Minimal $tRNA^{Ser}$ and $tRNA^{Sec}$ substrates for human seryl-tRNA synthetase: contribution of tRNA domains to serylation and tertiary structure

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Abstract The recognition process of $tRNA^{Ser}$ and $tRNA^{Sec}$ by human seryl-tRNA synthetase (SerRS) was studied using T7 transcripts representing defined regions of human $tRNA^{Ser}$ or $tRNA^{Sec}$ and the influence of the tRNA elements on serylation and tertiary structure was elucidated. The anticodon arms of both tRNAs showed no contribution to serylation in contrast to the acceptor stems and the long extra arms. D and T arms were only involved in formation of the L-shaped tRNA structure, not in the recognition process between tRNAs and SerRS. This is the first report of microhelices adapted from human tRNAs being aminoacylated by their homologous synthetase.

Key words: $tRNA^{Ser}$; $tRNA^{Sec}$; Seryl-tRNA synthetase; Microhelix; RNA structure; RNA structure probing

1. Introduction

The fidelity of protein biosynthesis is maintained by the correct interaction of aminoacyl-tRNA synthetases (AaRS) and their cognate tRNAs. The existing 20 different aminoacyl-tRNA synthetases are divided into two classes, each consisting of 10 members. Class I AaRS share two conserved sequence motifs HIGH and KMSKS and they contact the acceptor stems of their tRNAs at the minor groove site. The characteristics of class II AaRS are one or more of three structural motifs, contacting the acceptor stems of their tRNAs at the major groove site [1,2].

The overall three-dimensional structure of tRNAs consists of the acceptor-T arm axis and the anticodon-D arm axis in a nearly 90° angle to each other. This L-shaped structure involves tertiary interactions of nucleotides in the D and T loop. However, AaRS cannot use solely this L-shape to discriminate between single tRNAs. In addition they recognize typical structural and sequence peculiarities, called identity elements, of their cognate tRNAs. These elements are mostly present at the ends of the L-shape structure in the discriminator base position 73, in the acceptor arm, and in the anticodon loop, and less frequently in the extra arm [3–9]. For alanine [10,11], histidine [12] and serine [13,14] tRNAs, the anticodon sequence is not required for aminoacylation specificity; here the main identity elements are located in the acceptor stems and for serine additionally in the long extra arm.

Seryl-tRNA synthetase, a member of class II, is a very unusual enzyme compared with the other synthetases because of its substrate specificity. It not only serylates the four $tRNA^{Ser}$ isoacceptors but also the selenocysteine-incorporating $tRNA^{Sec}$. These tRNAs differ in sequence and structure, but share one structural element, a distinctive stem-loop structure located between the anticodon stem and the T stem called the long extra arm. $tRNA^{Ser}$ and $tRNA^{Sec}$ both fold into the L-shape with the long extra arm uncoupled from the rest of the molecule [15–17]. $tRNA^{Sec}$, however, is unique among all known tRNAs. Eukaryotic $tRNAs^{Sec}$ have a 6 bp long D stem instead of 3 or 4 bp in normal tRNAs, the acceptor stem is composed of 9 bp and the T stem of 4 bp [16,17]. Human $tRNA^{Sec}$ is serylated 10-fold less efficiently than $tRNA^{Ser}$; the possible reasons for this difference were discussed earlier [18].

Cusack et al. [19] have studied the tRNA-enzyme interactions in crystals of *Thermus thermophilus* SerRS with $tRNA^{Ser}$. As expected for class II synthetases, the motif 2 loop of the enzyme interacts with the major groove site of the acceptor stem of the tRNA. Here it is a ring-ring interaction of Phe^{262} of SerRS with U68 and C69 of the tRNA. Also, the N2 exocyclic amino group of the discriminator base G73 is in position to hydrogen bond with Glu^{258} of the enzyme, and several other interactions support the recognition process between the acceptor stem of the tRNA and SerRS [19]. The backbone of the long extra arm of $tRNA^{Ser}$ interacts with the α-helical coiled-coil arm of the synthetase [20].

