

Functional Importance of the 3'-Terminal Adenosine of tRNA in Ribosomal Translation*

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The universally conserved 3'-terminal CCA sequence of tRNA interacts with large ribosomal subunit RNA during translation. The functional importance of the interaction between the 3'-terminal nucleotide of tRNA and the ribosome was studied *in vitro* using mutant *in vitro* transcribed tRNA^{Val} A76G. Val-tRNA_{CCG} does not support polypeptide synthesis on poly(GUA) as a message. However, in a co-translation system, where Val-tRNA_{CCG} represented only a small fraction of total Val-tRNA, the mutant tRNA is able to transfer valine into a polypeptide chain, albeit at a reduced level. The A76G mutation does not affect binding of Val- or NAcVal-tRNA_{CCG} to the A- or P-sites as shown by efficient peptide bond formation, although the donor activity of the mutant NAcVal-tRNA_{CCG} in the peptidyl transfer reaction is slightly reduced compared with wild-type NAcVal-tRNA. Translocation of 3'-CCG-tRNA from the P- to the E-site is not significantly influenced. However, the A76G mutation drastically inhibits translocation of peptidyl-tRNA G⁷⁶ from the ribosomal A-site to the P-site, which apparently explains its failure to support cell-free protein synthesis. Our results indicate that the identity of the 3'-terminal nucleotide of tRNA is critical for tRNA movement in the ribosome.

The 3'-CCA sequence of tRNA is a universal ligand for protein biosynthesis; it is recognized by aminoacyl-tRNA synthetases, EF-Tu, and 23 S rRNA (1, 2). Experiments using *in vitro* transcribed tRNA^{Val} variants demonstrated the importance of the 3'-CCA sequence for aminoacylation (3, 4) and its significance in formation of the ternary complex between Val-tRNA, EF-Tu, and GTP (5). On ribosomes, the CCA end of tRNA interacts with 23 S rRNA at all ribosomal tRNA binding sites (6). The importance of the CCA end in ribosome-catalyzed peptide bond formation is well established (1, 7). Chemically synthesized aminoacyl oligonucleotides were used to demonstrate the significance of the 3'-CCA sequence as a peptide acceptor during peptide bond formation on ribosomes (8). *E. coli* tRNA^{Val} with mutations in the 3'-CCA sequence inhibits the peptidyltransferase activity of the ribosome (9). These findings were rationalized by showing functional base pairing of

C⁷⁴ with G²²⁵² of 23 S rRNA at the donor site of the ribosomal peptidyltransferase center (10) and of C⁷⁵ with G²⁵⁵³ at the acceptor site (11–13).

Mutation of C⁷⁴ to U⁷⁴ in tRNA^{His} derepresses the histidine biosynthetic operon of *Salmonella typhimurium* (14). Mutants of *Escherichia coli* tRNA^{Val}₁ with 3'-GCA or 3'-ACA promote –1 frameshifting and suppress a wide variety of nonsense mutants (14). It has also been proposed that substitution of the A⁷⁶ of tRNA affects binding of deacylated tRNA to the ribosomal E-site (15). These findings show the importance of the 3'-CCA end of tRNA in maintaining the reading frame during translation and suggest that the 3'-CCA end is involved in ribosomal translocation.

In this paper, we analyze the functional importance of the 3'-terminal A of tRNA for ribosomal translation. The results show that substitution of the 3' A of tRNA^{Val} with G blocks translocation of peptidyl-tRNA from the ribosomal A-site to the P-site and inhibits the peptidyl transfer reaction at the ribosomal donor (P) site.

MATERIALS AND METHODS

Preparation of tRNAs, Poly(GUA), and Enzymes

Transfer RNA^{Val} (anticodon UAC) was synthesized *in vitro* by T7 RNA polymerase-catalyzed run-off transcription from the phagemid pFVAL119 linearized by *FokI* (A76) or from pFVALG76 linearized by *MspI* (16). This method was shown to produce the expected nucleotide at the 3'-terminus (17). tRNA^{Val} variants were purified by size exclusion chromatography on Sephadex G-50 and gel electrophoresis in 7% urea-PAGE. Total *E. coli* tRNA was from Roche Molecular Biochemicals. tRNAs are designated as follows: tRNA_{CCA} (*in vitro* transcribed wild-type tRNA^{Val}), tRNA_{CCG} (*in vitro* transcribed tRNA^{Val} with the mutation A76G), tRNA_{bulk} (total (unfractionated) *E. coli* tRNA), and tRNA^{Val} (purified isoacceptors of valine-specific *E. coli* tRNA). 1 μg of tRNA^{Val} was taken as equal to 40 pmol.

DNA encoding the sequence poly(GTA)₄₄ was the kind gift of Dr. T. Tenson (University of Tartu). The poly(GTA)₄₄ was amplified by PCR and cloned under the control of the T7 late promoter in pLITMUS38 between the *ApaI* and *EcoRV* sites. The plasmid was cleaved by *BspTI*, and poly(GUA)₄₄ was prepared by *in vitro* transcription with T7 RNA polymerase. Poly(GUA) mRNA has the sequence 5'-GGCCCCGUA-GA(GUA)₄₄. Transcripts were purified by gel filtration chromatography on Sephadex G-50. 1 μg of poly(GUA)₄₄ was taken as equal to 25 pmol.

T7 RNA polymerase was purified as described by Davanloo *et al.* (18) from *E. coli* strain BL21(DE-3)/pAR1219 (kindly provided by W. Studier, Brookhaven National Laboratory, Upton, NY). To prepare ValRS(His)₆, the ValRS gene was PCR-amplified from *Thermus aquaticus* genomic DNA using primers GCCATATGGACCTGCCCAAGGCCTAC and CGGCGGCCGCCCTATTGGCTGAGGG. Amplified DNA was digested with *NdeI* and *NotI* and cloned into pET24A (Novagen) to produce pET24A-VTA. ValRS(His)₆ was expressed in BL21(DE-3) and purified using a Ni²⁺-nitrilotriacetic acid column (Qiaexpressionist). The enzyme was stored at –80 °C in buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM MgCl₂, 6 mM 2-mercaptoethanol, and 50% glycerol.

