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Antideterminants present in minihelix^{Sec} hinder its recognition by prokaryotic elongation factor Tu

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During protein biosynthesis, all aminoacylated elongator tRNAs except selenocysteine-inserting tRNA^{Sec} form ternary complexes with activated elongation factor. tRNA^{Sec} is bound by its own translation factor, an elongation factor analogue, e.g. the SELB factor in prokaryotes. An apparent reason for this discrimination could be related to the unusual length of tRNA^{Sec} amino acid-acceptor branch formed by 13 bp. However, it has been recently shown that an aspartylated minihelix of 13 bp derived from yeast tRNA^{Asp} is an efficient substrate for *Thermus thermophilus* EF-Tu·GTP, suggesting that features other than the length of tRNA^{Sec} prevent its recognition by EF-Tu·GTP. A stepwise mutational analysis of a minihelix derived from tRNA^{Sec} in which sequence elements of tRNA^{Asp} were introduced showed that the sequence of the amino acid-acceptor branch of *Escherichia coli* tRNA^{Sec} contains a specific structural element that hinders its binding to *T.thermophilus* EF-Tu·GTP. This antideterminant is located in the 8th, 9th and 10th bp in the acceptor branch of tRNA^{Sec}, corresponding to the last base pair in the amino acid acceptor stem and the two first pairs in the T-stem. The function of this C_7·G_{66}/G_{49}·U_{65}/C_{50}·G_{64} box was tested by its transplantation into a minihelix derived from tRNA^{Asp}, abolishing its recognition by EF-Tu·GTP. The specific role of this nucleotide combination is further supported by its absence in all known prokaryotic elongator tRNAs.

Keywords: antideterminant/G·U mismatch/protein RNA recognition/RNA engineering/selenocysteine

Introduction

During the elongation step of protein biosynthesis, aminoacylated tRNAs (aa-tRNAs) are carried to the A-site of codon-programmed ribosomes by activated elongation factor. Biochemical investigations suggest that the major elements of tRNAs required for interaction with prokaryotic elongation factor EF-Tu·GTP are contained within the tRNA amino acid-acceptor branch composed of the amino acid-acceptor stem and the T-stem and loop (Boutorin *et al.*, 1981; Wikman *et al.*, 1982, 1987; Joshi *et al.*, 1984). Recently, it was found that a minihelix derived from the amino acid-acceptor branch of yeast tRNA^{Asp} is an efficient substrate of *Thermus thermophilus* EF-Tu·GTP (Rudinger *et al.*, 1994) and that conserved residues and modified bases are not required for ternary complex formation (Harrington *et al.*, 1993; Nazarenko *et al.*, 1994; Rudinger *et al.*, 1994).

The recognition by activated elongation factor concerns all aminoacyl-tRNAs present in a cell, except initiator tRNA^{Met} and selenocysteine-specific tRNA^{Sec}, which have specific functions during protein biosynthesis and are recognized by their own translational factors. Initiator tRNA^{Met} binds directly to the ribosomal P-site in a process mediated by initiation factor (IF_2 for prokaryotes and eIF_2 for eukaryotes; reviewed by RajBhandary and Chow, 1995). tRNA^{Sec} donates its selenocysteine residue to the nascent polypeptide chain at particular UGA stop codons under the control of specific mRNA sequences. During this process, tRNA^{Sec} is recognized by a specialized elongation factor, the SELB protein (reviewed by Baron and Böck, 1995). To prevent undesired functional interferences during elongation, nature had to select unique structural features or antideterminants within these tRNAs that restrict their recognition by canonical elongation factors. Concerning initiator tRNAs, unique structural features absent in elongator tRNAs have been identified for their discrimination by elongation factors. In prokaryotes, an unusual C_1·A_{72} mismatch at the end of the amino acid-acceptor stem of tRNA^{fMet} is responsible for the weak interaction of this tRNA with EF-Tu·GTP (reviewed by RajBhandary and Chow, 1995), since mutants with a 1–72 Watson–Crick base pair are active in elongation (Seong and RajBhandary, 1987; Seong *et al.*, 1989). Initiator tRNA^{Met} from plants and fungi are excluded from the elongation process by the presence of a bulky 2′-phosphoribosyl modification at purine 64 in the T-stem (Kiesewetter *et al.*, 1990), since removal of this modification permits these molecules to form ternary complexes with GTP and EF-1α (Förster *et al.*, 1993; Aström and Byström, 1994). In contrast to initiator tRNAs, little is known about the antideterminants that prevent tRNA^{Sec} species from being recognized by elongation factors (Baron and Böck, 1991). Whereas SELB is an amino acid-specific elongation factor since it interacts with Sec-tRNA^{Sec} but not with Ser-tRNA^{Sec} (Forchhammer *et al.*, 1989), EF-Tu recognizes neither Ser-tRNA^{Sec} nor Sec-tRNA^{Sec} (Förster *et al.*, 1990).