Studies with seryl-tRNA synthetase and $tRNA^{Ser}$ of *Escherichia coli* implied that the acceptor stem, the long extra arm and the D stem of $tRNA^{Ser}$ are important for serine identity [13,21]. Further experiments showed that the long extra arm of $tRNA^{Ser}$ makes the largest contribution to aminoacylation efficiency and that the acceptor stem is the second most important domain for recognition by SerRS [14]. For the acceptor stem of *E. coli* $tRNA^{Ser}$, base pairs 1:72 through 5:68 were identified to be recognized by *E. coli* SerRS, with the major identity elements clustered between positions 2:71 and 4:69 [22].

We have shown recently that the major identity elements for serylation of human $tRNA^{Ser}$ and $tRNA^{Sec}$ with homologous SerRS are the acceptor stem including the discriminator base G73 and the long extra arm [23,24]. In another class II tRNA, such as $tRNA^{Leu}$, which also has a long extra arm, the replacement of the leucine-specific discriminator base A73 by the serine-specific G73 converts leucine to serine acceptance [25]. Especially the exocyclic 2-amino group of G73 is absolutely required for serylation [26]. This is strong evidence for the importance of the long extra arm and of the acceptor stem for the recognition process between human $tRNA^{Ser}$/$tRNA^{Sec}$ and SerRS.

It is of great interest to further investigate the contribution of discrete domains of human $tRNA^{Ser}$ and human $tRNA^{Sec}$ to the recognition by human SerRS. For this purpose we have

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synthesized several RNAs representing defined domains of $tRNA^{Ser}$ and $tRNA^{Sec}$, and determined their in vitro aminoacylation with SerRS, using HeLa S100 extract as synthetase preparation. For a better understanding of the recognition process, we have used chemical structure probing of $tRNA^{Ser}$ and $tRNA^{Sec}$ derivatives in order to find out whether the intact L-shape or the D-T loop interaction is essential for serylation. This is the first report of aminoacylation of mini- and microhelix substrates derived from human tRNAs with homologous aminoacyl-tRNA synthetase.

2. Materials and methods

2.1. Materials

L-[^{3}H]Serine (1.04 TBq/mmol) and [γ-^{32}P]ATP (110 TBq/mmol) were purchased from Amersham-Buchler (Braunschweig, Germany) and Hartmann Analytics (Braunschweig, Germany). T7 RNA polymerase was prepared from an overproducing strain of *E. coli* kindly provided by Dr. F.W. Studier, with a protocol adapted from Weber and Gross [27]. HeLa cytoplasmic S100 extract (5.5 mg/ml), prepared and dialyzed as described by Dignam et al. [28], was used for aminoacylations since pure human SerRS is not yet available. DMS was from Aldrich (Steinheim, Germany), DEPC from Serva (Heidelberg, Germany), hydrazine from Merck (Darmstadt, Germany) and aniline from Riedel-de Haen (Seelze, Germany). All other enzymes and reagents were obtained from commercial suppliers.

2.2. Construction of tDNA clones

The templates encoding $tRNA^{Ser}$ with a UGA anticodon [24] and $tRNA^{Sec}$ [18], containing the T7 promoter and a *Bst*NI restriction site, have been described earlier [25]. All mutated templates derived from $tRNA^{Ser}$ or $tRNA^{Sec}$ were constructed by PCR-based mutagenesis using appropriate synthetic oligodeoxynucleotides as primers [29] and were cloned into pUC19. The templates coding for the mini- and microhelices were directly cloned in pUC19, using full-length oligodeoxynucleotides, including the T7 promoter and the *Bst*NI restriction site. The sequences of all constructs were confirmed by dideoxy sequencing [30]. 'U' mutants are $tRNA^{Sec}$ derivatives, 'S' mutants derive from $tRNA^{Ser}$.