Plasmid encoding *E. coli* elongation factor EF-Tu-His (pKECA) was kindly provided by Dr. B. Kraal (19). EF-G-His expression plasmid

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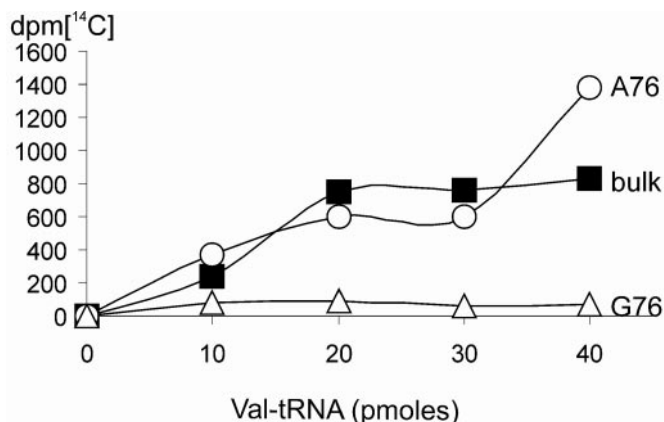


FIG. 1. Translation of poly(GUA) by Val-tRNA in a purified elongation factor-dependent system. *E. coli* ribosomes, poly(GUA), EF-Tu, and EF-G were incubated for 20 min with different concentrations of: bulk, unfractionated, native (modified) *E. coli* tRNA_{bulk} aminoacylated with [¹⁴C]Val (black squares); *in vitro* transcribed wild-type [¹⁴C]Val-tRNA_{CCA} (open circles); or *in vitro* transcribed G⁷⁶ mutant [¹⁴C]Val-tRNA_{CCG} (open triangles).

(pRSET) was a kind gift from Dr. M. Rodnina (20). The elongation factors were expressed and purified as described (19).

Aminoacylation

Transfer RNAs were aminoacylated by *T. aquaticus* ValRS as described by Liu and Horowitz (3), using [³H]Val or [¹⁴C]Val (Amersham Biosciences) with specific activities of 30,000 and 500 dpm/pmol, respectively. Charging levels were 20–25 pmol of Val/μg of tRNA for *in vitro* transcribed tRNA variants and 1.5 pmol of Val/μg of total *E. coli* tRNA. *N*-Acetylation of Val-tRNA variants was according to Haenni and Chapeville (21).

In Vitro Poly(GUA)-directed Translation

Ribosomes were isolated according to Rodnina and Wintermeyer (22). *In vitro* translation assays were carried out in buffer A (20 mM Tris-HCl, pH 7.6, 160 mM NH₄Cl, 12 mM MgCl₂, 5 mM 2-mercaptoethanol), essentially as described by Gavrilova *et al.* (23). Translation initiation complexes (mix R) were prepared by mixing 25 pmol of poly(GUA)₄₄ as mRNA, with 10 pmol of 70 S ribosomes in 15 μl of buffer A and incubating at 37 °C for 10 min. Mix T, which contained (in 20 μl of buffer A) 0–40 pmol of Val-tRNA variant, 10 μg of EF-Tu, 2 μg of EF-G, and 2.5 mM GTP, was incubated for 5 min at 30 °C. In co-translation experiments, mix R contained 0–8 pmol of ribosomes, 1 pmol of [³H]Val-tRNA variant, and 25 pmol of poly(GUA); mix T contained 20 pmol of *E. coli* tRNA_{bulk}, charged with [¹⁴C]Val, elongation factors, and GTP as above. If NAcVal-tRNA variants were used to initiate translation, mix R contained 20 pmol of NAc[³H]Val-tRNA_{CCA} or NAc[³H]Val-tRNA_{CCG}, 10 pmol of ribosomes, and 25 pmol of poly(GUA); mix T contained 0–40 pmol of [¹⁴C]Val-tRNA variant, elongation factors, and GTP. Both elongation factors were at saturating concentrations to avoid factor-dependent effects.

In all translation experiments, reactions were started by combining mix R with mix T. After a 20-min incubation at 37 °C, the reactions were stopped by the addition of 1.5 ml of 5% trichloroacetic acid, and the samples were heated at 95 °C for 20 min. Precipitates were collected on glass fiber filters, which were dried and counted in a scintillation spectrometer.

Dipeptide Synthesis Assay

Acceptor and donor activities of Val-tRNA in ribosomal peptide bond formation were analyzed by measuring dipeptide synthesis (in the absence of EF-G) using *in vitro* transcribed Val-tRNA and NAcVal-tRNA variants. Two mixes were prepared. Mix R, containing (per 30 μl) 25 pmol of poly(GUA)₄₄, 5 pmol of 70 S ribosomes, and 10 pmol of NAc[³H]Val-tRNA in buffer A, was preincubated for 10 min at 37 °C. Mix T, containing (per 20 μl) 0–8 pmol of [¹⁴C]Val-tRNA, 220 pmol of EF-Tu, 2.5 mM GTP, 2.5 mM PEP, and 10 units of PEP kinase in buffer A, was preincubated 5 min at 30 °C. Dipeptide bond formation was initiated by combining mix R and mix T. After incubating for 10 min at 37 °C, the reaction was stopped by the addition of NaOH to a final concentration of 0.6 M. Samples were further incubated for 20 min at 37 °C to hydrolyze aminoacyl- and peptidyl-tRNA and 200 μl of 5 N

