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Transfer RNA recognition by the *Escherichia coli* Δ^2-isopentenyl-pyrophosphate:tRNA Δ^2-isopentenyl transferase: Dependence on the anticodon arm structure

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ABSTRACT

To elucidate the sequence elements required in the anticodon stem for the recognition of *Escherichia coli* tRNA^{Ser} (GGA) by the *E. coli* isopentenyl-tRNA:A_{37} transferase (IPTT), which result in the conversion of A_{37} into isopentenylated i^6A_{37}, we have tested and characterized in vitro T7-runoff transcripts of 17 variants of *E. coli* tRNA^{Ser}(GGA) and 7 other tRNAs from *E. coli* and yeast. Our results indicate that, instead of a stringent specific anticodon stem and loop sequence, the key feature required for the recognition of *E. coli* tRNAs by IPTT is the $A_{36}A_{37}A_{38}$ sequence occurring within the seven-membered anticodon loop, and the retention of the standard helical structure and flexibility, especially in the proximal anticodon stem. The G_{30}*U_{40} mismatch base pair close to the anticodon loop is strictly avoided. The frequent occurrence of a C-G base pair in the three stem locations closest to the loop (positions 29–41, 30–40 and 31–39) or the occurrence of even one such C-G base pair along with some other similarly less suited, but individually tolerated deviations can also totally abolish the A_{37} isopentenylation of tRNA. For the position 30–40, the G-C base pair is shown uniquely suited, whereas for the adjoining 29–41 stem location, a purine–pyrimidine base pair with pyrimidine on the 3′-side is strongly preferred. Retention of the overall 3D tRNA structure is favorable for isopentenylation and allows some tolerance of proximal stem sequence deviations. Our data suggest a recognition mode that implies the interaction of IPTT with the strictly conserved $A_{36}A_{37}A_{38}$ sequence and the other functional groups located in the minor groove of the anticodon stem.

Keywords: anticodon; dimethylallyl diphosphate:tRNA dimethylallyltransferase; identity elements; maturation; isopentenyladenosine; modifying enzymes; transferase; tRNA; tRNA prenyl transferase

INTRODUCTION

Naturally occurring transfer RNAs always contain a variety of characteristic nucleosides that are formed by enzymatic modifications of the polynucleotide transcripts during the complex process of tRNA maturation (Limbach et al., 1994; reviewed in Björk, 1995a). Some of these modified nucleotides are common to all tRNA molecules, whereas others have been found in only one tRNA species. tRNA-modifying enzymes catalyzing the formation of these modified nucleotides are sensitive to different identity elements of the tRNA molecules. For those enzymes having a broad specificity, possibly general gross features and/or few common elements of the tRNA molecules are required, whereas for the most specific ones, tRNA recognition may be more complex and require unique identity elements of selected tRNA molecules (discussed in Grosjean et al., 1996). The possibility exists that few of the enzymes having a broad specificity also catalyze the same reaction at different locations of the same tRNA molecule and/or of different classes of RNAs. Such cases of "dual specificity" have been demonstrated recently in *Escherichia coli* (Wrzesinski et al., 1995; reviewed in Ofengand et al., 1995), as well as in yeast (Simos et al., 1996). This situation may be analogous to the recognition of tRNA isoacceptor species, tRNA-like structures in plant viral RNA, and artificial minihelices by selected aminoacyl-tRNA synthetases or elongation factors (reviewed in Giegé et al., 1993).

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In the present work, we focus our attention on the enzymatic formation of N^6-Δ^2-isopentenyladenosine (i^6A). This modified nucleoside is invariably found in position 37 (3′ adjacent to anticodon) of almost all eukaryotic and bacterial tRNAs that read codons starting from U (Sprinzl et al., 1996). The enzyme responsible for the formation of i^6A_{37} was shown to catalyze the transfer of the Δ^2-isopentenyl group of Δ^2-isopentenylpyrophosphate, derived from mevalonic acid, to the amino group on C_6 of adenosine-37 (reviewed in Hall, 1970). This Δ^2-isopentenylpyrophosphate:tRNA-Δ^2isopentenyltransferase (EC 2.5.1.8, isopentenyl-tRNA:A_{37} transferase, abbreviated as IPTT, also referred as tRNA prenyl transferase or dimethylallyl diphosphate:tRNA dimethylallyltransferase, DMAPP-tRNA transferase) is encoded by the *miaA* (previously called *trpX*) gene in *E. coli* and *MOD5* gene in yeast: they both have been cloned and sequenced (Dihanich et al., 1987; Najarian et al., 1987; Caillet & Droogmans, 1988; Connolly & Winkler, 1989). In some eubacteria (*Escherichia*, *Proteus*, and *Bacillus*) and chloroplasts, i^6A_{37} in tRNAs is thiomethylated to 2-methylthio derivative ($ms^2i^6A_{37}$), whereas under certain physiological conditions, $ms^2i^6A_{37}$ in tRNAs from plants and other eubacteria (*Salmonella*, *Pseudomonas*, or *Klebsiella)* is further hydroxylated to $ms^2io^6A_{37}$ (Agris et al., 1975; Buck & Ames, 1984). The production of these i^6A_{37} derivatives requires, respectively, *miaB*, *miaC*, and *miaE* (reviewed in Persson et al., 1994 and Björk, 1995b).

Although the isopentenyl-tRNA:A_{37} transferase (IPTT) was identified a long time ago, it has been purified only partially from *E. coli* (Kline et al., 1969; Bartz et al., 1970; Rosenbaum & Gefter, 1972) and yeast (Fittler et al., 1968; Gefter, 1969). Very recently, the corresponding protein was cloned, tagged at C- or N-terminal, and overexpressed in *E. coli*, thus allowing the detailed study of its biochemical and enzymatic properties (Leung et al., 1997; Moore & Poulter, 1997). The specificity and recognition of tRNAs by this enzyme has been postulated to depend in part on the nucleotides that are located 5′ adjacent to the modifiable nucleotide A_{37}. Indeed, all tRNAs containing i^6A_{37} (or its derivatives) have an anticodon ending with A_{36} (Nishimura, 1972; Yarus, 1982). Moreover, studies on missense and nonsense suppressors reveal that C to A or U to A change at position 36 of the anticodon of *E. coli* $tRNA^{Gly}$ (anticodon GCC, Carbon & Fleck, 1974; or anticodon UCC, Prather et al., 1981b) or of *E. coli* $tRNA^{Lys}$ (anticodon mam^5s^2UUU, Prather et al., 1983) was accompanied by the appearance of hypermodification of A_{37} to i^6A_{37} and further to $ms^2i^6A_{37}$. However, the sequencing analysis revealed that the yield of A_{37} modification was rather low (see also Yarus et al., 1986). Also in one case, A_{37} was totally unmodified in vivo, even though the suppressor tRNAs had the same anticodon loop sequence as that of identical suppressors derived from other tRNAs (Murgola et al., 1984, reviewed in Murgola, 1995). These observations demonstrate not only the importance of dinucleotide $A_{36}A_{37}$ for the enzymatic formation of $ms^2i^6A_{37}$, but also point out that tRNA recognition by the isopentenyl-tRNA:A_{37} transferase is more complex and depends on other identity elements in the tRNA molecule.

By inspection of the available data on nucleotide sequences of 42 tRNAs from *E. coli* and bacteriophage T_4, Yarus (1982) pointed out that the structure of the anticodon loop and the nearby base pairs in the anticodon stem are clearly related (extended anticodon theory). A consensus sequence for the anticodon loop and the proximal anticodon stem was proposed for each group of tRNAs harboring either an A, U, G, or C at position 36 (the cardinal nucleotide). Such consensus sequences not only may match specific structural requirements of the decoding machinery on the ribosome (as suggested by Yarus, 1982), but also may be part of tRNA recognition for selected posttranscriptional modifications within the anticodon stem-loop. In agreement with this hypothesis, almost all *E. coli* and phage T_4 tRNAs harboring an A_{36} at the third anticodon position contain a hypermodified nucleoside i^6A_{37} or $ms^2i^6A_{37}$. A similar conclusion about the necessity of loop sequence $A_{36}A_{37}A_{38}$ and anticodon stem with five Watson–Crick base pairs for i^6A modification was made by Tsang et al. (1983).

