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Aminoacyl RNA domain of turnip yellow mosaic virus Val-RNA interacting with elongation factor Tu

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ABSTRACT

Turnip yellow mosaic virus (TYMV) Val-RNA forms a complex with the peptide elongation factor Tu (EF-Tu) in the presence of GTP: the Val-RNA is protected by EF-Tu·GTP from non-enzymatic deacylation and nuclease digestion. The determination of the length of the shortest TYMV Val-RNA fragment that binds EF-Tu·GTP leads us to conclude that the valylated aminoacyl RNA domain equivalent in tRNAs to the continuous helix formed by the acceptor stem and the T arm is sufficient for complex formation. Since the aminoacyl RNA domain is also sufficient for adenylation by the ATP(CTP):tRNA nucleotidyltransferase, an analogy can be drawn between these two tRNA-specific proteins.

INTRODUCTION

The genome of several plant RNA viruses can be aminoacylated at the 3' terminus with a specific amino acid. Interestingly, folding of the tRNA-like regions of viral RNAs differs greatly from that of tRNAs (for reviews see ref. 1,2).

The secondary structure of the tRNA-like region of the valine-accepting turnip yellow mosaic virus (TYMV) RNA (3-5) is presented in fig. 1. In this structure, the acceptor stem (stem I) is formed by folding of only the 3' part without participation of the 5' part of the tRNA-like region. Therefore, the aminoacyl RNA domain, equivalent in tRNAs to the continuous helix formed by the acceptor stem and the T arm, constitutes an independent part of the molecule. Because of this unique feature, the determination of the length of the shortest 3'-terminal viral RNA fragment that can be adenylated led us to conclude (5,6) that the aminoacyl RNA domain is sufficient for adenylation by the ATP(CTP):tRNA nucleotidyltransferase (CCA enzyme).

Despite numerous investigations, the structural features in the tRNA molecule required for ternary complex formation between aminoacyl-tRNA and EF-Tu·GTP are still not entirely elucidated.

In the present communication, we demonstrate that TYMV Val-RNA forms a complex with EF-Tu·GTP since EF-Tu·GTP protects TYMV Val-RNA from non-enzym-

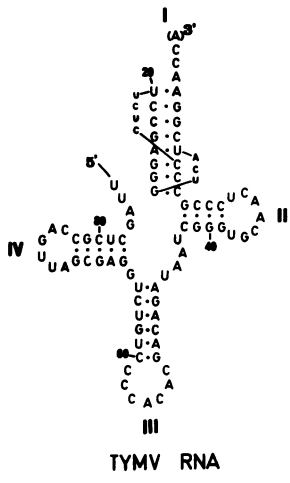

Fig. 1. Secondary structure of the tRNA-like region of TYMV RNA. I to IV correspond to stems and loops analogous in tRNAs to the acceptor, the T, the anticodon and the D stems and loops. We consider here the aminoacyl RNA domain as composed of regions I and II, and the anticodon RNA domain as composed of regions III and IV. Every 20th nucleotide is numbered.

atic deacylation and from nuclease digestion. The determination of the length of the shortest TYMV Val-RNA fragment that forms a complex with immobilized EF-Tu·GTP enables us to conclude that the valylated aminoacyl RNA domain fulfills the requirements for complex formation.

MATERIALS AND METHODS

Materials

TYMV-infected Chinese cabbage leaves were generously supplied by S. Astier-Manifacier and P. Cornuet (I.N.R.A., Versailles) and TYMV was purified by the method of Leberman (7). The viral RNA was extracted (8) under RNase-free conditions and stored at -70°C. Bulk yeast tRNA was from Sigma.

The Escherichia coli Val-tRNA synthetase (EC 6.1.1.9) was a partially or a highly purified preparation kindly supplied by S. Blanquet (Ecole Polytechnique, Palaiseau). Purified E. coli CCA enzyme (EC 2.7.7.25) was a generous gift of D. Eusèbe-Carré. EF-Tu·GDP from E. coli (specific activity 22 kU/mg) and EF-Tu·GDP from Thermus thermophilus (specific activity 12 kU/mg) were purified according to Gulewicz et al. (9). RNase T_1 (EC 3.1.27.3) was from Sankyo, and RNase A (EC 3.1.27.5) and pyruvate kinase (EC 2.7.1.40) were from Boehringer. The ^{3}H-valine was either from Amersham (30 Ci/mmol) or from New England Nuclear (60 Ci/mmol).