2.3. Preparation of tDNA transcripts

Transcription of *Bst*NI-linearized tDNA templates with T7 RNA polymerase yielded unmodified tRNAs, which were used for aminoacylation studies and chemical structure probing. The conditions for transcription with T7 RNA polymerase were as described by Achsel and Gross [23]. The resulting tRNAs, containing a correct 3′ terminus (CCA) and a 5′ triphosphate, were purified by gel electrophoresis, eluted from gel slices with NH_4OAc buffer [31] and precipitated. tRNAs and 3′-labelled tRNAs for all following experiments were denatured by heating for 5 min to 65°C in 5 mM $MgCl_2$ and cooling slowly (20 min) to room temperature.

2.4. In vitro aminoacylation

Aminoacylation was performed at 37°C in 36 μl containing 100 mM Tris-HCl (pH 7.6), 20 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 0.5 mM CTP, 0.5 mM DTT, 3.6 μl cytoplasmic HeLa S100 extract and 5 μM serine (including 2.5 μM [^{3}H]serine) or 15 μM [^{3}H]serine (for tRNA mutants with very low aminoacylation efficiency). tRNA concentrations ranged from 0.3 to 1.5 μM or 15 μM (for those with very low aminoacylation efficiency). Aliquots of 6 μl were transferred onto pieces of glass fiber papers which were washed on ice with 10% and twice with 5% trichloroacetic acid and three times with ethanol to remove free [^{3}H]serine. Radiolabelled aminoacyl tRNA was then quantitated by liquid scintillation counting. Apparent K_M and V_{max} values were obtained by Lineweaver-Burk analyses of the initial rates by using five different tRNA concentrations [25]. For mutant tRNAs with very low aminoacylation efficiency, aminoacyl tRNA formation after 25 min reaction time was measured and percent product formation was calculated relative to the amount of input RNA.

2.5. Chemical structure probing

tRNAs were labelled with [^{32}P]pCp at their 3′ ends [32]. Modification at N-3 of cytosine with DMS and N-7 of adenine with DEPC were performed under native, semi-denaturing and denaturing conditions, respectively. Methylated cytosine bases were cleaved with hydrazine at 0°C. Chain scission was induced by aniline at 60°C [33]. Control experiments were performed without DMS or DEPC, but including the same treatment with hydrazine and aniline, respectively. Additionally, 3′-labelled tRNAs were used for acid cleavage to generate a ladder for counting nucleotides, and a partial digest with RNase T1 was performed under denaturing conditions [32]. Reaction products were analyzed by gel electrophoresis on 10% polyacrylamide/8 M urea gels and visualized by autoradiography.

3. Results

3.1. Design of $tRNA^{Ser}$ and $tRNA^{Sec}$ derivatives

To investigate the contribution of discrete domains of human $tRNA^{Ser}$ and $tRNA^{Sec}$ towards the recognition by human SerRS and to stabilization of the L-shape, we have constructed several mutants of $tRNA^{Ser}$ and $tRNA^{Sec}$ (Fig. 1). S-A and U-A represent the tRNA structure without anticodon arm, S-E and U-E are constructs with a short extra arm derived from a class I tRNA extra arm consensus, and S-D and U-D lack the D arm. For the other variants more than one tRNA domain has been deleted: S-A/E and U-A/E have no anticodon and extra arm, and S-D/A, U-D/A are lacking the D and anticodon arm. SMini and UMini are composed of the acceptor and T stem, the helix being closed with the T loop, thus comprising one axis of the L-shaped tRNA structure. The minimal domains of tRNAs used for aminoacylation are the acceptor stems; the corresponding constructs are called SMicro and UMicro, respectively. These stems were extended by an additional C:G base pair in order to mimic the structural stability of the mature domain, and were connected by the well-characterized UUCG tetraloop [34].

3.2. Aminoacylation kinetics of wildtype $tRNA^{Ser}$, $tRNA^{Sec}$, and their variants without anticodon arm (S-A, U-A)

The initial serylation rates of the tRNA variants $tRNA^{Ser}$, $tRNA^{Sec}$, S-A, and U-A were determined by aminoacylation in a HeLa S100 extract as described in Section 2. According to our recent studies [18] the serine acceptance (relative V_{max}/K_M) of $tRNA^{Sec}$ showed a 10-fold reduction as compared with $tRNA^{Ser}$ (Table 1). In order to focus on the contribution of different tRNA domains towards the recognition by SerRS, the influence of the anticodon arm in the mutants S-A and U-A was investigated. Deletion of this domain caused only very little effect on serine acceptance. K_M increases clearly in the case of S-A and U-A (Table 1) compared with the wildtype