Previously, the nucleotide sequences of two *E. coli* $tRNAs^{Ser}$ (both with anticodon GGA) were determined (Grosjean et al., 1985; see also Fischer & Sprinzl, 1985). These two serine tRNAs differ only at position 20, where a cytosine was found in the minor form ($tRNA^{Ser}$(V)) and a dihydrouridine at the same position in the major form ($tRNA^{Ser}$(I)) (Fig. 1A). Surprisingly, neither of these two $tRNAs^{Ser}$ contained a modified adenosine in the position 3′ adjacent to the anticodon. However, due to the presence of mispair G_{30}*Ψ_{40} in $tRNA^{Ser}$ (I and V), as compared with all other $ms^2i^6A_{37}$ containing *E. coli* tRNAs that were sequenced at that time (Tsang et al., 1983; Sprinzl et al., 1996), it was suggested that such a nonconventional wobble base pair in the proximal anticodon stem could perturb the enzymatic isopentenylation of adenosine-37 (Grosjean et al., 1985).

In the present paper, we have tested this hypothesis. In order to determine the substrate specificity of the i^6A-forming enzyme from *E. coli*, we have constructed a collection of synthetic genes of *E. coli* $tRNA^{Ser}$(anticodon GGA) harboring various combinations of base pairs (including a standard G_{30}-C_{40} pair) in the proximal anticodon stem and tested in vitro whether the resulting transcripts become modified by the *E. coli* isopentenyl-tRNA:A_{37} transferase. The results indicate that only certain combinations of base pairs in the proximal anticodon stem, together with a major identity sequence $A_{36}A_{37}A_{38}$, allow for adenosine-37 to be modified enzymatically into i^6A_{37} derivatives.

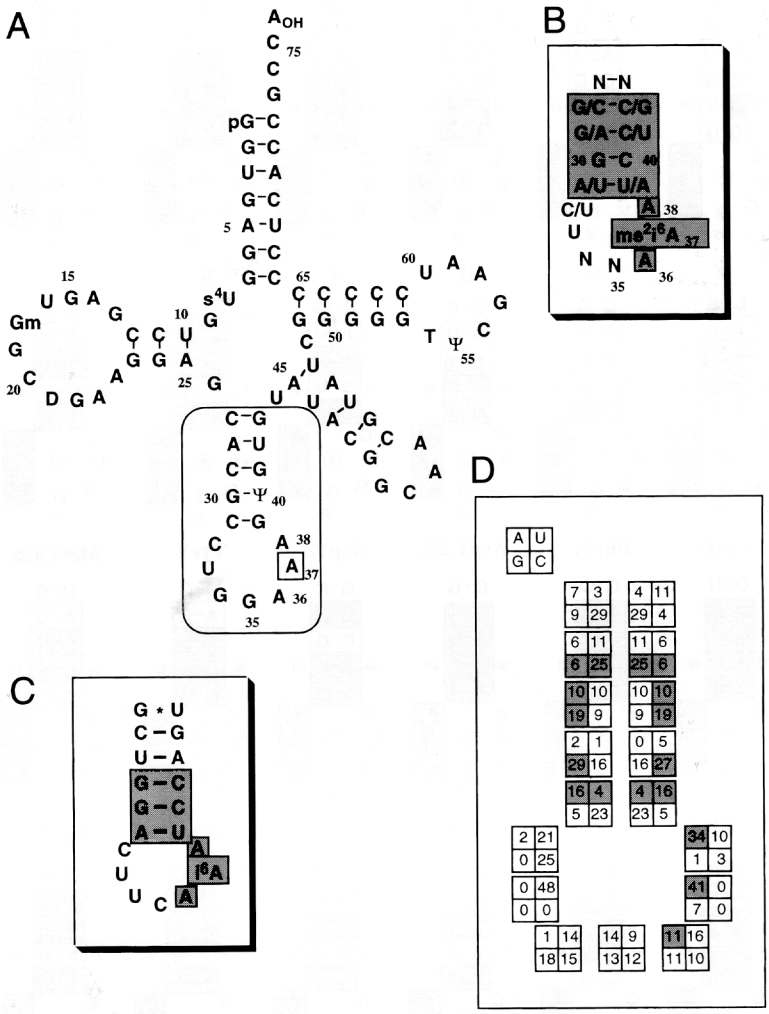

FIGURE 1. **A:** Cloverleaf presentation of wild-type *E. coli* $tRNA^{Ser}$(GGA). Abbreviations for modified bases are as in Limbach et al. (1994), putative site of modification to i^6A_{37} is boxed. **B:** Consensus sequence of eight *E. coli* tRNAs modified to ms^2i^6A in position 37, N stands for any nucleotide acceptable in this position. **C:** Structure of anticodon stem and loop of selenocysteine-inserting *E. coli* tRNA, modified only to i^6A_{37}. Nucleotides of consensus sequence are boxed. **D:** Frequency of bases and base pairs in the anticodon stem/loop of 48 *E. coli* tRNAs sequenced so far. In shadowed boxes are the nucleotides of the consensus sequence (B)

RESULTS

Analysis of *E. coli* tRNA database

To date, there are eight *E. coli* tRNAs for which the nucleotide sequence revealed the presence of $ms^2i^6A_{37}$ (Sprinzl et al., 1996). One other tRNA (selenocysteine-inserting serine tRNA) harbors the hypermodified nucleotide i^6A_{37} instead of $ms^2i^6A_{37}$ (Schön et al., 1989). The comparison of these tRNA nucleotide sequences, as was noticed first by Yarus (1982), reveals a consensus sequence at the 3′ side of the so-called cardinal nucleotide A_{36} and in the proximal anticodon stem (Fig. 1B,C, to be compared with the compilation of Fig. 1D). This sequence motif is composed of the conserved $A_{36}A_{37}A_{38}$ anticodon loop sequence coupled to the stem with five Watson–Crick base pairs having remarkable conservation at positions 28–42, 29–41, 30–40, and 31–39 (shaded in Fig. 1B,C). The corresponding nucleotides in the anticodon-proximal stem of naturally occurring unmodified *E. coli* $tRNA^{Ser}$ (GGA) deviate from the above consensus at almost each of the base pairs close to the anticodon loop (compare nucleotide composition of anticodon stem in Fig. 1A with those in the shadowed boxes in Fig. 1B,C). *E. coli* $tRNA^{Ser}$ (GGA) also includes a wobble $G_{30}{*}\Psi_{40}$ base pair, which could disrupt the local geometry of the anticodon stem.