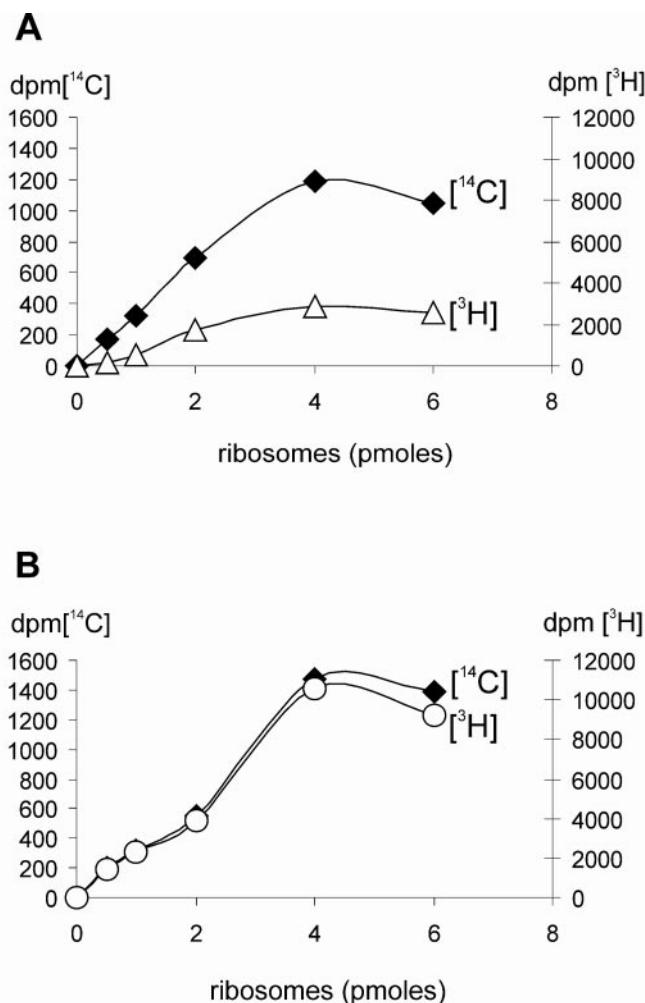


FIG. 2. Co-translation of poly(GUA) by wild-type tRNA^{Val} and the G⁷⁶ mutant of tRNA^{Val}. Translation of poly(GUA) was carried out in the presence of [¹⁴C]Val-tRNA_{bulk} (black diamonds), [¹⁴C]Val-tRNA_{bulk} plus [³H]Val-tRNA_{CCG}^{Val} (open triangles) (A), or [¹⁴C]Val-tRNA_{bulk} plus [³H]Val-tRNA_{CCA}^{Val} (open circles) (B) as described under "Materials and Methods."

H₂SO₄ were then added to lower the pH to <1. Finally, 1 ml of ethyl acetate was added with vigorous mixing. At the low pH, free NAc[³H]Val and the dipeptide NAc[³H]Val-[¹⁴C]Val are extracted into the organic phase, whereas [¹⁴C]Val remains in the water phase. Therefore, [¹⁴C] counts in the ethyl acetate phase are a measure of the NAc[³H]Val-[¹⁴C]Val formed. Under conditions of the experiment, no trichloroacetic acid-precipitable peptides were found, indicating that longer peptide chains are not formed.

Translocation

EF-G-dependent translocation was analyzed according to Watanabe (24).

A- to P-site Translocation—To assay the effect of G⁷⁶ on the translocation of tRNA from the ribosomal A-site to the P-site, the ribosomal P- and E-sites were filled with deacylated tRNA_{CCA} in the presence of poly(GUA) by incubating 20 μl of mix R, containing 5 pmol of 70 S ribosomes, 25 pmol of poly(GUA)₄₄, 20 pmol of tRNA_{CCA} in buffer A (final Mg²⁺ concentration, 12 mM) at 37 °C. After 10 min, 10 pmol of either NAc[³H]Val-tRNA_{CCA} or NAc[³H]Val-tRNA_{CCG} were added and allowed to bind to the ribosomal A-site by incubation at 37 °C for 20 min. The amount of NAc[³H]Val-tRNA_{CCA} bound was determined by nitrocellulose filter assay (25). Translocation was promoted by the addition of variable amounts of EF-G (0–2.5 pmol) and 1 mM GTP (final concentration) and incubating for either 10 min at 37 °C or 2 h on ice (final volume, 35 μl). At the end of the incubation period, all tubes were placed on ice. Similar results were obtained when the experiment was performed at 15 or 20 mM Mg²⁺.

The amount of translocation was determined by measuring P-site bound NAc[³H]-Val-tRNA_{CCA} or NAc[³H]-Val-tRNA_{CCG} with the puro-