Table 1
Serylation kinetics of unmodified $tRNA^{Ser}$ and $tRNA^{Sec}$ and derivatives

Substrate	App. K_M (μM)	App. V_{max} (μM/min)	V_{max}/K_M (1/min)	Rel. V_{max}/K_M ($tRNA^{Ser}$ = 1)
$tRNA^{Ser}$	0.54	2	3.7	1
S-A	1.1	1.5	1.4	0.38
$tRNA^{Sec}$	3.3	1.25	0.4	0.1
U-A	4	1.1	0.3	0.08

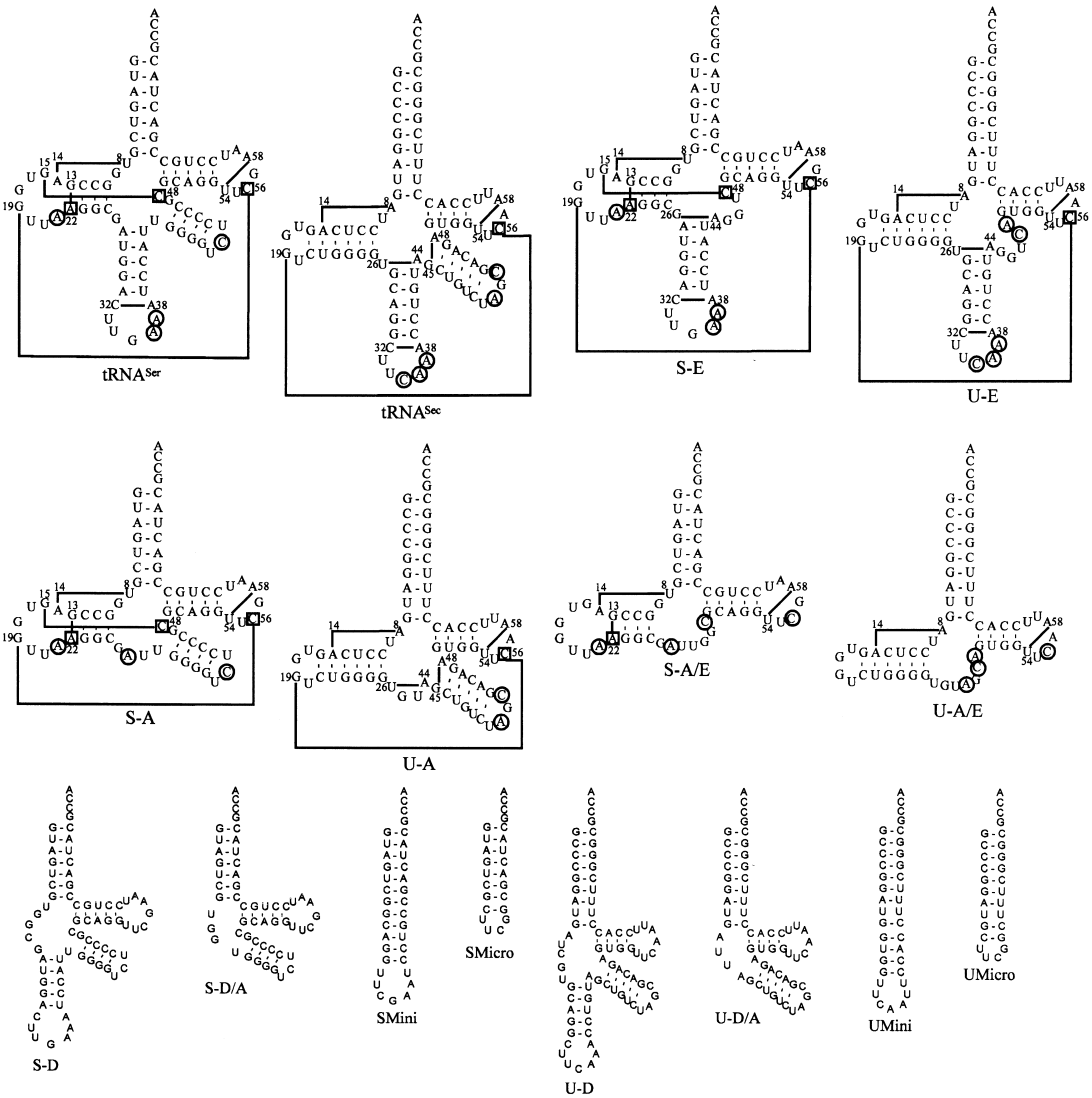

Fig. 1. Secondary structure of human tRNA^{Ser} and tRNA^{Sec} and their derivatives produced by deletion of secondary structure domains. Tertiary interactions identified by chemical structure probing are indicated by lines. Adenines reactive towards DEPC and cytosines towards DMS under native conditions are marked: circles = strong, squares = weak reactivity. 'S' and 'U' identify derivatives of tRNA^{Ser} and tRNA^{Sec}, respectively.

tRNAs; the small difference in the relative V_{max} indicates that the recognition of S-A and U-A is slightly hampered, but the rate of product formation (Table 2) is nearly the same as for the wildtype tRNAs. Therefore, whenever a productive interaction of the anticodon arm deficient mutants with the synthetase has occurred, the enzymatically catalyzed reaction takes place. Thus we conclude that the anticodon arm of human tRNA^{Ser} and tRNA^{Sec} is not a recognition element for human SerRS. The influence of anticodon arm deletion on the overall tRNA structure will be discussed later.

3.3. *Deletion of any but the anticodon domain of tRNA^{Ser}/tRNA^{Sec} causes a dramatic loss of serine acceptance*