Construction of $tRNA^{Ser}$ (GGA) variants

Figure 2 (first row) shows the different mutations introduced in the sequence of wild-type *E. coli* $tRNA^{Ser}$(I) (variant A). $tRNA^{Ser}$(GGA) was stepwise converted into

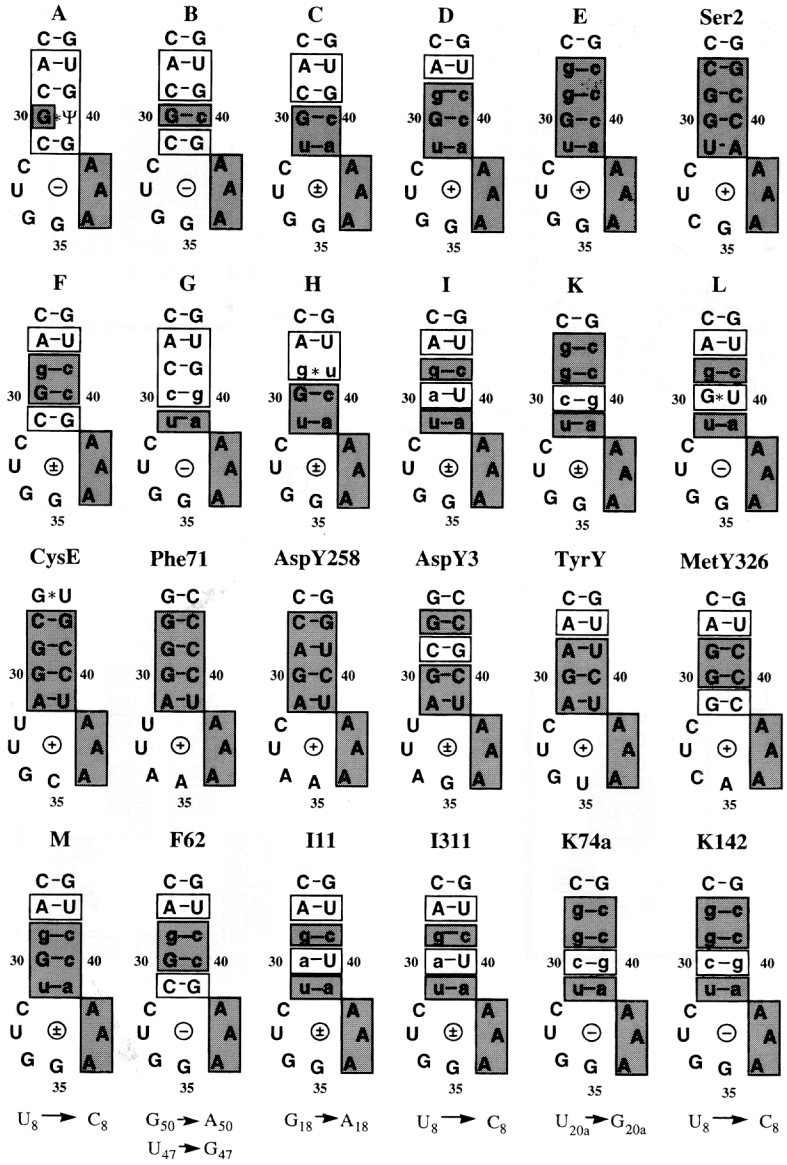

FIGURE 2. Sequences of anticodon stem of $tRNA^{Ser}(GGA)$ mutants and other tRNAs tested for i^6A formation in *E. coli* extract. The rest of the sequence is identical to wild-type $tRNA^{Ser}(GGA)$ except for mutants in 3D structure (bottom row) and wild-type and mutant tRNAs *E. coli* and yeast (third row). Mutations, introduced in wild-type $tRNA^{Ser}(GGA)$ are shown in small letters, nucleotides that conform the consensus sequence are in shadowed boxes, "negative identity elements" are in open boxes. ⊕, modification as in control wild-type $tRNA^{Ser}(CGA)$ (100%); ±, low level of modification even after incubation for 2 h; −, complete absence of modification.

variants B, C, D, and E harboring the same consensus sequence in the proximal stem (shadowed boxes) found in the naturally modified *E. coli* $tRNA^{Ser}$ (anticodon CGA, named Ser 2 in Fig. 2). The mutated nucleotides and those corresponding to the consensus nucleotides are shown in small letters and the base pairs that deviate from the ones shown in the consensus sequence (Fig. 1B) are in boxes without shading. Additional variants F–L of *E. coli* $tRNA^{Ser}$ (row 2 in Fig. 2) were made from the variant D to test further each individual base pair, as well as the G*U wobble pair within the anticodon-proximal stem for i^6A_{37} formation and its efficiency.

Enzymatic formation of Δ^2-isopentenyladenosine in tRNA variants

Each of the 24 tRNA genes described above were cloned downstream from a T7 promoter and transcribed in vitro using [α-^{32}P]-labeled ATP. The resulting purified run-off radiolabeled transcripts were incubated at 37 °C with a S100 *E. coli* extract in the presence of Δ^3-isopentenyl-pyrophosphate and ATP as described in Materials and Methods. After incubation, the radiolabeled tRNA transcripts were hydrolyzed with nuclease P1 to generate radiolabeled 5′-adenosine monophosphate or

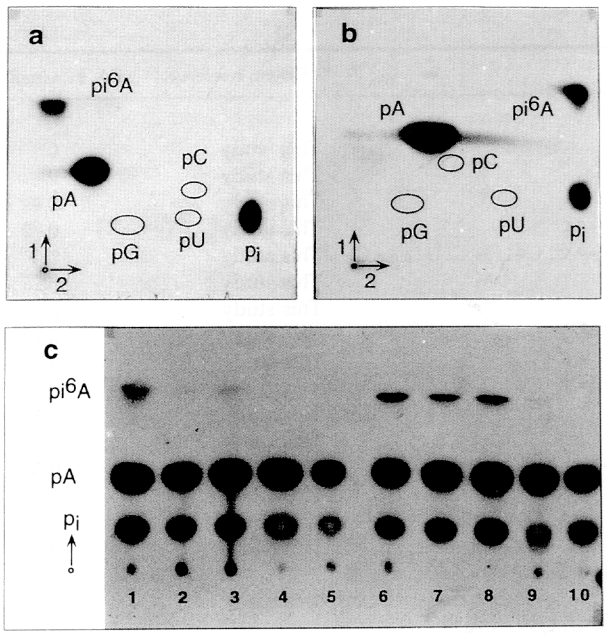

FIGURE 3. Thin-layer chromatography of reaction products. **A,B:** Two-dimensional separation using solvent system I and II, respectively (as described in Materials and Methods). **C:** One-dimensional resolution of reaction products for different mutants of $tRNA^{Ser}$ (GGA) and control tRNA transcripts. Lane 1, $tRNA^{Ser}$(CGA); lane 2, mutant M; lane 3, mutant C; lane 4, wild-type tRNASer(GGA) (variant A); lane 5, mutant B; lane 6, mutant D; lane 7, mutant of $tRNA^{Phe}$ (Phe 71); lane 8, mutant of yeast $tRNA^{fMet}$ (MetY326); lane 9, mutant G; lane 10, mutant L.

its modified counterpart i^6AMP. Upon resolution on thin-layer cellulose plates, the radioactivity in the AMP and i^6AMP spots were quantified. Figure 3A and B shows typical examples of such autoradiographies with P1 hydrolyzate of the *E. coli* $tRNA^{Ser}$ (mutant D). Figure 3C shows the autoradiography of a 1D chromatography plate where the P1-hydrolyzates of 10 different tRNA transcripts were compared (see figure legend). Figure 4A and B shows the time-course of i^6A_{37} formation in *E. coli* $tRNA^{Ser2}$, a natural substrate of the isopentenyl-tRNA:A_{37} transferase, as well as the time-course in several variants of the *E. coli* $tRNA^{Ser}$(GGA). Table 1 lists the molar amounts of i^6A_{37} formed in each of these 24 tRNA transcripts after 2 h of incubation at 37 °C with *E. coli* S100 extract.