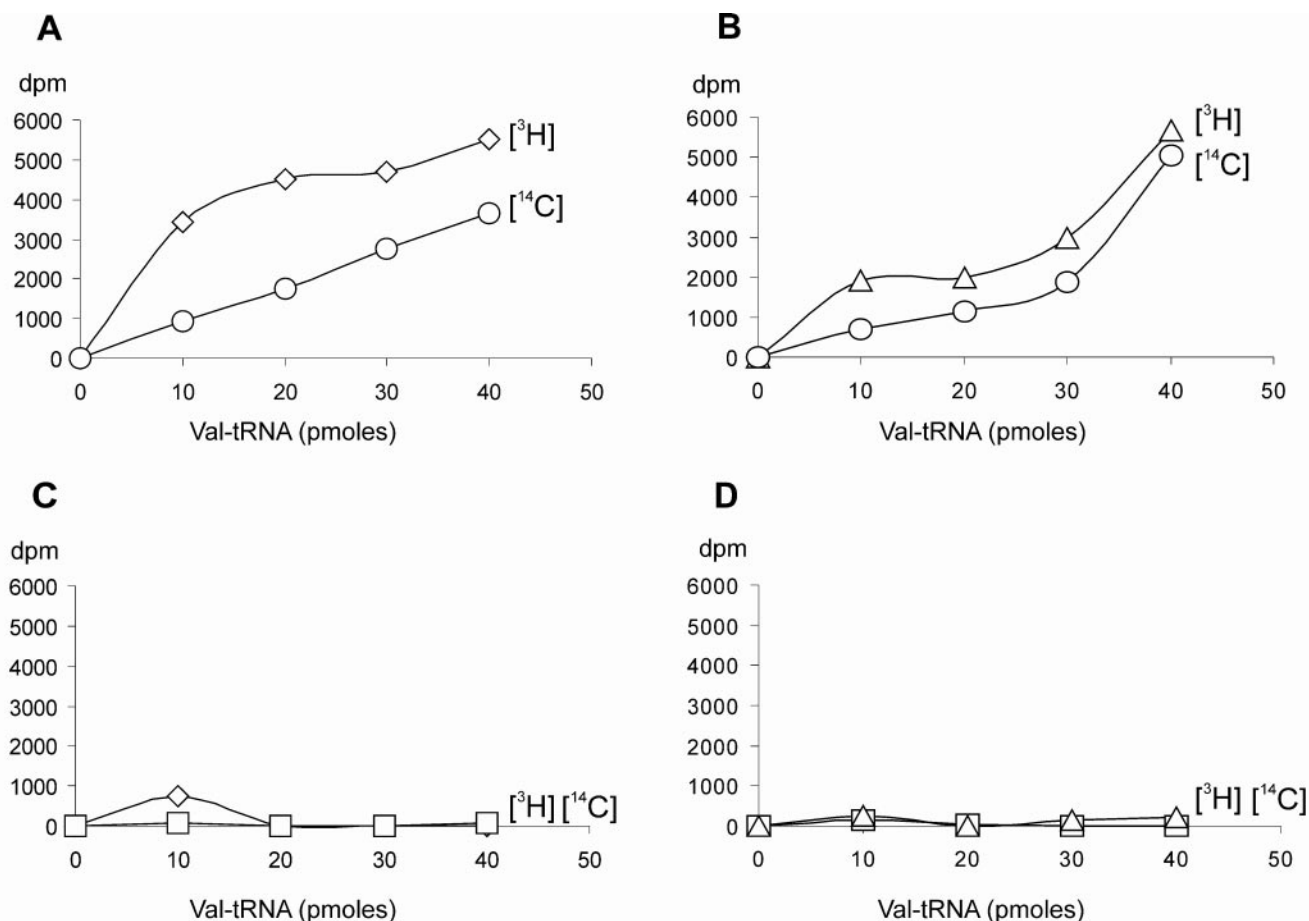


FIG. 3. Activity of *in vitro* transcribed tRNA^{Val} variants as initiator and elongator substrates in poly(GUA)-directed translation. Translation initiation complexes were formed by incubating NAc³H]Val-tRNA^{Val} variants with ribosomes and poly(GUA). Elongation occurred after the addition of [¹⁴C]Val-tRNA_{CCA} or [¹⁴C]Val-tRNA_{CCG} together with elongation factors and GTP. A, NAc³H]Val-tRNA_{CCA} (open diamonds) and [¹⁴C]Val-tRNA_{CCA} (open circles). B, NAc³H]Val-tRNA_{CCG} (open triangles) and [¹⁴C]Val-tRNA_{CCA} (open circles). C, NAc³H]Val-tRNA_{CCA} (open diamonds) and [¹⁴C]Val-tRNA_{CCG} (open squares). D, NAc³H]Val-tRNA_{CCG} (open triangles) and [¹⁴C]Val-tRNA_{CCG} (open squares).

mycin reaction. Mixes were incubated in the presence of 1 mM puromycin at 0 °C for 30 min. The reaction was stopped by the addition of sodium hydroxide (0.6 M final concentration) and incubated 15 min at 37 °C to hydrolyze any remaining aminoacyl-tRNA. After neutralization with 0.2 ml of 1 M potassium phosphate (pH 7.6) NAc³H]Val-puromycin was extracted with 1 ml of ethyl acetate. The amount of NAc³H]Val-puromycin formed was determined by scintillation counting of the ethyl acetate phase.

P- to E-site Translocation—This assay is similar to that of the A- to P-site translocation assay except that the ribosomal P- and E-sites were occupied with either deacylated tRNA_{CCA} or deacylated tRNA_{CCG} in the presence of poly(GUA) by preincubating 25 μ l of mix R (5 pmol of 70 S ribosomes, 25 pmol of poly(GUA)₄₄, 20 pmol of tRNA_{CCA} or tRNA_{CCG} in buffer A) at 37 °C for 10 min. Subsequently, 10 pmol of NAc³H]Val-tRNA_{CCA} were added and allowed to bind to the ribosomal A-site at 37 °C for 20 min. Translocation was initiated as described for the A- to P-site translocation assay, and the amount of NAc³H]Val-tRNA_{CCA} translocated to the P-site was measured by the puromycin reaction. The A-site tRNA can only go to the P-site after P to E translocation has occurred; thus, if tRNA_{CCG} blocks this movement, then A to P translocation cannot occur.

RESULTS

Translation of Poly(GUA) by *in Vitro* Transcribed Val-tRNA^{Val} Variants—Prior work showed that *in vitro* transcribed tRNA^{Val} variants C⁷⁶ and U⁷⁶, while readily accepting valine, are inactive in polypeptide synthesis (3, 9) largely because of a decreased affinity for EF-Tu·GTP (5). In contrast, tRNA^{Val} with a 3'-terminal guanine (G⁷⁶), which is a poor substrate for valyl-tRNA synthetase but can be aminoacylated at high enzyme concentrations, does function in translation as shown by its ability to transfer valine into poly(Val,Phe) in a poly(U₃,G)-

directed reaction, but only if Phe-tRNA is also present (3). We have therefore restricted our studies to the G⁷⁶ variant of tRNA^{Val}. The ability of this mutant tRNA to support synthesis of poly(Val) on *E. coli* 70 S ribosomes in an *in vitro* poly(GUA)-directed system was compared with that of wild-type tRNA^{Val} (A⁷⁶). Aminoacylated tRNAs were utilized to eliminate effects due to differences in the rates of aminoacylation (3). Purified *E. coli* translation elongation factors EF-Tu and EF-G were used in these experiments, and no aminoacyl-tRNA synthetases or CCA-adding enzyme activity were present. Therefore, incorporation of valine into polypeptides is exclusively from added Val-tRNA.

Valine is incorporated into polypeptide from both native (modified) *E. coli* [¹⁴C]Val-tRNA_{bulk} and from *in vitro* transcribed [¹⁴C]Val-tRNA_{CCA} at levels proportional to the [¹⁴C]Val-tRNA added (Fig. 1). In control experiments without poly(GUA), no trichloroacetic acid-precipitable valine incorporation was observed (results not shown). Under conditions of the experiment the unmodified tRNA^{Val} transcript is quite active in poly(Val), indicating that modified nucleotides are not essential for translation on the ribosome. Others have observed that tRNAs lacking modified nucleotides are only marginally less efficient in translation than native (modified) tRNAs (26–29) (for a review, see Ref. 30).

