</td>
<td>&lt;0.01\textsuperscript{a}</td>
</tr>
<tr>
<td>R7</td>
<td>25</td>
<td>7.92×10\textsuperscript{-3}</td>
<td>2.09</td>
</tr>
<tr>
<td>R8</td>
<td>187</td>
<td>7.17×10\textsuperscript{-3}</td>
<td>0.26</td>
</tr>
<tr>
<td>R9</td>
<td>393</td>
<td>6.15×10\textsuperscript{-3}</td>
<td>0.10</td>
</tr>
<tr>
<td>R10</td>
<td>87</td>
<td>1.07×10\textsuperscript{-2}</td>
<td>0.84</td>
</tr>
<tr>
<td>R11</td>
<td>137</td>
<td>5.94×10\textsuperscript{-3}</td>
<td>0.28</td>
</tr>
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</table>

ND: activity was not detected, even if 40 pmole of the transcripts was subjected to the aminoacylation mixture (a).
magnitude for various mutations (R1–R3, and R7–R11). Thus, these residues do not seem to be involved in the recognition sites. In these mutants, D arm/T loop interaction is not completely lost as compared with the mutations of Q1 and Q2, which show no serine acceptance and in which the D arm/T loop interaction is completely disrupted. The sequences in the truncated D arm are not conserved in serine tRNAs of animal mitochondria (Fig. 2, 29), so only the formation of D arm/T loop interaction may be required for the aminoacylation reaction.

All these results suggest that both of the interactions, between the D arm and T loop, and between A58 and U54, play an important role in recognition by seryl-tRNA synthetase. It is likely that the synthetase recognizes the A58 residue only when its position is fixed by D arm/T loop interaction as well as by base-pairing with the U54 residue.

DISCUSSION

In this study we have demonstrated the significance of the T-loop region of mitochondrial serine tRNA, by homologous seryl-tRNA synthetase, using various tRNA transcripts. The same residues corresponding to A58 and U54 in tRNA(AGY) are also found in another isoacceptor tRNA(UCN), as m'A and U, respectively (39). Thus, it is most probable that both tRNA(AGY) and tRNA(UCN) have the same recognition site(s) at the same position.

Furthermore, sequence analyses of various mitochondrial genes have revealed that animal mitochondrial tRNA(UCN) appears to have conserved the invariant GG sequence in the D loop and the T+C sequence in the T loop, which are considered to participate in the D loop/T loop interaction. On the other hand, most other animal mitochondrial tRNAs have lost these sequences (29), suggesting that D loop/T loop interaction in these tRNAs has become unnecessary in the evolutionary process. Thus, the presence of highly conserved GG and TTC sequences in the D and T loops, respectively, involved in D loop/T loop interaction in animal mitochondrial tRNA(UCN)’s strongly suggests that this tertiary interaction plays a significant role in the biological function of serine tRNAs in the organella.

Moreover, the adenosine residue in the middle of the T loop (A58) is highly conserved in mitochondrial serine tRNAs, as shown in Fig. 2 (29). This suggests that this residue could be a recognition site of mitochondrial seryl-tRNA synthetase. An intriguing fact is that bovine mitochondrial seryl-tRNA synthetase is also able to aminoacylate E. coli tRNA with most amino acids including serine, indicating that this enzyme does not discriminate individual tRNAs of E. coli so strictly (34). This also suggests that these E. coli tRNAs possess recognition site(s) for mitochondrial seryl-tRNA synthetase.

The significance of the T-loop region of mitochondrial serine tRNAs for serine acceptor activity may also be supported by the fact that only two serine tRNA isoacceptors possess the T arm but all the other tRNAs lack this structure in nematode mitochondria, as inferred from the DNA sequences of Caenorhabditis elegans and Ascaris suum mitochondria (26, 27). This suggests that the T arm region of tRNA is not necessarily important for the translational process, but is indispensable for the serine acceptor activity of serine tRNAs in the organella.

Conservation of the T arm structure in serine tRNAs of all animal mitochondria strongly supports the view that the T arm serves as a major determinant in animal mitochondria.

E. coli serine tRNAs are tRNAs whose recognition sites were first investigated by using the amber suppressor (1); however, the recognition sites were not so simple as those of other tRNAs. Several regions, but not the anticodon have been considered to have a synergistic effect on the serine acceptor activity of serine tRNAs. It is most plausible that conformation of the D, T and variable loops together with the acceptor stem is important for the recognition by E. coli seryl-tRNA synthetase. Amongst these regions, the orientation of the long variable loop can be considered to be the most decisive recognition site in E. coli tRNA(AGY)’s (17). However, this cannot be the case for mitochondrial serine tRNAs, because they do not conserve this long variable loop (29). Considering that the recognition sites of serine tRNAs are different for various organisms, it may be concluded that the recognition sites of serine tRNAs are variable, and that the seryl-tRNA synthetase and serine tRNA isoacceptors may have co-evolved during the evolution of organisms.

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REFERENCES