Lack of i^6A_{37} in naturally occurring *E. coli* $tRNA^{Ser}$ (GGA) cannot be explained solely by the presence of the wobble G_{30}*Ψ_{40} base pair in the anticodon stem

As shown in Figure 4A and Table 1, no trace of i^6A_{37} was detected in mutant B of *E. coli* $tRNA^{Ser}$(GGA) where U_{40} was changed into C_{40}, thus generating a tRNA harboring $A_{36}A_{37}A_{38}$ and an anticodon stem with five Watson-Crick base pairs. If, in addition to this U_{40} to C_{40} mutation, the base pair C_{31}-G_{39} was replaced by U_{31}-A_{39}, then the resulting $tRNA^{Ser}$ mutant C became a substrate for the *E. coli* isopentenyl-tRNA:A_{37} transferase. However, as shown in Figure 4A, the rate of i^6A_{37} formation in this tRNA variant remains substantially lower than the rate of isopentenylation in the natural substrate $tRNA^{Ser2}$(CGA). Only when the base pair C_{29}-G_{41} was "reversed" into G_{29}-C_{41}, in addition to the two above mutations as in variants B and C, was the resulting mutant D almost as good a substrate as wild-type $tRNA^{Ser2}$(CGA). In this latter tRNA variant, three of four base pairs in the proximal anticodon stem were exactly as in the consensus sequence presented in Figure 1B. Additional mutation as in mutant E to create a G_{28}-C_{42} base pair in place of A_{28}-U_{42} does not improve significantly the ability of *E. coli* $tRNA^{Ser}$ (GGA) to become entirely modified into i^6A_{37}.

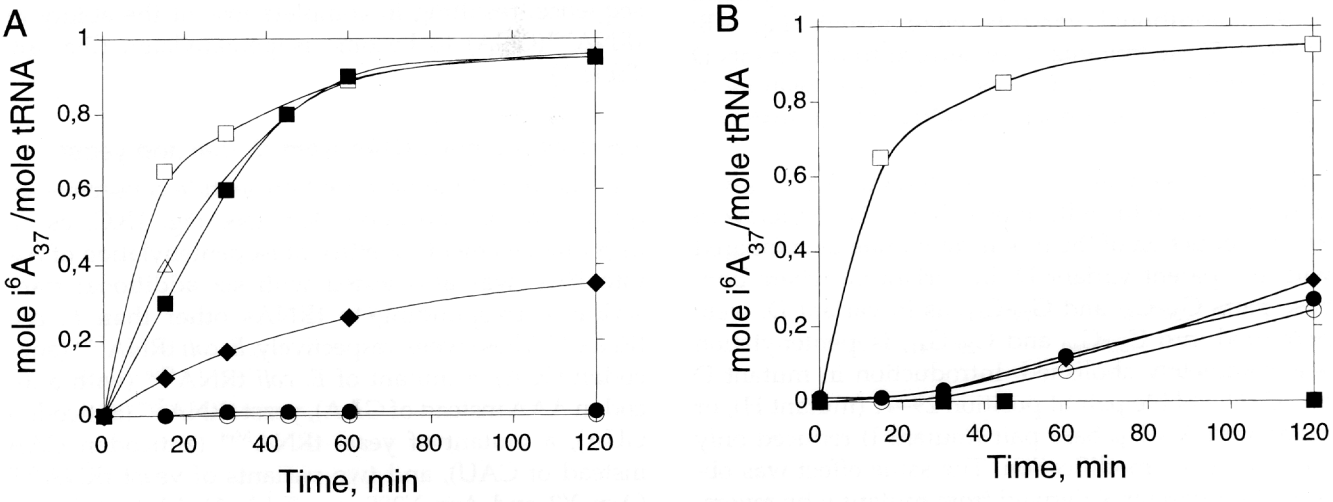

FIGURE 4. Kinetics of modification for different mutants of $tRNA^{Ser}$(GGA), with wild-type $tRNA^{Ser}$(CGA) and *E. coli* $tRNA^{Cys}$ used as controls. **A:** Mutants A (○); B (●); C (♦); D (□); E (■); wild-type $tRNA^{Ser}$(CGA) (△). **B:** Mutants I (●); H (○); K (♦); L (■); and *E. coli* $tRNA^{Cys}$ (□).

TABLE 1. tRNAs and tRNA mutants used in this work.

Name	tRNAs and tRNA Mutants	Origin, reference	i^6A, mol/mol tRNA
Variants of *E. coli* $tRNA^{Ser}$ (GGA)			
Var A	$tRNA^{Ser}_{(GGA)}$ wild-type	This study	0
Mut B	$tRNA^{Ser}_{(GGA)}$, U40 → C	This study	0
Mut C	$tRNA^{Ser}_{(GGA)}$, U40 → C, C31 → U, G39 → A, A76 → C	This study	0.45
Mut D	$tRNA^{Ser}_{(GGA)}$, C29 → G, C31 → U, G39 → A, U40 → C, G41 → C	This study	0.90
Mut E	$tRNA^{Ser}_{(GGA)}$, A28 → G, C29 → G, C31 → U, G39 → A, U40 → C, G41 → C, U42 → C	This study	1.0
Mut F	$tRNA^{Ser}_{(GGA)}$, C29 → G, U40 → C, G41 → C	This study	0.15
Mut G	$tRNA^{Ser}_{(GGA)}$, G30 → C, C31 → U, G39 → A, U40 → G	This study	0
Mut H	$tRNA^{Ser}_{(GGA)}$, C29 → G, C31 → U, G39 → A, U40 → G, G41 → U	This study	0.25
Mut I	$tRNA^{Ser}_{(GGA)}$, C29 → G, G30 → A, C31 → U, G39 → A, G41 → C	This study	0.35
Mut K	$tRNA^{Ser}_{(GGA)}$, A28 → G, C29 → G, G30 → A, C31 → U, G39 → A, U40 → G, G41 → C, U42 → C	This study	0.35
Mut L	$tRNA^{Ser}_{(GGA)}$, C29 → G, C31 → U, G39 → A, G41 → C	This study	0
Mut M	$tRNA^{Ser}_{(GGA)}$, U8 → C, C29 → G, C31 → U, G39 → A, U40 → G, G41 → C	This study	0.30
Mut F62	$tRNA^{Ser}_{(GGA)}$, C29 → G, U40 → C, G41 → C, U47 → G, G50 → A	This study	0
Mut I11	$tRNA^{Ser}_{(GGA)}$, G17 → A, C29 → G, G30 → A, C31 → U, G39 → A, G41 → C	This study	0.30
Mut I311	$tRNA^{Ser}_{(GGA)}$, U8 → C, C29 → G, G30 → A, C31 → U, G39 → A, G41 → C	This study	0.2
Mut K74a	$tRNA^{Ser}_{(GGA)}$, U20a → G A28 → G, C29 → G, G30 → C, C31 → U, G39 → A, U40 → G, G41 → C, U42 → C	This study	0
Mut K142	$tRNA^{Ser}_{(GGA)}$, U8 → C, A28 → G, C29 → G, G30 → C, C31 → U, G39 → A, U40 → G, G41 → C, U42 → C	This study	0
Other tRNAs (wild-type or mutants)			
Phe 71	$tRNA^{Phe}$ *E. coli*, mutant G34 → A	Dr. O. Uhlenbeck	1.0
Ser 2	$tRNA^{Ser}_{(CGA)}$ *E. coli*, ser-2, wild-type	This study	1.0
CysE	$tRNA^{Cys}_{(AGA)}$ *E. coli*, wild-type	Dr. Y.-M. Hou	0.85
AspY3	$tRNA^{Asp}$ yeast, C31 → A, G34 → A, U35 → G, C36 → A, G37 → A, C38 → A, G39 → U, U40 → C, U1 → G, A72 → C	This study	0.30
AspY258	$tRNA^{Asp}$ yeast, G27 → C, G28 → C, C29 → A, C31 → A, U32 → C, G34 → A, U35 → A, C36 → A, G37 → A, C38 → A, G39 → U, U40 → C, G41 → U, C42 → G, C43 → G, U1 → G, A72 → C	Dr. S. Auxilien	0.75
MetY326	$tRNA^{Met}_i$ yeast, A1 → G, U36 → A	Dr. F. Fasiolo	1.0
TyrY	$tRNA^{Tyr}_{(GUA)}$ yeast, wild-type	Dr. C. Florentz	0.90

A set of standard base pairs rather than the specific nucleotides in the anticodon stem enable isopentenylation of tRNA at A_{37}

To clarify the nature of identity determinants in the anticodon stem that are required for isopentenylation of A_{37}, additional variants were tested (Fig. 4B; Table 1). These mutants were derived from variants D and E which, as shown above, were fairly well modified by the *E. coli* isopentenyl-tRNA:A_{37} transferase (Fig. 4A).