In contrast to wild-type Val-tRNA^{Val}, the mutant [¹⁴C]Val-tRNA_{CCG} is completely inactive in poly(GUA)-directed translation (Fig. 1). Although the G⁷⁶ variant of tRNA^{Val} alone is not active, it does transfer valine into polypeptides in a co-trans-

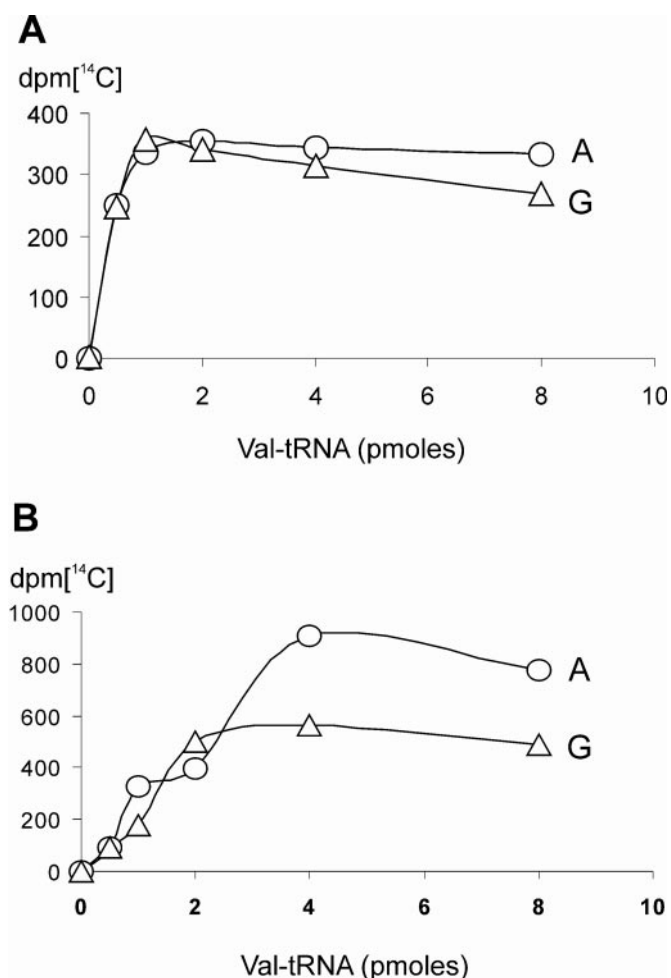


FIG. 4. Activity of Val-tRNA variants in dipeptide formation on ribosomes. A, acceptor activity of Val-tRNA variants in dipeptide formation. NAc[³H]Val-tRNA_{CCA} was bound to the ribosomal P-site in the presence of poly(GUA), and increasing amounts of [¹⁴C]Val-tRNA_{CCA} (open circles) or [¹⁴C]Val-tRNA_{CCG} (open triangles) complexed with EF-Tu and GTP were added. Dipeptide formation was determined as described under "Materials and Methods." B, donor activity of NAcVal-tRNA variants in dipeptide formation. NAc[³H]Val-tRNA_{CCA} (open circles) or NAc[³H]Val-tRNA_{CCG} (open triangles) was bound to the P-site of poly(GUA)-programmed ribosomes, and increasing amounts of [¹⁴C]Val-tRNA_{CCA} complexed with EF-Tu and GTP were added. Dipeptide formation was determined as described under "Materials and Methods."

lation reaction with wild-type Val-tRNA^{Val}. When [³H]Val-tRNA_{CCG} is mixed with an excess of [¹⁴C]Val-tRNA_{bulk} (molar ratio 1:20) in the poly(GUA)-directed translation system, [³H]Val from the mutant tRNA_{CCG} transcript is incorporated into polypeptides (Fig. 2A). This result is consistent with the finding of Liu and Horowitz (3) that the Val-tRNA_{CCG} transcript can participate in ribosomal translation *in vitro*. Comparison with incorporation of [³H]Val from wild-type [³H]Val-tRNA_{CCA} into polypeptides in the co-translation system demonstrates that the G⁷⁶ mutant is considerably less active (Fig. 2, compare A and B). Furthermore, incorporation of [¹⁴C]Val (from wild-type [¹⁴C]Val-tRNA_{bulk}) into peptides is one-third lower in the presence of the G⁷⁶ tRNA^{Val} mutant (Fig. 2, A and B), indicating that Val-tRNA_{CCG} inhibits poly(GUA) translation by native (A⁷⁶) Val-tRNA^{Val}.

The possibility that valine is transesterified from tRNA_{CCG} to tRNA_{CCA} was examined by adding deacylated wild-type tRNA_{CCA} to a reaction in which [¹⁴C]Val-tRNA_{CCG} is the only source of valine. If transesterification is responsible for incorporation of radioactivity from Val-tRNA_{CCG} into poly(Val) in

the co-translation assay, incorporation should be stimulated by the added deacylated tRNA_{CCA}. Such stimulation was not observed (data not shown), and we conclude that transesterification of valine from mutant to wild-type tRNA does not occur in our system.

To test the donor activity of Val-tRNA variants in poly(GUA)-directed translation, NAcVal-tRNA was added to the reaction. N-acetyl aminoacyl-tRNA can serve only as a donor substrate for peptide bond formation and acts as an initiator tRNA in this assay. For these experiments, ribosomal initiation complexes were formed by incubating NAc[³H]Val-tRNA_{CCA} or NAc[³H]Val-tRNA_{CCG} with ribosomes and poly(GUA), followed by a second incubation step in the presence of either [¹⁴C]Val-tRNA_{CCA} or [¹⁴C]Val-tRNA_{CCG}, elongation factors, and GTP. It is evident, from the results, shown in Fig. 3, that [³H]Val is incorporated into polypeptides when either NAc[³H]Val-tRNA_{CCA} or NAc[³H]Val-tRNA_{CCG} serves as the initiator tRNA, as long as wild-type [¹⁴C]Val-tRNA_{CCA} is the elongator tRNA (Fig. 3, A and B). In contrast, polypeptide synthesis does not occur when [¹⁴C]Val-tRNA_{CCG} serves as the elongator tRNA, independent of which tRNA^{Val} variant is used for initiation (Fig. 3, C and D). These results show that the G⁷⁶ mutant of tRNA^{Val} is active as a donor substrate for ribosomal peptide bond formation. It is not clear, however, whether the G⁷⁶ tRNA^{Val} mutant can serve as an acceptor at the peptidyltransferase center, because reactions other than peptide bond formation may be affected by the mutation. The results suggest that the G⁷⁶ tRNA^{Val} mutant cannot participate in poly(GUA)-directed translation due to a defect in peptide chain elongation.