Here, we address the question of the discrimination of prokaryotic *Escherichia coli* tRNA^{Sec} by EF-Tu·GTP and of the identification of the antideterminants responsible for this discrimination. At first glance one would guess the antideterminants are defined by an unusual secondary structure of tRNA^{Sec} (Figure 1A). The amino acid-accepting branch of *E.coli* tRNA^{Sec} has 13 bp instead of 12 in canonical tRNAs, an extra arm which is the longest known to date, and an unusual set of tertiary interactions

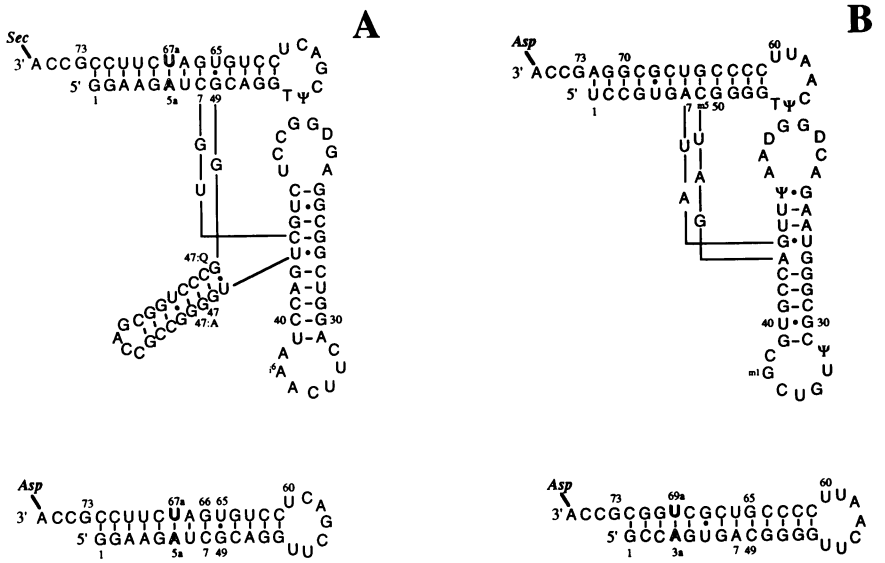

Fig. 1. Comparison of the structures of *E.coli* tRNA^{Sec} and the aspartylatable minihelix^{Sec} (**A**) with those of yeast tRNA^{Asp} and minihelix$^{Asp\text{-}13bp}$ (**B**). Sequence data on tRNA^{Sec} are from Schön *et al.* (1989) and on tRNA^{Asp} from Gangloff *et al.* (1971).

stabilizing its L-shaped structure (Schön *et al.*, 1989; Baron *et al.*, 1993). Removal of 1 bp from the amino acid-acceptor branch of this molecule allows the interaction with EF-Tu·GTP and prevents recognition by SELB (Baron and Böck, 1991). This led to the conclusion that the length of the amino acid-accepting branch is important for the discrimination between both translational factors. However, this discrimination does not occur in the case of minihelices corresponding to the amino acid-acceptor branch of yeast tRNA^{Asp}, since aspartylated minihelices containing either 12 or 13 bp are recognized with the same affinity by EF-Tu from *T.thermophilus* (Rudinger *et al.*, 1994) (Figure 1B). This suggests that other features are involved in the rejection process. While the antideterminants can conceptually correspond to either structural elements (e.g. conformations, tertiary interactions) or to combinations of nucleotides unique to the tRNA^{Sec}, we favour the hypothesis that they are found primarily within the first 10 bp of the amino acid-accepting branch of the tRNA corresponding to the helical domain in elongator tRNAs known to interact with EF-Tu·GTP (Rudinger *et al.*, 1994).

To test this hypothesis we used aminoacylated minihelices, either derived from *E.coli* tRNA^{Sec} or from yeast tRNA^{Asp}. The experiments became feasible since the minihelices can be efficiently aminoacylated by yeast aspartyl-tRNA synthetase (AspRS) (Frugier *et al.*, 1994). By mutational analysis of a minihelix derived from the amino acid-acceptor stem of *E.coli* tRNA^{Sec} we show that a particular base pair combination is responsible for the discrimination process. This conclusion was corroborated by a transplantation experiment in which the antideterminants present in tRNA^{Sec} were introduced into a 13 bp minihelix^{Asp}.

Results

Interaction of minihelices derived from tRNA^{Sec} and tRNA^{Asp} with EF-Tu·GTP

A minihelix^{Sec}, displayed in Figure 1A, corresponding to a RNA hairpin mimicking the amino acid-acceptor branch of *E.coli* tRNA^{Sec} and formed by the stacking of the 8 bp of the amino acid-acceptor stem over the 5 bp of the T-stem was prepared by *in vitro* transcription. This molecule possesses the discriminator base G_{73} and is thus expected to be efficiently aspartylated (Frugier *et al.*, 1994). Indeed, plateau levels up to 25% aminoacylation were found with yeast AspRS.

An estimate of a possible interaction of the aminoacylated minihelix^{Sec} with EF-Tu·GTP by two complementary experimental approaches was first performed. Spontaneous hydrolysis of the aspartate residue from minihelix^{Sec} was similar in the presence and in the absence of EF-Tu·GTP (data not shown), indicating that the RNA–protein complex is not formed to a significant extent. This conclusion was confirmed by fluorescence measurements in which a competition of Asp-minihelix^{Sec} for the binding of fluorescence-labelled Tyr-tRNA^{Tyr} to EF-Tu·GTP was determined. A K_d > 190 nM was obtained (Table I), showing that Asp-minihelix^{Sec} interacts only weakly with EF-Tu·GTP. As a control, under strictly identical conditions, deacylation protection and fluorescence titration experiments with Asp-minihelix^{Asp} containing 13 bp were performed. A significant protection of deacylation by EF-Tu·GTP was seen (Figure 2A) and correspondingly, a K_d value of 4 nM was calculated from the titration curve shown in Figure 2B. This is at least 45-fold lower than that for Asp-minihelix^{Sec} (Table I). It was verified, and already known (Rudinger *et al.*, 1994), that uncharged minihelices are poor competitors for recognition of charged minihelices by EF-Tu·GTP. Altogether these results indicate that the main features within tRNA^{Sec} accounting for the lack of interaction with EF-Tu·GTP are located within its acceptor branch. This implies that the antideterminant features have to be absent in all other elongator tRNAs.