Back reversion of U_{31}-A_{39} in variant D to C_{31}-G_{39} (mutant F) as in the wild-type $tRNA^{Ser}$(GGA), shows a drastic reduction of the modification rate as compared with the parent variant D. In variant G, where both base pairs G_{30}-C_{40} and G_{29}-C_{41}, as in variant D, were permuted into C_{30}-G_{40} and C_{29}-G_{41}, isopentenylation was completely abolished. Introduction in mutant D of a G*U wobble pair at position 29–41 (mutant H), or a standard A_{30}-U_{40} base pair (mutant I) reduced only the rate of A_{37} modification. The same effect was observed for variant K derived from mutant E by reversing G_{30}-C_{40} to C_{30}-G_{40} base pair. These three last tRNA mutants (H, I, K) become modified only partially after incubation for 2 h. The key role of the Watson–Crick G_{30}-C_{40} pair as major identity element in the proximal anticodon stem is seen clearly in variant L, which is derived from mutant D by mutation of C_{40} to U_{40}, thus creating a G_{30}*U_{40} wobble pair within the consensus sequence, resulting in complete loss of the ability of $tRNA^{Ser}$(GGA) to become isopentenylated (Fig. 4B; Table 1).

Tests with other tRNAs from *E. coli* and yeast

The validity of the proposed nucleotide consensus sequence in the anticodon stem (see Fig. 1B,C) as an important element for efficient isopentenylation of A_{37} into i^6A_{37} was also tested with six additional transcripts corresponding to tRNAs other than *E. coli* $tRNA^{Ser}$. These were, respectively, *E. coli* $tRNA^{Cys}$ (anticodon GCA), a mutant of *E. coli* $tRNA^{Phe}$ (with anticodon AAA instead of GAA), yeast $tRNA^{Tyr}$ (anticodon GUA), a mutant of yeast $tRNA^{Met}$ (anticodon CAA instead of CAU), and two variants of yeast $tRNA^{Asp}$ (Asp Y3 and Asp Y258, see Table 1). Mutant AspY3 derived from yeast $tRNA^{Asp}$ had a proximal anticodon stem that deviates from the consensus motif as defined

above at positions 29 and 41, whereas yeast $tRNA^{Tyr}$ (TyrY) had an A-U base pair at position 28–42 and the variant of yeast $tRNA^{Met}$ (MetY326) had both A_{28}-U_{42} and G_{31}-C_{39}, which deviate from the consensus motif (see Fig. 1B). Except for variant AspY3, which was modified less efficiently, all these tRNA molecules were isopentenylated efficiently (Table 1). Interestingly, *E. coli* $tRNA^{Cys}$, which contains a wobble G*U pair at position 27–43, had no influence on both the rate and the level of A_{37} isopentenylation (Table 1). Similarly, results with tRNA variants TyrY (wild-type yeast $tRNA^{Tyr}$) and MetY326 (mutant of yeast $tRNA^{Met}$) allow us to conclude that the presence of an A_{28}-U_{42} pair instead of the consensus G_{28}-C_{42} pair or C_{28}-G_{42} pair is well tolerated by the enzyme.

Mutations affecting 3D structure

To test the effect of mutations that alter the overall 3D tRNA structure on its suitability as a substrate for the *E. coli* isopentenyl-tRNA:A_{37} transferase, we introduced additional mutations in a few of the above variants of *E. coli* $tRNA^{Ser}$(GGA) (Fig. 2, bottom row). Variant M is derived from $tRNA^{Ser}$ mutant D by substituting C_8 in place of U_8 and thus destroying the tertiary U_8-A_{14} pair, which is known to play a major role in tRNA folding (Pan et al., 1991; Major et al., 1993). The results (Table 1) show that this mutation reduces significantly the level of A_{37} modification. In the case of tRNA variants K142 and K74a, both of which are derived from mutant K, no isopentenylation of A_{37} was found, whereas with variants I11 and I311, both of which were derived from tRNA mutant I, the effect appeared to be not too dramatic. These results allow us to conclude that i^6A formation is dependent on the overall tRNA architecture.

In vitro modification pattern of wild-type *E. coli* $tRNA^{Ser}$(GGA) and its variants

Naturally modified wild-type $tRNA^{Ser}$(GGA) contains several modified nucleotides (s^4U_8, Gm_{17}, D_{20}, D_{20a}, Ψ_{40}, T_{54}, and Ψ_{55}). Analysis of the wild-type $tRNA^{Ser}$ (GGA) transcript incubated in *E. coli* S100 extract indicates that, under experimental conditions used for i^6A_{37} formation, only U_{40}, U_{54}, and U_{55} become modified to Ψ_{40}, T_{54}, and Ψ_{55}. The positions 8 (s^4U), 17 (Gm), 20, and 20a (D) remain unmodified in vitro (results not shown). The modification pattern was also determined for several other $tRNA^{Ser}$ variants (mut D, mut L, mut I, mut M, and mut I311). These constructs were chosen to explore the possible influence of the anticodon stem/loop sequence and of tRNA 3D structure. As in the case of wild-type $tRNA^{Ser}$(GGA), only T_{54} and Ψ_{55} were formed quantitatively. As far as uridine-40 is concerned, this base is located in a region of the anticodon stem where multiple mutations were introduced (see Fig. 2). Thus, only three $tRNA^{Ser}$ variants (mut I, mut L, and mut I311) remain to be potential substrates for the corresponding pseudouridine synthase (*hisT*). Two $tRNA^{Ser}$ variants (mut I, mut L) demonstrate the identical kinetics of Ψ_{40} formation compared with the wild-type tRNA, whereas for the mut I311 (3D structure mutant), only trace amounts of Ψ_{40} were found. Thus, no correlation between the appearance of Ψ_{40} and i^6A_{37} was observed. The influence of position 20 was also tested directly using two pairs of tRNA variants differing only at that position (wild type and mutant F). Again, no difference in modification efficiency was detected. Although the dependence of certain tRNA modifications on the presence of the other modified nucleotides is plausible, this phenomenon has never been demonstrated clearly up to now. Likewise, the data presented here for $tRNA^{Ser}$(GGA) modification indicate that the other modifications in $tRNA^{Ser}$ are not affecting the outcome of the isopentenylation of A_{37}.

DISCUSSION

E. coli IPTT is sensitive to the type of base pairs in the proximal anticodon stem and to the overall architecture of tRNA

In the present work, we demonstrate that the base pairs at positions 29–41 and 30–40 in the anticodon stem are the most important ones for the efficient isopentenylation of A_{37} (see Fig. 1B). Both of these are preferably the purines at positions 29 and 30 (5′ side of the anticodon stem) and the pyrimidines at positions 40 and 41 (3′ side of the anticodon stem). The inversion of these two base pairs completely abolishes the ability of the tRNA to become isopentenylated at its A_{37}, whereas the inversion of only one of them considerably reduces the isopentenylation level achieved. Base pairs U-A or A-U or G-C at location 31–39 serve equally well. The top two locations of the five-base pair anticodon stem, namely 27–43 and 28–42, may not be involved in the recognition of tRNA by IPTT because even the noncanonical G*U base pair serves well at 27–43 location in *E. coli* $tRNA^{Cys}$.

The overall tRNA 3D structure is also important for recognition of tRNA transcript by IPTT. This is noticeable also in tRNA variants having a less suited A-U base pair at location 30–40. However, in general, the effect of such potential 3D structure-disrupting mutations seems to become pronounced only when coupled with other deviations from the consensus sequence of the proximal anticodon stem. Thus, the anticodon stem with the consensus sequence seems to withstand better the likely disruption of the overall 3D structure when the destabilizing mutations were introduced.