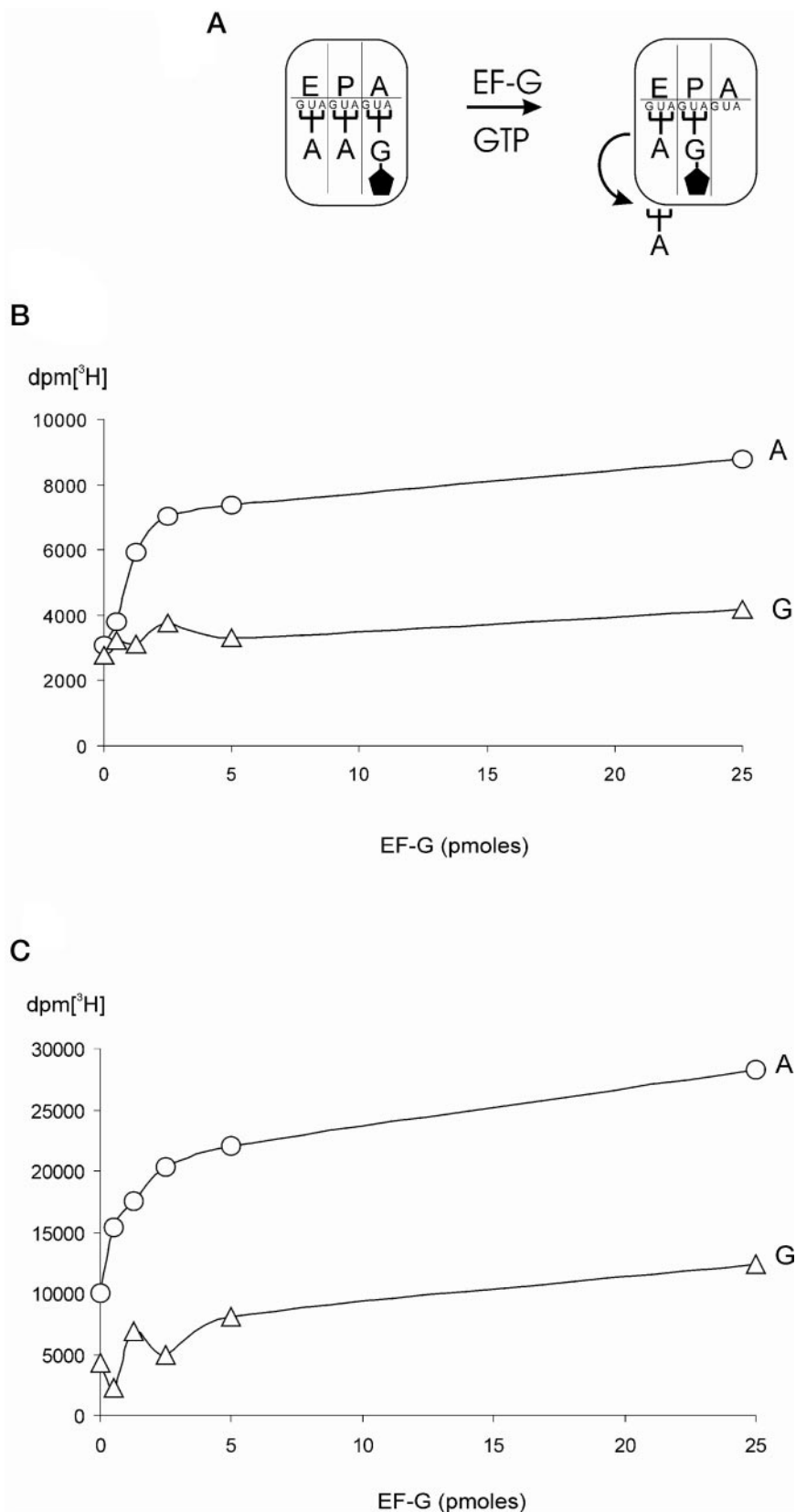
Dipeptide Synthesis—The inability of the G⁷⁶ tRNA^{Val} mutant to function in poly(GUA)-directed translation was examined further by analyzing the effects of this mutation on peptidyltransferase activity as measured by dipeptide formation.

To investigate the acceptor activity of Val-tRNA variants in peptide bond synthesis, NAc[³H]Val-tRNA_{CCA} (A⁷⁶) was bound to the ribosomal P-site in a codon-dependent manner, and dipeptide synthesis was followed after the addition of increasing amounts of either [¹⁴C]Val-tRNA_{CCA} or [¹⁴C]Val-tRNA_{CCG}, complexed with EF-Tu and GTP. Fig. 4A shows that wild-type tRNA^{Val} and the G⁷⁶ variant are active as acceptors in peptide bond formation. Clearly the A to G transition at position 76 of tRNA^{Val} does not affect acceptor activity of tRNA^{Val} in our system. This result agrees with the observation that substitutions in the CCA sequence have only small effects on the acceptor activity of chemically synthesized aminoacyl-oligonucleotides in the ribosomal peptidyltransferase reaction (8).

Donor activity of the G⁷⁶ tRNA^{Val} mutant, was determined by binding either NAc[³H]Val-tRNA_{CCG} or wild-type NAc[³H]Val-tRNA_{CCA} to poly(GUA)-programmed 70 S ribosomes and measuring the extent of dipeptide synthesis after incubation with increasing concentrations of [¹⁴C]Val-tRNA_{CCA}-EF-Tu-GTP. The results show that both substrates, NAcVal-tRNA_{CCG} and NAcVal-tRNA_{CCA} are active as donors in peptide bond formation, although the G⁷⁶ variant is slightly less efficient (Fig. 4B). This result agrees with the observation that NAc[³H]Val-tRNA_{CCG} can serve as initiator tRNA in poly(GUA)-directed translation (Fig. 3B). NAcVal-tRNA_{CCG} was also shown to be active as the donor in ribosome-catalyzed peptide bond formation when puromycin was the acceptor substrate (9).

The dipeptide synthesis experiments show that the G⁷⁶ tRNA^{Val} mutant can participate in ribosomal peptide bond formation both as an acceptor and as a donor substrate. Therefore, the inability of tRNA_{CCG} to function in poly(GUA)-directed translation cannot be attributed to a defect in peptide bond formation *per se*.

FIG. 5. Effect of the G⁷⁶ mutation on the EF-G-dependent translocation of NAcVal-tRNA from the ribosomal A-site to the P-site. A, diagrammatic representation of the experiment to test the effect of G⁷⁶ on translocation from the A- to the P-site; the control experiment using only wild-type (A⁷⁶) tRNA is not depicted. The identity of the 3'-terminal nucleotide of tRNA^{Val} is shown; the *pentagon* represents the NAcVal moiety. B and C, after preincubation with deacylated wild-type (A⁷⁶) tRNA^{Val} to saturate the ribosomal P- and E-sites, NAc^[3H]Val-tRNA_{CCA} (*open circles*) or NAc^[3H]Val-tRNA_{CCG} (*open triangles*) was bound to the ribosomal A-site, and EF-G was added at the concentrations indicated. Translocation of the peptidyl-tRNA analog from the A-site to the ribosomal P-site at 0 °C (B) or 37 °C (C) was determined by the puromycin reaction.