Search of antideterminants

The strategy consists in studying the interaction with EF-Tu·GTP of various minihelices derived from tRNA^{Sec} in which sequence elements from tRNA^{Asp} were inserted. Minihelix^{Sec} was first roughly mutated with the expectation

Table I. Equilibrium dissociation constants (K_d) of Asp-minihelices with EF-Tu·GTP measured by fluorescence titration according to Ott *et al.* (1989)

Asp-minihelices	K_d (nM)	L[a]
Asp-13bp	4	1
Sec	190	45
Mutated minihelices^{Sec}		
Sec1(Asp)	5	1.3
Sec2(Asp)	136	34
Sec3(Asp)	49	12
Sec4(Asp)	10	2.5
Sec5(Asp)	10	2.5
Sec6(Asp)	37	9
Sec7(Asp)	100	25
Sec(shift)	5	1.3
Transplanted minihelix^{Asp}		
Asp1(Sec)	81	20
Asp2(Sec)	25	6

[a]Loss of affinity (L) relative to minihelix$^{Asp\text{-}13bp}$ (-fold) is defined as the ratio $K_{d\,(mutant)}/K_{d\,(minihelix\ Asp\text{-}13bp)}$. L-values for duplicated experiments varied by <20%; bp, base pairs.

that some of the variants will interact efficiently with EF-Tu·GTP and thus are deprived of antideterminant nucleotides. The mutations introduced in tRNA^{Sec} cover base pairs 2–10 (base pair 1 was not considered since both aspartate and selenocysteine minihelices contain a common $G_1 \cdot C_{72}$ pair). Depending on the results of the functional assays, further mutants will be designed to restrict the possibilities for antideterminant locations. Such process might be reiterated until a unique combination is established experimentally. Explicit proof of its functional role will be verified in a transplantation experiment in which the antideterminant box will be introduced in a tRNA^{Asp} minihelix which in turn should become rejected by EF-Tu·GTP.

Interaction of minihelix^{Sec} containing sequence elements of minihelix$^{Asp\text{-}13bp}$ with elongation factor Tu·GTP

In the first series of experiments, chimeric molecules derived from minihelix^{Sec} and containing 5 bp from minihelix$^{Asp\text{-}13bp}$, inserted either as continuous or discontinuous stretches were constructed (Figure 3A). (For the nomenclature of minihelices, see legend to Figure 3.) These minihelices can be aspartylated to plateau levels up to 20% by yeast AspRS. Their interaction with EF-Tu·GTP was determined using both hydrolysis protection and fluorescence titration assays. The two methods led to the same conclusions and data corresponding to fluorescence titration are summarized in Table I. Asp-minihelix$^{Sec2(Asp)}$ and Asp-minihelix$^{Sec3(Asp)}$ show high K_d values (136 and 49 nM) and thus, similarly to Asp-minihelix^{Sec}, are poor substrates for EF-Tu·GTP. In contrast, Asp-minihelix$^{Sec1(Asp)}$ and Asp-minihelix$^{Sec4(Asp)}$ are characterized by low K_d values (5 and 10 nM, respectively) and thus acquired the potential to be recognized by EF-Tu·GTP as this is the case for Asp-minihelix$^{Asp\text{-}13bp}$. These data are confirmed by strong protections against deacylation of the two latter minihelices while those with high K_d values are not, or only moderately, protected.