Altogether the results show that the modification enzyme responsible for the synthesis of i^6A has a marked preference for the G-C base pair at location 30–40, along with a canonical purine–pyrimidine base pair at location 29–41 in the anticodon stem. Thus, in spite of some distinct preferences indicated by the i^6A_{37} yield data, the absolute requirement for some specific base pair(s) at specific location(s) in the anticodon stem is not shown. This suggests that, for the recognition of tRNA substrates and possible anchoring of the IPTT in the anticodon stem, the avoidance of significant structural deviation due to mismatch and the retention of the appropriate stem flexibility close to the anticodon loop may be the key requirement. Thus, a range of alternative stem sequences and base pairings are permissible.

E. coli IPTT is also dependent on sequence and size of anticodon loop

Our data are consistent with the earlier findings from several laboratories showing that the sequence $A_{36}A_{37}A_{38}$ within a seven-membered anticodon loop is the major identity determinant for the *E. coli* isopentenyl-tRNA:A_{37} transferase. This conclusion arose essentially from systematic analysis of modification pattern for suppressor tRNA molecules (Carbon & Fleck, 1974; Roberts & Carbon, 1974; Prather et al., 1981a, 1983; Yarus et al., 1986; Raftery & Yarus, 1987), as well as for missense tRNA molecules (Murgola et al., 1983, 1984). However, depending on the type of mutated tRNAs, the yield of $i^6A_{37}/ms^2i^6A_{37}$ as formed under in vivo conditions varied significantly and, in some cases, was even very low (discussed in Murgola, 1985; Yarus et al., 1986). In retrospect, such observations can now be rationalized on the basis of the influence of other architectural peculiarities of the tRNA molecule, including the anticodon stem base pairs close to the anticodon loop.

Comparison with in vivo data

We report here that A_{37} in the transcript of a variant of yeast initiator $tRNA^{fMet}$ (anticodon CA**A**) harboring the three characteristic G-C pairs in anticodon stem, which corresponds to the consensus sequence, was isopentenylated quantitatively after incubation in *E. coli* extract. A similar situation has been reported recently for an *E. coli* strain transformed by a plasmid carrying the gene for *E. coli* initiator $tRNA^{fMet}$ harboring an amber anticodon C**UA** instead of the normal CA**U**. The resulting suppressor $tRNA^{Met}$ that appeared under such in vivo conditions contained the hypermodified nucleoside $ms^2i^6A_{37}$ at apparently high yield, whereas the parental $tRNA^{fMet}$ (anticodon CA**U**) had its A_{37} unmodified (Mangroo et al., 1995). Surprisingly, in a subsequent paper by the same group (Mandal et al., 1996), it was shown that the inversion of base pair G_{30}-C_{40} in this *E. coli* suppressor $tRNA^{Met}$ apparently did not affect the yield of $ms^2i^6A_{37}$ formation, whereas such a reversion in our $tRNA^{Ser}$ mutants resulted in appreciably lower i^6A_{37} yield in vitro in the present study (mutant K). This discrepancy may be attributed to the structural differences between the initiator and the elongator tRNAs, as well as to the presence of three consecutive G-C base pairs close to the anticodon loop, which may confer enhanced tolerance of C-G base pair at location 30–40 in the *E. coli* initiator $tRNA^{fMet}$. On the other hand, our data show that the $tRNA^{Ser}$ mutant with inverted C_{30}-G_{40} base pair displays a clearly reduced, but well measurable level of i^6A modification in vitro. It may be argued that, if the mutant of *E. coli* $tRNA^{fMet}$ could be modified in a similar way, possibly such a mutant molecule could be fully modified in vivo, because the half-life of tRNA molecule in the cell is significantly higher than 2 h. Also, unlike in the in vitro test, the increased intracellular concentration of the reaction components may overcome the reduced substrate activity of the mutant tRNA.

Earlier, using recombinant missense *E. coli* initiator $tRNA^{fMet}$, which had an anticodon change from CA**U** to CA**A**, we also observed some isopentenylation of A_{37} after its microinjection into the cytoplasm of *Xenopus laevis* oocyte (Grosjean et al., 1987). In this case, the yield of i^6A_{37} that formed in such a heterologous system was very low (less than 10% after 70 h incubation), whereas the parental *E. coli* initiator $tRNA^{fMet}$ (anticodon CA**U**) had its A_{37} modified quantitatively (100%) into threonylcarbamoyl adenosine (t^6A) after incubation for only a few hours. Thus, the yield of A_{37} modification in a given tRNA depends not only on the type of tRNA used, but also the type of mutation introduced in the tRNA and the origin of the modification enzyme.

Our results on tRNAs other than *E. coli* $tRNA^{Ser}$ (GGA) demonstrate that the trends discussed above apply substantially to these various tRNAs because they were modified predictably at A_{37} in *E. coli* extract. Nevertheless, one cannot exclude the existence of subtle structural differences in specific tRNAs that may somewhat modulate the tolerance specifics of tRNA recognition by IPTT.

Putative mode for tRNA recognition by the *E. coli* IPTT enzyme

Some analogy may exist between i^6A_{37} formation in *E. coli* $tRNA^{Ser}$(GGA) variants and queuosine formation by the prokaryotic tRNA:G_{34} transglycosylase (EC 2.4.2.29, also called Q-insertase). Like IPTT, this enzyme recognizes a trinucleotide sequence $U_{33}G_{34}U_{35}$ within a seven-membered anticodon loop and works only if the target sequence is part of a stem-loop struc-

ture (Nakanishi et al., 1994; Curnow & Garcia, 1995). The Q-insertase from *Zymomonas mobilitis*, a 23-kDa protein, has been crystallized and its structure was solved at 1.85 Å resolution. Remarkably, the authors could dock the target G_{34} into the active site of the enzyme without significant perturbation of the anticodon stem/loop (Romier et al., 1996).

If this model of recognition also applies to *E. coli* isopentenyl-tRNA:A_{37} transferase (35 kDa protein), then one may speculate as to how the enzyme interacts with the tRNA molecule. Figure 5A and B shows the spatial location of the amino group on C_6 of A_{37} within the structure of the anticodon stem-loop of yeast $tRNA^{Ser}$ (Dock-Bregeon et al., 1989), which is naturally