All tRNA^{Ser} and tRNA^{Sec} mutants except the S-A/U-A variants are almost devoid of serine acceptance and it was impossible to determine the kinetic parameters K_M and V_{max} with HeLa S100 extract as synthetase preparation; thus, for those future experiments, pure human SerRS, which is currently not available, will be essential. To allow a comparison between the serine aminoacylation efficiency of the remaining mutants, the relative product formation after 25 min reaction time was measured (Table 2). Almost no serine acceptance was found for the derivatives with a short extra arm, S-E (0.22%) and U-E (0.57%). For S-A/E (0.21%) and U-A/E (0.22%) the aminoacylation efficiency was as low as for S-E and U-E.

The deletion of the D arm alone of tRNA^{Ser} and tRNA^{Sec} (S-D, 4.4%, and U-D, 1.8%), or deletion of the D arm in combination with the anticodon arm (S-D/A, 3.31%, and U-D/A, 2.42%) creates mutants which are better substrates for SerRS than those discussed above, even though they do not have the L-shaped structure due to the missing D-T loop interactions.

The derivatives SMini (1.33%), UMini (1.57%), SMicro (1.31%) and UMicro (1.33%) showed a detectable serine acceptance, which is nearly identical for these four substrates. These results lead to the assumption that the minimal required

Table 2
Product formation

Substrate	Product formation (nM)	% Product formation
S-D	660	4.4
S-E	33	0.22
S-D/A	497	3.31
S-A/E	31	0.21
SMini	199	1.33
SMicro	196	1.31
U-D	270	1.8
U-E	86	0.57
U-D/A	363	2.42
U-A/E	33	0.22
UMini	236	1.57
UMicro	199	1.31
$tRNA^{Ser}$	999.99	99.99
S-A	958.30	95.83
$tRNA^{Sec}$	672.12	67.21
U-A	606.15	60.62

The concentration of RNA in the assay was 1 μM (= 100%) for $tRNA^{Ser}$, S-A, $tRNA^{Sec}$ and U-A and 15 μM (= 100%) for the remaining derivatives. Product formation was measured after 25 min reaction time.

identity element for aminoacylation of $tRNA^{Ser}$ and $tRNA^{Sec}$ comprises the acceptor stem and the discriminator base.