The second set of variant minihelices (Figure 3B) was

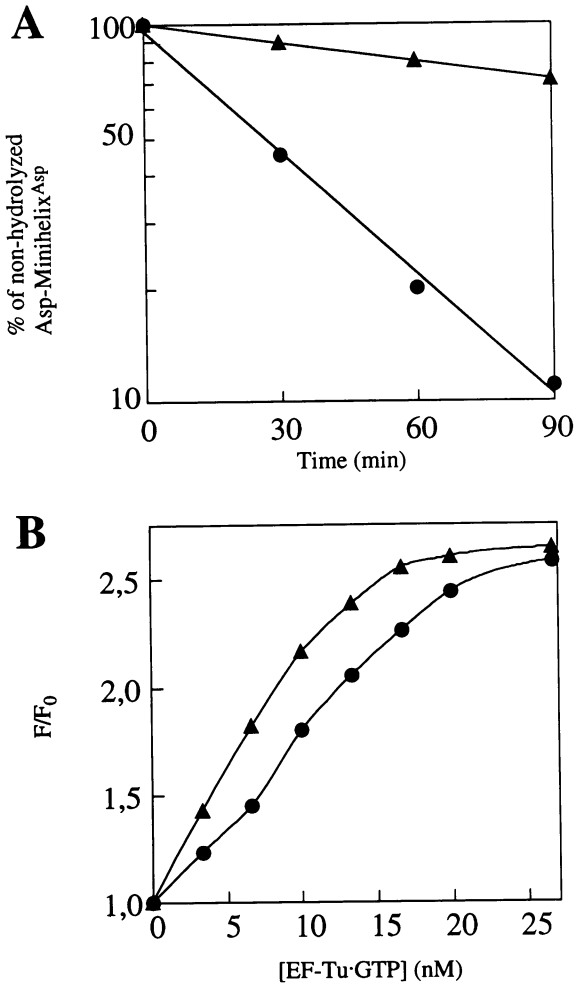

Fig. 2. Interaction of the activated elongation factor Tu with Asp-minihelix$^{Asp\text{-}13bp}$. (**A**) Hydrolysis protection assays. Rates of hydrolysis of the aspartate residue from Asp-minihelix$^{Asp\text{-}13bp}$ in the presence (▲) and absence (●) of EF-Tu·GTP. The concentration of the RNA was 0.2 μM and that of EF-Tu·GTP 4 μM. (**B**) Fluorescence titration of 10 nM [AEDANS-s^{2}C]Tyr-tRNA^{Tyr} with EF-Tu·GTP (▲) and titration of a mixture of 10 nM [AEDANS-s^{2}C]Tyr-tRNA^{Tyr} and 35 nM minihelix$^{Asp\text{-}13bp}$ (●) with EF-Tu·GTP. The relative fluorescence F/F_0 of [AEDANS-s^{2}C]Tyr-tRNA^{Tyr} at 485 nm was plotted against the concentration of EF-Tu·GTP.

aimed to define more precisely the antideterminants of minihelix^{Sec} preventing its efficient interaction with EF-Tu·GTP. The mutants were constructed taking into account the functional properties of minihelix$^{Sec1(Asp)}$ and minihelix$^{Sec4(Asp)}$ that imply that these antideterminants are located in a distal position to the accepting CCA-end. Mutations were reduced to 3 bp in minihelix$^{Sec5(Asp)}$ and to 2 bp in minihelix$^{Sec6(Asp)}$ and minihelix$^{Sec7(Asp)}$. These mutants are readily aspartylated to plateau levels of ~20% and their K_ds for interaction with *T.thermophilus* EF-Tu·GTP are listed in Table I. Of the three molecules tested, only minihelix$^{Sec5(Asp)}$, mutated in 3 bp, interacts efficiently with the protein (K_d = 10 nM). The two other minihelices with 2 bp inserted present an intermediate behaviour, with K_d values of 37 and 100 nM, respectively (Table I). These results were again confirmed by protection assays against deacylation. Altogether, these investigations show explicitly that introduction of sequence elements from tRNA^{Asp} at positions 7·66, 49·65 and 50·64 into

Fig. 3. Chimeric aspartylatable minihelices derived from *E.coli* tRNA^{Sec} with boxes derived from yeast tRNA^{Asp} (shaded). In (**A**), 5 bp were mutated and 3 or 2 bp were mutated in the variants displayed in (**B**). In the nomenclature of minihelices, e.g. in minihelix$^{Secx(Asp)}$, the superscript 'Sec' corresponds to the basic sequence, namely that of tRNA^{Sec} from which the minihelix was derived; 'x' designates the number of a mutant in a series and the superscript 'Asp' refers to sequence features derived from tRNA^{Asp} that were introduced into this minihelix.

minihelix^{Sec} allows this latter to interact efficiently with EF-Tu·GTP. Accordingly, these base pairs in minihelix^{Sec} are those responsible for the lack of interaction with EF-Tu·GTP, namely base pairs C_7·G_{66} (the last base pair of the amino acid-acceptor arm), G_{49}·U_{65}, and C_{50}·G_{64} (the two first base pairs of the T-arm).

Interaction of Asp-minihelix$^{Asp\text{-}13bp}$ containing sequence elements of minihelix^{Sec} with elongation factor Tu·GTP

The role of the three contiguous base pairs (C_7·G_{66}, G_{49}·U_{65}, C_{50}·G_{64}) as antideterminants was confirmed by a transplantation experiment where these elements were inserted into minihelix$^{Asp\text{-}13bp}$ (Figure 4A). As expected (and in agreement with the results obtained in the tRNA^{Sec} context) the chimeric Asp-minihelix$^{Asp1(Sec)}$ does not interact efficiently with EF-Tu·GTP and behaves similarly to Asp-minihelix^{Sec}. This conclusion was confirmed by protection assays in which Asp-minihelix$^{Asp1(Sec)}$ and Asp-minihelix^{Sec} behave identically.