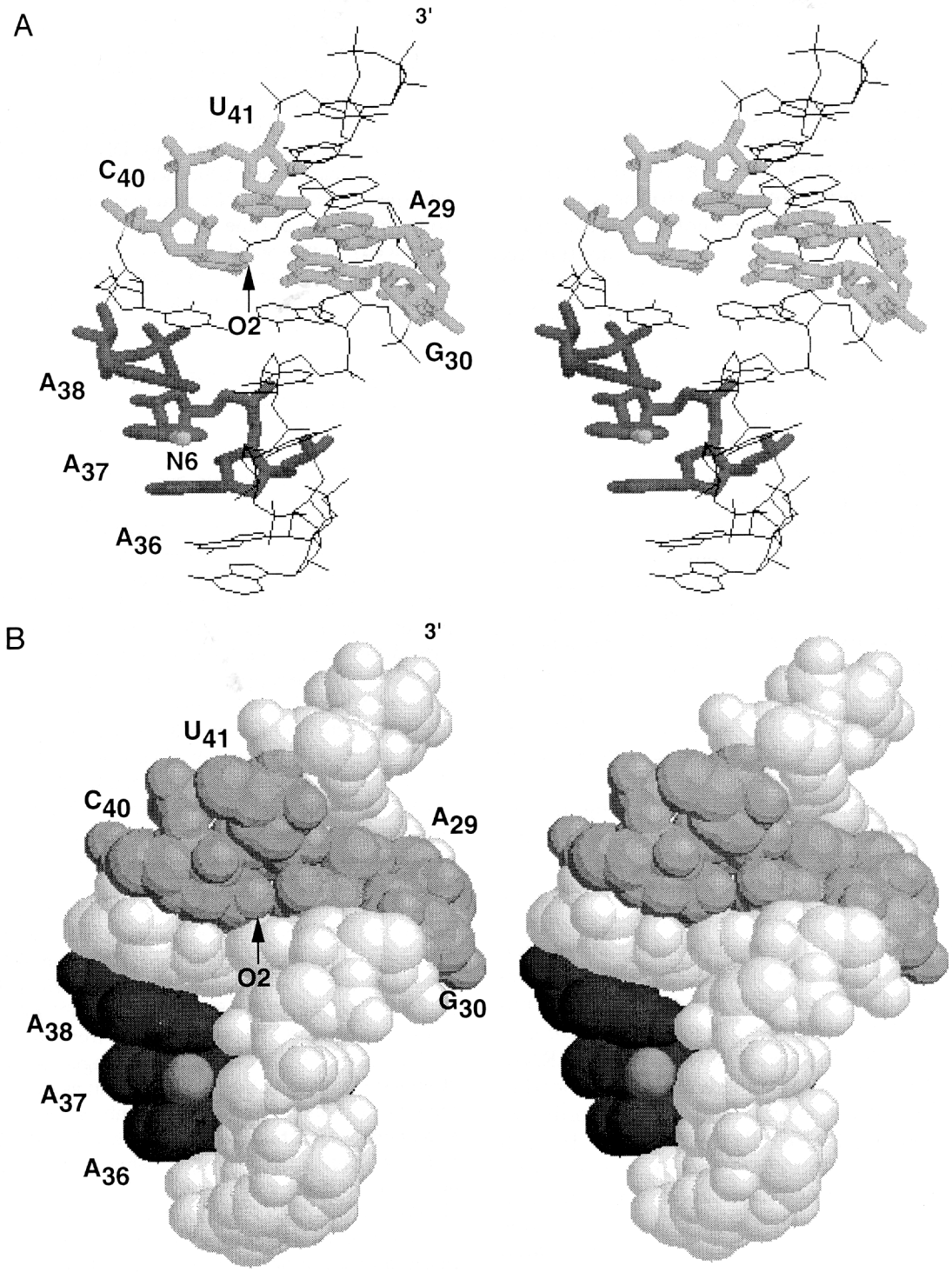

FIGURE 5. Stereo pairs for the structural model of the anticodon stem-loop of yeast $tRNA^{Ser}$ showing the bases and functional groups in tRNA participating in recognition by *E. coli* isopentenyl-tRNA:A_{37} transferase. **A:** Wireframe presentation. **B:** Space-filled presentation. In gray are the base pairs most likely involved directly in recognition by IPTT enzyme.

isopentenylated at position 37. To modify A_{37}, the enzyme may contact some additional bases of the RNA substrate, probably from the minor groove of the anticodon stem having A helical form RNA structure. Taking into account the observations of the present study, one may propose that the isopentenyl-tRNA:A_{37} transferase requires chemical details that are common to purines in positions 29, 30, and/or to pyrimidines in positions 40, 41, besides the preferred G_{30}-C_{40} base pair. These structural details have to be presented adequately in space in order to contact specific amino acids of the modifying enzyme. If this scenario is correct, then the most likely groups for such potential interactions are the exocyclic 2-NH_2 of guanosine-30 and the O_2 of cytosine-40 (see Fig. 5A,B). Indeed, in the cases where uridine replaces cytosine at position 40, due to the spatial displacement of the functional groups within the wobble G*U pair, potential loss of discrimination may occur. Also the inversion of G_{30}-C_{40} to C_{30}-G_{40} or its replacement by A_{30}-U_{40} may lead to a similar situation for reasons of the dislocation or absence of the exocyclic 2-amino group of G_{30} and O_2 of C_{40}. This model is reminiscent of the recognition of the G_3*U_{70} wobble base pair in the acceptor stem of $tRNA^{Ala}$ by alanyl-tRNA synthetase (Hou & Schimmel, 1988; Musier-Forsyth & Schimmel, 1993; Limmer et al., 1996; Liu et al., 1996). In the latter case, one single unpaired exocyclic amino group in the minor groove of the acceptor stem plays an essential role in the specific interaction with the cognate synthetase. However, one can also invoke an indirect recognition of the helix distorted due to the presence of G*U mismatch (Gabriel et al., 1996, see also Green, 1991). Modulation of the amino acid identity by a U_{30}*G_{40} pair in the anticodon stem has also been described for yeast amber $tRNA^{Ile}$ in *E. coli* (Buttcher et al., 1994).

The possibility also exists that IPTT binding induces substantial alterations of the anticodon loop and stem conformation. Such changes have been observed in the crystal structures of several tRNAs in complex with their cognate aminoacyl-tRNA synthetases (Rould et al., 1989; Ruff et al., 1991), as well as in solution by NMR analysis of the secondary and tertiary structures of *E. coli* $tRNA^{Ile}$ in a free and synthetase-bound state (Nureki et al., 1993). Likewise, upon binding to the sequence $A_{36}A_{37}A_{38}$, the *E. coli* isopentenyl-tRNA:A_{37} transferase may distort considerably the anticodon loop and the anticodon-proximal stem in order to maximize contact(s) with the active site. If such "conformational flexibility" of the anticodon stem-loop is essential for the efficiency of the reaction, one could also understand why different combinations of selected nucleotides having the same relative flexibility, rather than any particular nucleotide sequence, would allow the enzymatic reaction to occur or to abort.

MATERIALS AND METHODS

Enzymes and chemicals

α-[^{32}P]-radiolabeled adenosine triphosphate (400 Ci/mmol) was from Amersham (UK). Tris, DTE, nucleoside triphosphates, *Penicillium citricum* nuclease P1, phenylmethylsulfonylfluoride (PMSF), and Δ^3-isopentenyl-pyrophosphate were from Sigma (USA). Diethylpyrocarbonate (DEPC), diisopropylfluoro-phosphate (DFP), restriction enzymes, T_4 polynucleotide kinase, and T_4 DNA ligase were from Boehringer-Mannheim (Germany). Vector pGEM9Z-f(−) used for cloning, bacteriophage T_7 RNA polymerase, and RNasin were from Promega (USA). Thin-layer cellulose plates were from Schleicher & Schuell (Germany). DNA T_7 sequencing kits were from Pharmacia P-L Biochemicals (Sweden). MORPH™ site-specific plasmid DNA mutagenesis kit was purchased from 5prime → 3prime Inc. (Boulder, USA). Deprotected, chemically synthesized oligodeoxynucleotides were purchased from Eurogentec (Belgium) or Genset (France). All other chemicals were from Merck Biochemicals (Germany).