A- to P-site Translocation—To determine the effects of the 3'-terminal nucleotide of tRNA^{Val} on the movement of tRNA from the ribosomal A-site to the P-site, the P- and E-sites were preincubated with deacylated wild-type tRNA_{CCA} under conditions where the P- and E-sites are known to bind tRNA (6, 31–34). Then either NAc^[3H]Val-tRNA_{CCA} or NAc^[3H]Val-

tRNA_{CCG} was bound to the A-site (see Fig. 5A). The efficiency of NAc^[3H]Val-tRNA^{Val} binding to ribosomes, as determined by a nitrocellulose filter-binding assay (25), was the same for both NAc^[3H]Val-tRNA^{Val} variants (data not shown). In each case, 20–30% of the NAc^[3H]Val-tRNA^{Val} was bound to the P-site without addition of EF-G, as determined by the ability to react

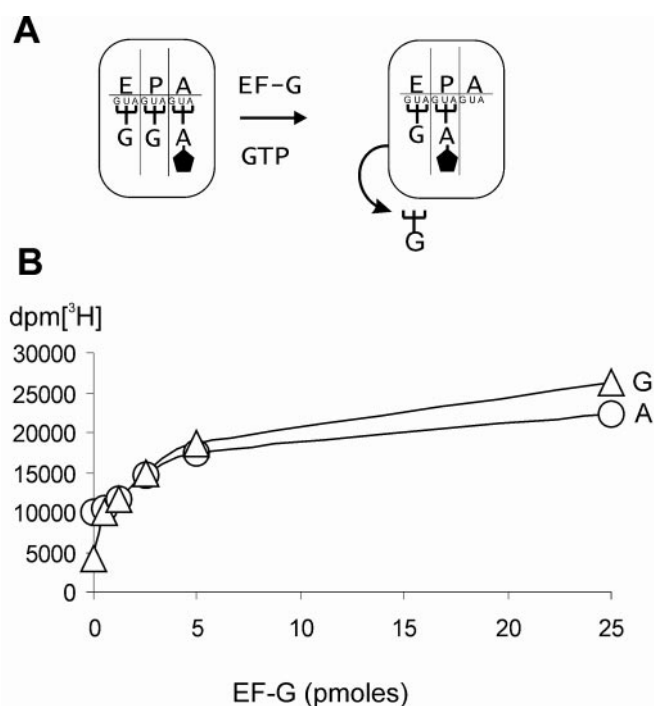


FIG. 6. Effect of the G⁷⁶ mutation on the EF-G-dependent translocation of NAcVal-tRNA from the ribosomal P-site to the E-site. A, diagrammatic representation of the experiment to test the effect of G⁷⁶ on translocation from the P- to the E-site; the control experiment using only wild-type (A⁷⁶) tRNA is not depicted. Identity of the 3'-terminal nucleotide of tRNA^{Val} is shown; the pentagon represents the NAcVal moiety. B, deacylated tRNA_{CCA} (open circles) or tRNA_{CCG} (open triangles) were bound, in a codon-dependent manner, to the ribosomal P- and E-sites, followed by the binding of NAc³H]Val-tRNA_{CCA} to the A-site. The indicated concentrations of EF-G were then added, and translocation of NAc³H]Val-tRNA_{CCA} to the ribosomal P-site was determined by the puromycin reaction.

with puromycin, whereas 70–80% was bound to the A-site. Movement of the NAc³H]Val-tRNA^{Val} variants from the ribosomal A- to the P-site was determined by measuring P-site bound NAc³H]Val-tRNA^{Val} by the puromycin reaction, after incubation with increasing concentrations of EF-G and GTP (see “Materials and Methods”). At 0 °C, EF-G-stimulated translocation occurs only with wild-type NAc³H]Val-tRNA_{CCA}; the mutant NAc³H]Val-tRNA_{CCG} is not translocated from the A-site to the P-site (Fig. 5B). At 37 °C, EF-G-dependent translocation of NAc³H]Val-tRNA_{CCG} does occur, but at a much lower level than that of wild-type NAc³H]Val-tRNA_{CCA} (Fig. 5C).

In control experiments, NAc³H]Val-tRNA_{CCA} or NAc³H]Val-tRNA_{CCG} bound directly to the P-site readily react with puromycin to the same degree (data not shown). Thus, the failure of NAc³H]Val-tRNA_{CCG} to react with puromycin is due to inefficient translocation and not to its inability to react with puromycin from the P-site.

P- to E-site Translocation—The 3'-terminal nucleotide of tRNA has been shown to play a role in the binding of deacylated tRNA to the ribosomal exit site (E-site) (15, 32). Therefore, substitution of A⁷⁶ may inhibit movement of deacylated tRNA from the ribosomal P-site to the E-site during translocation and thereby inhibit translation.

The effect of the G⁷⁶ mutation in tRNA^{Val} on the translocation of tRNA from the ribosomal P-site to the E-site was tested in a manner similar to that for A- to P-site translocation. Poly(GUA)-programmed 70 S ribosomes were incubated with either deacylated tRNA_{CCA} or tRNA_{CCG} under conditions where the P- and E-sites are known to bind tRNA (6, 31–34). In a second incubation step, wild-type (A⁷⁶) NAc³H]Val-tRNA_{CCA} was bound to the ribosomal A-site (see Fig. 6A). Similar

amounts of NAc³H]Val-tRNA_{CCA} were bound to ribosomes with both prebound tRNA^{Val} variants (10,000 dpm with tRNA_{CCA} and 12,000 dpm with tRNA_{CCG}), as determined by nitrocellulose binding assay (data not shown). Translocation of NAc³H]Val-tRNA_{CCA} from the A-site to the puromycin reactive P-site was promoted by the addition of increasing concentrations of EF-G. Elongation factor-dependent translocation of NAc³H]Val-tRNA_{CCA} from the A-site to the P-site, as measured by the puromycin reaction, is essentially the same, regardless of which tRNA^{Val} variant is present at the P- and E-sites (Fig. 6B), clearly indicating that the G⁷⁶ tRNA^{Val} mutant is displaced from the ribosomal P- and E-sites as readily as wild-type (A⁷⁶) tRNA^{Val}.