As a control, we investigated the interaction with EF-Tu·GTP of a chimeric Asp-minihelix$^{Asp2(Sec)}$ with only the 8th and 9th bp from tRNA^{Sec} transplanted into the aspartate framework (Figure 4A). This molecule should lose some of its antideterminant properties. As expected, the measurements indicated a behaviour intermediate between that of Asp-minihelix^{Sec} and Asp-minihelix$^{Asp\text{-}13bp}$ (Table I). This

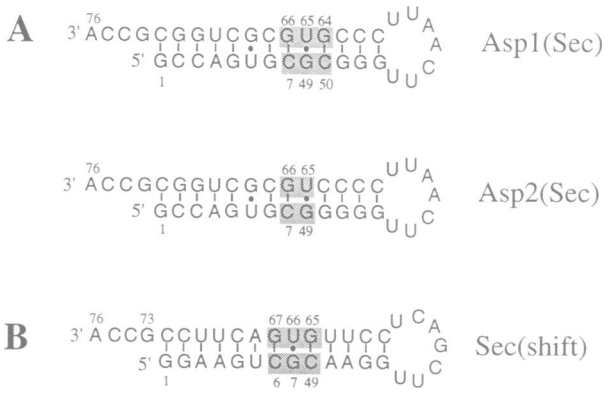

Fig. 4. (**A**) Chimeric aspartylatable minihelices derived from yeast tRNA^{Asp} with base pairs from tRNA^{Sec} (shaded) and (**B**) minihelix^{Sec} presenting the antideterminant box (shaded) shifted by 1 bp towards the CCA-end [named minihelix$^{Sec(shift)}$].

Table II. Aspartylation levels and interaction of aspartylated RNA with activated elongation factor as measured by hydrolysis protection assays

Asp-RNAs	Aspartylation level[a] (%)	Protection level[b] (%)
tRNAs		
Wild-type tRNA^{Sec}	30	5–10
Variant tRNA$^{Sec(Asp)}$	30	100
Minihelices		
Sec	25	0
Asp-13bp	20	40–65

[a]Aspartylation plateaux, obtained under comparative conditions in the presence of an excess of AspRS, are expressed as the percentage of charged RNA molecules present in the aminoacylation media.
[b]Hydrolysis protection assays; protection were measured after 90 min in the presence of a 10- to 20-fold molar excess of activated EF-Tu·GTP. For minihelix^{Sec} with 13 bp and wild-type tRNA^{Sec}, the two values of protection percentages correspond to mean values measured with a 10- and 20-fold excess of elongation factor, respectively.

shows that the 3 bp have to be present to fulfil their antideterminant role.

Role of the position of the antideterminant box in Asp-minihelix^{Sec} for interaction with elongation factor Tu·GTP

As a general conclusion of the above results, it appears that the antideterminant box has to be located precisely within the 8th, 9th and 10th bp of the amino acid-acceptor branch of tRNA^{Sec} to fulfil its role. To confirm this assumption, we prepared an Asp-minihelix^{Sec} in which the three antideterminant base pairs were shifted by 1 bp towards the CCA-terminus (Figure 4B). This molecule is aspartylated to 25% and interacts with EF-Tu·GTP with a K_d of 5 nM (Table I). Thus, as anticipated, the behaviour of this molecule is similar to that of Asp-minihelix$^{Asp\text{-}13bp}$.

Interaction with elongation factor Tu·GTP of aspartylated tRNA^{Sec} with or without the antideterminant box

Two tRNA^{Sec} transcripts, either wild-type or mutated at the 8th, 9th and 10th bp so as to introduce the antideterminant box as in minihelix$^{Sec5(Asp)}$ (see Figures 1A and 3B), could be aspartylated to 30% plateau levels (Table II).

This efficient charging is accounted for by the presence of the G_{73} discriminator residue in both $tRNA^{Sec}$ molecules and the absence of any aspartate determinant in the anticodon. Thus, these molecules behave as minihelices in the presence of AspRS because the contacts with the anticodon binding domain of the synthetase are lost. This behaviour is reminiscent of the efficient charging of a $tRNA^{Asp}$ variant in which the aspartate anticodon was changed to a methionine anticodon (Pütz *et al.*, 1993). The aspartylation properties of $tRNA^{Sec}$ and its variants will be described elsewhere (J.Rudinger *et al.*, in preparation). Using these aspartylated $tRNA^{Sec}$ molecules, it could be shown by the hydrolysis protection assay that EF-Tu·GTP strongly protects the $tRNA^{Sec}$ variant from which the antideterminant box was removed, whereas only poor protection is observed with the wild-type molecule (Table II). This result is comparable with the protection percentages observed under identical experimental conditions on the corresponding minihelices charged with the same amino acid (Table II).

Discussion

A moderate K_d discrimination

This work demonstrates that rejection of Asp-minihelix^{Sec} by EF-Tu·GTP is triggered by the presence of antideterminants in its 8th, 9th and 10th bp. This conclusion is based on protections against deacylation of variant minihelices by EF-Tu·GTP and on determination of K_ds for the interaction of aspartylated minihelices with EF-Tu·GTP. The K_d variation observed for minihelices interacting well or for those not interacting (or only weakly interacting) with EF-Tu·GTP is of ~2 orders of magnitude. Although this variation in K_d values might appear low, it is significant and compares well with similar determinations obtained with complete tRNAs interacting or not interacting with EF-Tu·GTP. This is in particular the case for the discrimination between Ser-$tRNA^{Sec}$ and Ser-$tRNA^{Ser}$ by EF-Tu·GTP (Förster *et al.*, 1990) and that between methionylated initiator and elongator tRNAs (Janiak *et al.*, 1990; Kiesewetter *et al.*, 1990). However, this binding discrimination linked to the nature of the tRNA of 2 orders of magnitude is rather moderate as compared with that brought by the presence of the aminoacyl residue at the 3′-end of the tRNA which is of 3–4 orders of magnitude (Januak *et al.*, 1990).