Construction and cloning of synthetic tRNA genes

The variants of synthetic yeast $tRNA^{Ser}$(GGA) gene were cloned in the multicloning site of a pGEM9Z-f(−) vector using *Sfi* I restriction site at the 5′-end and *Hin*d III site at the 3′-end. Constructions were such that all tRNA genes had an upstream T_7 RNA polymerase promoter and downstream *Eco*T 22I restriction site. Most synthetic variants of the yeast $tRNA^{Ser}$(GGA) genes (mutants A–K, F62, I11, K74a) were prepared as described by Perret et al. (1990), by stepwise hybridization and ligation of two sets of five complementary and partially overlapping synthetic oligonucleotides. Prior to the hybridization, all synthetic oligonucleotides were purified by electrophoresis on a 20% denaturing acrylamide gel then phosphorylated at their 5′ ends with ATP and T_4 polynucleotide kinase according to standard procedures described in Sambrook et al. (1989). Hybridization of the complementary oligonucleotide pairs was done at 80 °C followed by slow cooling to room temperature (in about 2–3 h). Ligation reactions were performed overnight at 16 °C with 1 unit of T_4 DNA ligase with doubly *Sfi* I/*Hin*d III-digested pGEM9Z-f(−) vector. Transformation was performed with competent cells derived from *E. coli* strain JM103 (D(*lac-proAB*), *thi*, *strA*, *supE*, *endA*, *sbcB* 15, *hsdR* 4, F′[*traD* 36, *proAB*$^+$, *lacI*^{q} *lacZ* DM15]). Other $tRNA^{Ser}$(GGA) variants (mut L, M, I311, K142) were derived from the mutants described above by site-directed mutagenesis on double-stranded DNA using the MORPH™ kit as described by the manufacturer and by Lacks and Greenberg (1977) and Kramer et al. (1984). Mutations were confirmed in each case by the dideoxy sequencing technique using T_7 DNA polymerase according to (Sanger et al., 1977). The oligonucleotide TGT GCTGCAAGGCGATT, complementary to the 3′-end of lac Z, was used as a sequence primer. Large- and small-scale plasmid preparations were done according to standard procedures (Sambrook et al., 1989). The gene for $tRNA^{Ser}$(CGA) (Ser-2) was recloned by PCR using plasmid pTOSer, kindly provided by Dr. R. Leberman (EMBL Outstation, Grenoble

France), with simultaneous addition of T7 polymerase promoter and linearization site (*Mva* I). The PCR product was cloned in pUC119 using *Hind* III and *Bam*H I sites. Plasmids carrying wild-type *E. coli* $tRNA^{Cys}$ and yeast $tRNA^{Tyr}$(GUA) were gifts from Dr. Y.-M. Hou (Univ. Thomas Jefferson, Philadelphia, Pennsylvania, USA) and Dr. C. Florentz (IBMC, Strasbourg, France) respectively. *E. coli* $tRNA^{Phe}$ mutant Phe 71 and yeast $tRNA^{fMet}$ (mutant MetY326) were gifts from Dr. O. Uhlenbeck, (Boulder, Colorado, USA), and Dr. F. Fasiolo (IBMC, Strasbourg, France), respectively.

In vitro transcription of the tRNA genes

All the tRNA substrates used in this work were radiolabeled with [^{32}P]ATP. They were produced by in vitro transcription of the corresponding genes in linearized plasmids by bacteriophage T7 polymerase as described previously (Perret et al., 1990). Routinely, 1 μg of the digested plasmid was lyophilized with 50–100 μCi [α-^{32}P]ATP. The lyophilized products were redissolved in 10 μL of reaction mixture containing 40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 10 mM DTE, 2 mM spermidine, 10 units RNasin, 1 mM each of GTP, UTP, and CTP, but only 100 μM final concentration of ATP. The reactions were initiated by the addition of 20 units T_7 RNA polymerase, and were incubated for 2–3 h at 37 °C. After transcription, the reaction was terminated by addition of EDTA to a final concentration of 10 mM and an equal volume of 8 M urea, 30% sucrose, 0.1% bromophenol blue, and 0.1% xylene cyanol. The tRNA transcript was denatured by heating for 2 min at 65 °C. The transcription product was further purified by electrophoresis on a 6% denaturing polyacrylamide gel. Only the full-length transcripts, as revealed by autoradiography of the gel, were used for further experiments. The tRNA transcripts were recovered from the gel by overnight elution at room temperature with a salt solution containing 0.5 M NH_4 acetate buffer, pH 5.0, 10 mM Mg-acetate, 0.1 mM EDTA, 0.1% (w/v) SDS. The eluted RNAs were ethanol-precipitated at −20 °C, washed with cold 70% ethanol, dried under a vacuum and redissolved in DEPC-treated water. They were stored at −20 °C until used.

E. coli S100 extract

The *E. coli* strain *MRE 600* (deficient in RNase A) was grown overnight at 37 °C in Luria–Bertani medium containing 0.5% bacto-yeast extract, 0.5% bacto-tryptone, and 1% NaCl. All the following operations were performed at 4 °C. Cells were harvested by low-speed centrifugation and resuspended in twice their weight of lysis buffer (25 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 10 mM Mg-acetate, 10% glycerol, 2 mM DTE, 1 mM PMSF, 1 mM DFP). The frozen cell suspension was passed through a French press at about 4,000–5,000 psi and the resulting homogenate was sonicated briefly (15 s) to destroy high-molecular mass DNA and centrifuged at 12,000 × *g* for 10 min to remove cell debris. The supernatant was harvested and centrifuged for 1 h at 100,000 × *g* in a Beckman TL-100 ultracentrifuge. The resulting supernatant "S100" (about 3–5 mg of total protein/mL) was quickly frozen in liquid nitrogen and kept at −80 °C until used.

In vitro system for testing A_{37} conversion into i^6A_{37} in tRNA

As was shown previously (Zeevi & Daniel, 1976), completely unmodified tRNA precursors, which can be produced easily in vitro from tRNA genes by purified RNA polymerase, are fairly good substrates for several tRNA modifying enzymes, including the IPTT enzyme, that are present in a *E. coli* S100 extract. The S100 extract also contained the enzyme that catalyzes the isomerization of the stable Δ^3-isopentenylpyrophosphate into the less stable Δ^2-isomer that is used by the isopentenyl-tRNA:A37 transferase (Caillet & Droogmans, 1988).

The standard reaction mixture for the detection of the isopentenyladenosine formation in tRNAs was composed of 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 0.034 mM Δ^3-isopentenylpyrophosphate, 1 mM ATP, 10% glycerol, 50–100 fmol (5–10 nM, final concentration) of [^{32}P]-labeled tRNA substrate in a final volume of 10 μL. Reaction was initiated by the addition of *E. coli* S100 extract to a final concentration about 0.3–0.5 mg of total protein/mL. After incubation at 37 °C, the reaction mixture was diluted to 0.1 mL, an equal volume of phenol:chloroform:isoamyl alcohol (50:48:2) was added, and denatured proteins were removed by centrifugation for 5 min at 10,000 × *g*. The traces of phenol remaining in the supernatant were extracted twice with diethyl ether. The nucleic acids were then ethanol-precipitated in the presence of 0.3 M sodium acetate at −20 °C, collected by centrifugation, dried, redissolved in 10 μL of 30 mM Na-acetate, pH 5.3, and hydrolyzed overnight at 37 °C into nucleoside 5′-phosphates with nuclease P_1 (0.3 U/μL).

Detection and quantification of isopentenyladenosine in tRNA transcripts

The presence of 5′-[^{32}P]isopentenyl AMP in tRNA hydrolyzates was detected by 1D or 2D thin-layer chromatography on cellulose plates, followed by autoradiography as described (Droogmans & Grosjean, 1991). The chromatographic systems were: first dimension developed with isobutyric acid/ammonia 25%/water (66:1:33, v/v/v), second dimension developed with 0.1 M sodium phosphate, pH 6.8/solid ammonium sulfate/*n*-propanol for the system 1 and with 2-propanol/HCl 37%/water (68:17.6:14.4, v/w/v) for the system 2. The relative amounts of i^6AMP and AMP were measured by cutting out the radiolabeled spots from the thin-layer chromatography plates and counting radioactivity by liquid scintillation techniques. To improve the accuracy, counting was performed with 3% error cut-off. Alternatively, quantification was performed using PhosphorImager (Molecular Dynamics, USA) with integrated software ImageQuant. Knowing the nucleotide composition of each of the tRNA substrates, the molar ratio of isopentenyladenosine per mole of tRNA formed during incubation with the extract was calculated. The experimental error was estimated to be about 0.1 mol/mol of tRNA after liquid scintillation technique, and as low as 0.05 mol/mol after quantification using PhosphorImager. These errors were estimated using the identically treated tRNA transcripts repeated in triplicate.

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