Based on these results, we can conclude that replacing A⁷⁶ in tRNA^{Val} with G⁷⁶ interferes with EF-G-dependent ribosomal A- to P-site translocation, and this explains the failure of Val-tRNA_{CCG} to function in poly(GUA)-directed translation.

DISCUSSION

In this study, we have examined the functional role of the 3'-terminal nucleotide of tRNA^{Val} in ribosomal translation by comparing the activity of *in vitro* transcribed wild-type tRNA^{Val} (A⁷⁶) with that of the G⁷⁶ mutant in translation of the cognate (GUA) codon and in individual steps of the polypeptide elongation cycle. Our results show that the G⁷⁶ Val-tRNA^{Val} mutant is completely inactive in poly(GUA)-directed translation when present alone (Fig. 1), suggesting that the A76G mutation affects a step in polypeptide synthesis on the ribosome. Co-translation experiments, carried out in the presence of excess native (A⁷⁶) tRNA^{Val}, demonstrate that the mutant tRNA_{CCG} can function in poly(GUA)-directed translation although with reduced efficiency (Fig. 2A).

To identify the step affected by the G⁷⁶ mutation, each phase of the polypeptide elongation cycle was individually investigated. Studies of the activity of tRNA_{CCG} in dipeptide synthesis show that the G⁷⁶ mutation does not significantly affect the acceptor activity of Val-tRNA in the peptidyltransferase reaction (Fig. 4A). Substitutions in the CCA sequence have also been shown to have only small effects on the acceptor activity of aminoacyl-oligonucleotides in the peptidyl transfer reaction (8).

In contrast, the donor activity of NAcVal-tRNA_{CCG} is reduced by 20–30% compared with that of NAcVal-tRNA_{CCA} in both poly(GUA)-directed translation (Fig. 3) and in the dipeptide synthesis assay (Fig. 4B). Evidently, the identity of the 3'-nucleotide of tRNA is more important at the donor site and less important at the acceptor site of the ribosomal peptidyltransferase center. Crystallographic data of aminoacyl- and peptidyl-tRNA analogs bound to *Haloarcula marismortui* 50 S ribosomes (7) indicate that N-1 of A⁷⁶ of the tRNA at the donor site acts as a hydrogen bond acceptor from the 2'-OH of A²⁴⁵⁰ of the 23 S rRNA and the base stacks on the ribose of A²⁴⁵¹. A guanine at position 76 of tRNA can still stack efficiently on the ribose of A²⁴⁵¹ but will have difficulty forming the appropriate hydrogen bonds with the 2'-OH group of A²⁴⁵⁰ because its N-1 is protonated, unlike that of A⁷⁶. This may account for the reduced donor activity of the G⁷⁶ variant of tRNA^{Val} at the peptidyl transfer center.

It has been proposed that binding of tRNA to the E-site promotes movement of the tRNA-mRNA complex with respect to the ribosome (15). The removal or substitution of A⁷⁶ decreases the affinity of the ribosomal E-site for tRNA at least 100-fold (15, 32, 35). Failure of the G⁷⁶ mutant of tRNA^{Val} to translate poly(GUA) may be due to the inability of the G⁷⁶ mutant to bind correctly to the E-site, thus blocking movement of tRNA from the P- to the E-site. Our results, however, reveal that the A76G mutation does not interfere with the translocat-

tion of tRNA from the P-site to the E-site (Fig. 6B). In this connection, it is interesting to note that the 3'-end of deacylated tRNA that is formed after transpeptidation does not immediately progress to the E-site but remains temporarily at the peptidyltransferase center, as shown by recent cross-linking experiments (6). It is possible that the G⁷⁶ tRNA^{Val} mutant can dissociate from ribosomal P-site without stably binding to the E-site.

The most striking result of the A⁷⁶ to G⁷⁶ substitution in tRNA^{Val} is the nearly complete inhibition of A- to P-site tRNA translocation. The experiments presented in Fig. 5 clearly demonstrate that translocation of the peptidyl-tRNA analog, NAc[³H]Val-tRNA, from the A- to the P-site is severely inhibited by the A76G mutation, thus accounting for the inability of the G⁷⁶ mutant of tRNA^{Val} to translate poly(GUA). This result is in contrast to the ability of the G⁷⁶ mutant of tRNA^{Val} to bind to both the A- and the P-sites, as inferred from its activity both as an acceptor and a donor in peptide bond formation (Fig. 4). According to the hybrid state model, the 3'-end of tRNA moves concomitantly with peptide bond formation from the acceptor to the donor site leading to the P/A hybrid state (36). This movement and the related conformational changes (37) are expected to depend on correct interaction between the CCA end of tRNA and 23 S rRNA. The affinity of aminoacyl-oligonucleotides for the ribosomal donor site is higher by 1 order of magnitude compared with the acceptor site (38, 39). This affinity gradient can be essential for movement of the CCA end of peptidyl-tRNA during translocation (40). If the G⁷⁶ variant of peptidyl-tRNA does not bind correctly to the ribosomal donor site, as implied by its reduced donor activity (Figs. 3 and 4B), it can affect movement of the CCA end of tRNA and thereby inhibit translocation. On the other hand, it is possible that A⁷⁶ of peptidyl-tRNA is specifically recognized by 23 S rRNA or a ribosomal protein during the translocation reaction. One candidate for such recognition is nucleotide A²⁶⁰², which is disordered in the empty 50 S subunit and becomes positioned between the CCA bound at the A-site and the CCA bound at the P-site after tRNA binding (7). A second candidate is ribosomal protein L²⁷, which can be cross-linked to A⁷⁶ at both the A- and the P-sites (41).

Feinberg and Joseph have recently identified two 2'-OH groups, at positions 71 and 76, which are required for tRNA translocation from the P- to the E-site (42). This result is in agreement with the finding that 2'-deoxy-A⁷⁶-substituted tRNA inhibits ribosomal translocation (43). We have shown that the adenosine at position 76 of tRNA is essential for translocation from the A- to the P-site (Fig. 5). The importance of specific functional groups of tRNA for movement from the A-site to the P-site and from the P-site to the E-site suggests an active ribosomal mechanism for translocation, with essential transient interactions between tRNA and the ribosome.

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