3.4. Chemical structure probing of the tRNA derivatives containing a D and T arm

We focussed our interest on most of the tertiary interactions maintaining the L-shape of $tRNA^{Ser}$ and $tRNA^{Sec}$. For this analysis we chose the modification at N-3 of cytidine with DMS and N-7 of adenine with DEPC under native, semi-denaturing and denaturing conditions [33].

Data of wildtype $tRNA^{Ser}$ and $tRNA^{Sec}$ were obtained in order to compare the results of the chemical structure probing of $tRNA^{Ser}$ and $tRNA^{Sec}$ derivatives (Fig. 1). $tRNA^{Ser}$ folds into a tertiary structure built by the following interactions: the Hoogsteen pairs U8:A14 and U54:A58, the *trans* G15:C48 pair, the purine-purine pair G13:A22, and the G19:C56 base pair. In the anticodon loop C32 and A38 are protected under native condition, possibly because they form an unusual base pair or because they are part of a magnesium-binding site [35,36]. For $tRNA^{Sec}$ the following interactions were detected: the Hoogsteen pairs A8:A14 and U54:A58, the base pair G19:C56, the standard U26:A44 base pair, and the *cis* Watson-Crick G45:A48. C32 and A38 showed the same reactivity as in $tRNA^{Ser}$. $tRNA^{Ser}$ derivatives S-E and S-A and $tRNA^{Sec}$ derivatives U-E and U-A formed the same tertiary interactions as described for the wildtype tRNAs and for S-E the additional base pair G26:A44 was detected which was not visible in the wildtype $tRNA^{Ser}$ sequence because of the U at position 44. Taken together, these results support an L-shaped model for S-E and U-E and D-T loop interaction for S-A and S-E. In contrast to these results, no D-T loop interactions were detected for the derivatives S-A/E and U-A/E. S-A/E shows only the U8:A14 and U54:A58 Hoogsteen base pairs and the anomalous purine-purine pair G13:A22. Only two tertiary interactions, the Hoogsteen base pairs A8:A14 and U54:A58, occur in the native structure of U-A/E.

4. Discussion

To advance our knowledge about the contribution of human $tRNA^{Ser}$ and $tRNA^{Sec}$ domains to homologous SerRS recognition, we constructed several tRNA derivatives. Since pure human SerRS is not yet available, HeLa S100 extract was employed for these studies. In order to obtain structural data for $tRNA^{Ser}$/$tRNA^{Sec}$ derivatives, we used modification with DMS at N-3 of cytosine and with DEPC at N-7 of adenine, which allowed the examination of important tertiary structural features. We have obtained similar results compared to those investigated for yeast $tRNA^{Ser}$ [15] and eukaryotic $tRNA^{Sec}$ [16,17] for the tertiary features of human $tRNA^{Ser}$ and $tRNA^{Sec}$ (Fig. 1).

We have now determined that the contribution of the anticodon arm of $tRNA^{Ser}$ or $tRNA^{Sec}$ to SerRS recognition is negligible. The same conclusion has been drawn for the *E. coli* $tRNA^{Ser}$-SerRS recognition, where the deletion of the anticodon arm creates no decrease of serylation [14]. The strongest evidence that the anticodon arm is no identity element for SerRS comes from the crystal structure of complexed *T. thermophilus* $tRNA^{Ser}$-SerRS. There the anticodon arm was not part of the contact regions between tRNA and SerRS [19,20]. As we show here, even for formation of the D-T loop interactions of $tRNA^{Ser}$ and $tRNA^{Sec}$ the anticodon arm is not necessary (Fig. 1).