Three base pairs as antideterminants

Our results demonstrate unambiguously that base pairs $C_7{\cdot}G_{66}$, $G_{49}{\cdot}U_{65}$ and $C_{50}{\cdot}G_{64}$ of the amino acid-acceptor branch of *E.coli* $tRNA^{Sec}$ are the antideterminants responsible for the rejection of Asp-minihelix^{Sec} by prokaryotic elongation factor Tu. Considering the interaction of aminoacyl-tRNA with this factor which is restricted to the first 10 bp of the amino acid-acceptor branch (Rudinger *et al.*, 1994), it can be expected that this base pair combination likewise will act as an antideterminant in the complete tRNA. In a first set of experiments we could demonstrate that this expectation is indeed correct. In the hydrolysis protection assay, an aspartylated wild-type *E.coli* $tRNA^{Sec}$ transcript is only poorly protected by EF-Tu·GTP against amino acid hydrolysis, while a $tRNA^{Sec}$ variant, mutated like minihelix$^{Sec5(Asp)}$ and thus deprived of the antideterminant box, is strongly protected, like a control elongator aspartylated $tRNA^{Asp}$ (Table II). Despite this result, we do not exactly know at the present stage of our work, to what extent subtle effects brought by the entire tRNA may modulate the quantitative effects seen in the minihelix context. The existence of a tRNA effect that would enhance the antideterminant properties of the minihelix^{Sec} is suggested by *in vivo* experiments with variants of prokaryotic $tRNA^{Sec}$ mutated in the D-arm and the variable region that permit translation of ordinary UGA codons (Li and Yarus, 1992). If so, the role of the entire tRNA structure would be to present the antideterminant box in such a way in the amino acid-accepting branch, allowing optimal rejection by EF-Tu·GTP. The importance of the complete $tRNA^{Sec}$ structure was also emphasized for the serine phosphorylation function of this tRNA (Wu and Gross, 1994).

Moreover, our data indicate that the location of these base pairs at the 8th, 9th and 10th bp positions is as important as their nature, since a shift of the antideterminant box by 1 bp towards the CCA-end renders the shifted variant a substrate of EF-Tu·GTP. This explains the results from the experiments of Baron and Böck (1991) aimed at finding the structural reasons for functional properties of *E.coli* $tRNA^{Sec}$ in elongation factor recognition. Sec-$tRNA^{Sec}$ with 12 bp in the amino acid-acceptor branch interacted with EF-Tu·GTP while losing its ability to bind to the specialized SELB elongation factor. An obvious conclusion was that the lack of interaction of Sec-$tRNA^{Sec}$ with elongation factor Tu was due to the unusual length of the amino acid-acceptor branch. In fact, in the variant studied, the removal of $U_{5a}{\cdot}U_{67a}$ (Figure 1A) shifted the antideterminant box by one position with respect to 3′-terminal adenosine.

The conclusion that the 3 bp $C_7{\cdot}G_{66}$, $G_{49}{\cdot}U_{65}$ and $C_{50}{\cdot}G_{64}$ at the 8th, 9th and 10th bp positions of the amino acid-acceptor branch of *E.coli* $tRNA^{Sec}$ are the antideterminants is strongly supported by the fact that this combination is unique among all elongator *E.coli* tRNAs (Steinberg *et al.*, 1993).

How does tRNA rejection operate?

It is a general belief that elongation factors do not recognize specific sequence features within elongator tRNAs, since these factors have to interact with complete sets of ~60 different tRNAs. Since it has been shown that the first 10 bp of aminoacylated elongator tRNAs are crucial for interaction with elongation factor (Rudinger *et al.*, 1994), it is likely that the protein has to recognize the sugar–phosphate backbone of this domain. Such mode of recognition implies that the mechanism of antideterminination leading to the rejection of a tRNA should be linked to the disturbance of the helical structure of its amino acid-acceptor branch. If this view is correct, the 3 bp we have identified as the antideterminant within minihelix^{Sec} should present peculiar conformational characteristics.

Structural features conferred to the antideterminant box by G·U bp

The sequence precluding the interaction of Asp-minihelix^{Sec} with EF-Tu·GTP contains a G·U pair which probably contributes together with the two flanking C·G

pairs to a special conformation responsible for tRNA^{Sec} rejection by EF-Tu·TP. Conformational alterations in RNA helices linked to the presence of a G·U bp have been reported in a number of crystallographic (Ladner *et al.*, 1975; Westhof *et al.*, 1985) and NMR (White *et al.*, 1992; Allain and Varani, 1995) studies. Chemical mapping of G·U-containing helices in tRNA also points to structural alterations in such helical domains (Romby *et al.*, 1987). Thermodynamic investigations indicate that RNAs with G·U pairs are destabilized when compared with those molecules presenting solely Watson–Crick base pairs (Rhodes, 1977). The extent of the destabilization might be dependent upon the nature of the nucleotides surrounding the mismatch (the C_7·G_{66} and C_{50}·G_{64} pairs in tRNA^{Sec}) as can be anticipated from studies on DNAs (Aboul-ela *et al.*, 1985). In the recent NMR study on a RNA helix derived from a ribozyme and containing a G·U wobble pair, a large deviation from a regular RNA A-helix conformation was observed (Allain and Varani, 1995). In this structure, the U of the G·U pair is surrounded by two purines, as in *E.coli* tRNA^{Sec}. Also noticeable are changes in the binding of water molecules and metal ions on an RNA helix linked to the presence of a G·U pair which where identified by a crystallographic (Holbrook *et al.*, 1991) and NMR (Limmer *et al.*, 1993) analysis, respectively.