The extra arm is one of the most important identity elements of human $tRNA^{Ser}$ and $tRNA^{Sec}$ [23,24]. The specific interaction of *E. coli* and *T. thermophilus* SerRS with their cognate $tRNA^{Ser}$ depends on the recognition of the backbone of the long extra arm of $tRNA^{Ser}$ by the remarkable α-helical coiled-coil (helical arm) of the synthetase [14,19,20]. The tRNA derivatives S-E and U-E show almost no serine acceptance, even though they contain an L-shaped structure and the other main identity element of the acceptor stem. Thus, we propose that human SerRS contains a similar structure for the specific recognition of the typical long extra arm element of human $tRNA^{Ser}$ and $tRNA^{Sec}$. The two mutants S-E and U-E exhibit the same overall structure as class I tRNAs. This may indicate that human SerRS possibly excludes class I tRNAs, i.e. the majority of the tRNAs, in the first step of the recognition process. Small $tRNA^{Ser}$ and $tRNA^{Sec}$ derivatives, which cannot form an L-shaped structure, are still aminoacylated if they contain the long extra arm as in S-D, U-D, S-D/A and U-D/A. Like S-E and U-E, the derivatives S-A/E and U-A/E show almost no serine acceptance. Surprisingly, structure probing revealed that the D and T loops cannot interact with each other if both the extra arm and the anticodon arm are absent, indicating that the remaining stem loops of these mutants possibly interfere with acceptor stem recognition by SerRS. In these derivatives special interactions compared to the wildtype tRNAs, which may be important to form the G19:C56 interaction, were missing. In S-A/E no G15:C48 base pair was detectable and in U-A/E no U26:A44 base pair, therefore the G26:U44 base pair in S-A/E is possibly also absent. The G15 is paired with C48 in S-E and S-A and the G26:A44 base pair in S-E and possibly the G26:U44 base pair exists in S-A. In U-E and U-A the U26:A44 base pair is detectable, suggesting a possible involvement of the 26:44 base pair and of the G15:C48 base pair in a correct D-T loop interaction (Fig. 1).

To investigate the role of the acceptor stem, the other important identity element of $tRNA^{Ser}$/$tRNA^{Sec}$ for SerRS recognition, mini- and microhelices of the two tRNAs were constructed and their serine acceptance was determined. For these

minimal substrates of human SerRS the acceptor stem including the discriminator base is the only required identity element. However, any microhelix with G in the discriminator base position does not act as a substrate for human SerRS, since we have attempted to serylate a microhelix derived from human $tRNA^{Leu}$ with the serine-specific G at the discriminator base position with no detectable aminoacylation [37]. Nevertheless this G is responsible for a specific destabilization of the G1:C72 base pair, which is identical in $tRNA^{Leu}$, $tRNA^{Ser}$ and $tRNA^{Sec}$. Possibly structural features, more than sequence elements of the acceptor stem, are important for the recognition by human SerRS, in contrast to the results obtained for the *T. thermophilus* and *E. coli* $tRNA^{Ser}$-SerRS interaction. These structural elements are not only present in the first base pair, but also in the remaining acceptor stem. Sequence comparisons between the acceptor stems of human $tRNA^{Ser}$, $tRNA^{Sec}$ and $tRNA^{Leu}$ show that only the 4:69 base pair varies between all three tRNAs; in $tRNA^{Ser}$ it is a G:C, in $tRNA^{Sec}$ a C:G and in $tRNA^{Leu}$ an A:U pair. For *E. coli* $tRNA^{Ser}$ minihelices, the 4:69 base pair is the major recognition element in the acceptor stem with G:C and A:U showing the best serylation results, whereas U:A and C:G decrease serine acceptance [22]. For human $tRNA^{Ser}$/$tRNA^{Sec}$-SerRS interaction the variable 4:69 base pair cannot play a sequence-specific role as in *E. coli*. Possibly, this base pair is responsible for a structural feature in the microhelices of human $tRNA^{Ser}$/$tRNA^{Sec}$ which supports SerRS recognition, and the microhelix of the $tRNA^{Leu}$ mutant with G at the discriminator base position lacks this special structure.

Considering all of the above data, we propose the following model for tRNA recognition by human SerRS. The enzyme first screens the overall structure of the tRNAs present in the cell, excluding all class I tRNAs with short extra arms. Of the remaining class II tRNAs with long extra arm, $tRNA^{Leu}$ is rejected because of the A in position 73 and of an unfavorable acceptor arm structure. Finally, the serylation reaction is performed with the remaining tRNAs, $tRNA^{Ser}$ and $tRNA^{Sec}$, which have the appropriate acceptor stem structure and the correct discriminator base G.

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