In this context, it is worth recalling that G·U pairs have been found important in the function of several RNAs. This is the case for tRNA^{Ala} where the G_3·U_{70} pair represents the major identity element specifying aminoacylation by alanyl-tRNA synthetase (Hou and Schimmel, 1988; McClain and Foss, 1988). Chemical nature of the G·U pair (Musier-Forsyth *et al.*, 1991), as well as the resulting changes in the conformation of the helix (McClain *et al.*, 1988) may play a role in the recognition of tRNA^{Ala} by alanyl-tRNA synthetase. Finally, G·U pairs have been shown to define the 5′ splice site of group I intron (Green *et al.*, 1991; Strobel and Cech, 1995) as well as the self-cleavage in hepatitis delta virus ribozyme (Been, 1994).

Altogether, these data support the view that the G·U bp present in the antideterminant box of tRNA^{Sec} contributes to a local distortion in the helical structure of its amino acid-acceptor branch and accordingly plays a pivotal role in the rejection mechanism by EF-Tu·GTP. However, this distortion and hence its functional consequences is not due solely to the unique presence of the G·U pair, since some elongator tRNAs contain G·U, U·G, U·U or C·A mismatches either at the 8th, 9th or 10th bp position. The present work demonstrates that the two flanking C·G pairs also participate in the antidetermination mechanism. Nevertheless, sequence combinations close to that found in tRNA^{Sec} might lead to a reduced binding ability with EF-Tu·GTP. This is actually the case of a tRNA^{Val} isoacceptor which presents the worst affinity for EF-Tu·GTP among the elongator tRNAs so far tested (Ott *et al.*, 1989) and as anticipated, contains a base pair arrangement at the 8th, 9th and 10th bp position resembling that present in tRNA^{Sec} (the 8th C·G pair replaced by a G·C pair and the 9th and 10th pairs similar to those in tRNA^{Sec}).

Has evolution retained a unique rejection mechanism for selenocysteine tRNAs against elongation factor recognition?

Selenocysteine-inserting tRNAs have been identified both in the prokaryotic and eukaryotic kingdoms. The comparison of the sequences of all prokaryotic tRNA^{Sec} reveals the presence of G·U at the 9th bp position in *Proteus vulgaris* as well as in *Desulfomicrobium baculatum* and a G·A pair at the 8th position in *Clostridium thermoaceticum* (Tormay *et al.*, 1994). The presence of these unusual base pairs at positions homologous to the antideterminant box of *E.coli* tRNA^{Sec}, suggests a similar discrimination process of these tRNAs against EF-Tu·GTP as is the case in *E.coli* tRNA^{Sec}. Further, the presence of a non-Watson–Crick base pair is evolutionarily maintained, since all amino acid-acceptor stems of eukaryotic tRNA^{Sec} possess a U·U located at the 8th bp position (Sturchler-Pierrat *et al.*, 1995). This strict conservation is indicative of a functional role and might be involved in the discrimination process against eEF-1α.

In conclusion, it is tempting to propose that the structural features preventing the binding of tRNA^{Sec} to prokaryotic and likely also eukaryotic elongation factors might play an additional role by contributing to the recognition of Sec-tRNA^{Sec} by their specialized elongation factors, SELB and its eukaryotic analogue. Such a dual strategy, with a unique structural feature in a tRNA used alternatively as a positive and a negative signal, exists for the aminoacylation identity of *E.coli* tRNA^{Ile} in which the modified base lysidine in the anticodon specifies recognition by isoleucyl-tRNA synthetase and rejection by methionyl-tRNA synthetase (Muramatsu *et al.*, 1988). Also discriminator residue G_{73} in tRNA^{Asp} which specifies the aspartate identity of all minisubstrates and of the tRNA^{Sec} variants studied in this work, can be considered as a negative signal against charging by synthetases specific, e.g. for alanine, glycine, proline and phenylalanine, since mutating the wild-type discriminator residue of the cognate tRNAs of these enzymes to G_{73} prevents their charging (reviewed by McClain, 1993). Related with these considerations is the problem of the rejection of initiator tRNAs by elongation factors. Here also, nature has selected a rejection mechanism based on a conformational alteration of the amino acid-acceptor branch of these tRNAs. In prokaryotic initiator tRNAs this alteration concerns the first base pair (RajBhandary and Chow, 1995), but interestingly in eukaryotic yeast and plant initiator tRNAs, antidetermination is brought about by a bulky post-transcriptional modification at ribose 64 (Kiesewetter *et al.*, 1990; Förster *et al.*, 1993) at the same location as the U residue of the G·U pair in the tRNA^{Sec} antideterminant box. Whether this similar location is fortuitous or reflects a structural property of elongation factors in RNAs is not yet understood and will require further investigations.

Materials and methods

Materials

Yeast AspRS (Lorber *et al.*, 1983) and T7 RNA polymerase (Wyatt *et al.*, 1991) were prepared as described previously. EF-Ts and EF-Tu·GDP were purified from *T.thermophilus* HB8 using established procedures (Peter *et al.*, 1990; Limmer *et al.*, 1992). Oligonucleotides were synthesized on an Applied Biosystem 381 A DNA synthesizer and purified on a Nucleosil 120-5-C18 column (Bischoff Chromatography,

Zymark-France, Paris). L-[^{14}C]aspartic acid (224 mCi/mmol) was purchased from Amersham (France); phosphoenolpyruvate, pyruvate kinase and all ribonucleotides were from Boehringer Mannheim.

Synthesis of minihelices and of tRNAs

Minihelices derived from *E.coli* $tRNA^{Sec}$ and from yeast $tRNA^{Asp}$ were obtained by *in vitro* transcription of single-stranded templates. The templates were obtained by annealing an 18mer oligonucleotide (minus strand of the T7 RNA polymerase promoter) to another oligonucleotide, complementary to the 18mer, followed by the complement of the RNA sequence desired. For this purpose, equimolar concentrations of the two oligonucleotides were mixed in 0.3 M Tris–HCl, pH 8.1, 0.15 M $MgCl_2$, incubated for 3 min at 65°C and then slowly cooled to room temperature. Transcription was performed for 3 h at 37°C in 40 mM Tris–HCl, pH 8.1, 22 mM $MgCl_2$, 1 mM spermidine, 5 mM dithioerythritol, 0.01% Triton X-100, 4 mM of each ribonucleoside triphosphate, 5 mM GMP, and the appropriate amount of T7 RNA polymerase. After phenol extraction, transcripts were ethanol-precipitated and purified to single nucleotide resolution by denaturing 15% PAGE. Full-length transcripts were eluted from gel by electroelution (Schleicher and Schuell, Dassel, Germany). Transcripts of *E.coli* $tRNA^{Sec}$ and of a variant mutated as $minihelix^{Sec5(Asp)}$ were prepared by established procedures (Perret *et al.*, 1990).

Aminoacylation reaction

Before aminoacylation, the eluted transcripts were heated at 65°C for 2 min and cooled for 5 min to allow native conformation. Aspartylation of minihelices and tRNAs was performed in 25 mM Tris–HCl, pH 7.5, 7.5 mM $MgCl_2$, 0.5 mM ATP, 0.1 mg/ml bovine serum albumin, 0.1 mM L-[^{14}C]aspartic acid and 0.8 μM pure yeast AspRS for 2 h at 20°C. All reactions were stopped by phenol extraction in the presence of sodium acetate, pH 4.5. Transcripts were ethanol-precipitated, washed, dried and resuspended in 10 mM sodium acetate, pH 4.5, in order to prevent spontaneous deacylation. Aspartylation levels were determined after 5% trichloroacetic acid precipitation on 3MM Whatman filters.

Hydrolysis protection assays

These experiments were conducted as described previously by Pingould and Urbanke (1980). They permit qualitative monitoring of ternary complex formation between aminoacylated RNA, GTP and EF-Tu, since EF-Tu·GTP when bound to aminoacyl-tRNA protects the labile amino acid ester bound from spontaneous base-catalysed hydrolysis. For this aim, EF-Tu·GDP was first converted into active EF-Tu·GTP. Thus, 1.4 nmol EF-Tu·GDP are incubated in the presence of 50 mM Tris–HCl, pH 7.5, 50 mM KCl, 50 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM GTP, 2 mM phosphoenolpyruvate, 5 U pyruvate kinase and 14 pmol EF-Tu for 20 min at 37°C in a final volume of 100 μl. The activated EF-Tu·GTP is kept on ice until use.

The protection assays for each aspartylated minihelix were performed by incubation of aspartylated transcripts (~20 pmol, taking into account the aspartylation level of each minihelix) freshly dissolved in 50 mM Tris–HCl, pH 7.5, 50 mM KCl and 10 mM $MgCl_2$ with a 10- and 20-fold excess of EF-Tu·GTP (200 and 400 pmol) in a final volume of 100 μl. An experiment without protein was carried out in parallel where EF-Tu was replaced by a buffer containing 50 mM Tris–HCl, pH 7.5, 50 mM KCl and 10 mM $MgCl_2$. Ternary complexes between EF-Tu·GTP and aspartylated minihelices were obtained after a 10 min incubation in ice. The temperature was increased to 37°C in order to favour spontaneous hydrolysis of the ester bound. Aliquots of 20 μl were removed at 0, 30, 60 and 90 min and spotted on 3MM Whatman filters. After trichloroacetic acid precipitation, residual radioactivity was determined by liquid scintillation counting.

For each set of experiments realized with minihelices, a positive control was performed in which aspartylated minihelices were replaced by yeast Asp-$tRNA^{Asp}$ and incubated in the presence of a 5-fold excess of EF-Tu·GTP.

K_d measurements

The equilibrium dissociation constants K_d of ternary complexes formed between aminoacylated minihelices, EF-Tu from *T.thermophilus*, and GTP were determined by fluorescence titration using (AEDANS-s^2C_{75})Tyr-$tRNA^{Tyr}$ (AEDANS, *N*-[(acetylamino)ethyl]-5-naphthylamine-1-sulphonic acid) as the fluorescent reporter ligand and the particular aspartylated minihelix as its competitor. Details concerning the method are given in Ott *et al.* (1989). The K_ds for all molecules were determined with the same preparation of fluorescent reporter and all experiments were repeated at least twice.

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