Aminoacylation

Aminoacylation was performed for 15 min at 37°C in 500 µl containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 2 mM ATP, 150 µg of CCA enzyme, 75 µg of partially purified Val-tRNA synthetase, 3.5 µM

^{3}H-valine (30 Ci/mmol) and 0.5 mg of TYMV RNA or of yeast tRNA; a 5 µl aliquot was removed to determine the cold trichloroacetic acid (TCA) precipitable radioactivity, the remainder was ethanol precipitated and the pellet dried and stored at -70°C.

The ^{3}H-Val-RNA of TYMV used for partial RNase T_1 digestion was prepared as described above except that the reaction was performed at 30°C and that the incubation mixture contained ^{3}H-valine at 60 Ci/mmol and 2 nM highly purified Val-tRNA synthetase.

Formation of EF-Tu·GTP

The E. coli EF-Tu·GTP complex was obtained by incubating EF-Tu·GDP (3 nmoles) with 75 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 75 mM NH_4Cl, 50 mM KCl, 10 mM DTT, 1 mM GTP, 5 mM phosphoenolpyruvate (PEP) and 20 µg of pyruvate kinase in 50 µl for 20 min at 37°C and transferring to ice. In these conditions the EF-Tu·GTP complex was stable for several hours.

Protection from RNase A

A solution (25 µl) containing 50 mM Na borate pH 7, 10 mM $MgCl_2$, 75 mM NH_4Cl, 1 mM DTT, 100 µM GTP and 0.25 µM TYMV ^{3}H-Val-RNA or yeast ^{3}H-Val-tRNA was incubated for 2 min at 30°C with E. coli EF-Tu·GTP as indicated in the legend of fig. 3 and transferred to ice for 2 min; 5 µl aliquots were removed to determine the cold TCA precipitable radioactivity (time 0). RNase A (1.5 mU or 2.5 mU for Val-tRNA or TYMV Val-RNA respectively) was added and 5 µl aliquots were removed after 1 and 2 min at 0°C to determine the cold TCA precipitable radioactivity.

Protection from deacylation

A solution (100 µl) containg 75 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 75 mM NH_4Cl, 5 mM DTT, 100 µM GTP, 2.5 mM PEP, 15 µg of pyruvate kinase and 0.25 µM TYMV ^{3}H-Val-RNA was incubated at 30°C with or without 5 µM E. coli EF-Tu·GTP. At different times, 5 µl aliquots were removed to determine the cold TCA precipitable radioactivity.

Partial RNase T_1 digestion of TYMV ^{3}H-Val-RNA

To obtain TYMV ^{3}H-Val-RNA fragments, 400 µg of TYMV ^{3}H-Val-RNA were preincubated in 600 µl of 20 mM Na citrate pH 5, 7 M urea and 5 mM EDTA for 5 min at 37°C and then cooled in ice. The sample was divided into four parts, RNase T_1 (1, 2, 5 or 10 mU/µg RNA) was added and incubation performed at 37°C for 20 min. After repeated phenol extraction, the aqueous phases were extracted with ether to remove residual phenol, pooled and ethanol precipitated; the dried pellet was stored at -70°C.

Affinity chromatography of TYMV ^{3}H-Val-RNA fragments

EF-Tu·GDP from Th. thermophilus (∿25 mg) covalently bound to cyanogen bromide-activated Sepharose 4B (∿1.5 ml) as described by Derwenskus et al. (10) was used for affinity chromatography. Matrix-bound EF-Tu·GDP was converted to EF-Tu·GTP by washing the column (0.3 x 5 cm) with buffer I (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 50 mM NH_4Cl, 50 mM KCl, 1 mM DTT and 1 mM GTP) for about 2 h at room temperature.

The TYMV ^{3}H-Val-RNA fragments (∿100 µg) in 250 µl of buffer II (10 mM HEPES pH 7.5, 10 mM $MgCl_2$, 10 mM NaCl, 1 mM DTT and 50 µM GTP) were applied to the EF-Tu·GTP column and incubated for 10 min at 30°C. The column was then successively washed step-wise at room temperature with buffer II, buffer III (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 50 mM NH_4Cl, 1 mM DTT and 50 µM GTP) and buffer IV (100 mM Na borate pH 7.5, 10 mM $MgCl_2$, 1 M NaCl, 1 mM DTT and 50 µM GTP). In a separate experiment, following buffer II, the column was wahsed with buffer II' (identical to buffer II except that it contained 50 mM NaCl) before applying buffer III. A constant flow-rate of ∿100 µl/min was maintained and fractions of 450 µl were collected.

The elution profile was monitored by determining the radioactivity contained in 5 µl aliquots from each fraction. The appropriate fractions were pooled and ethanol precipitated; the dried pellets were dissolved in 50 µl of sample buffer (5 mM Tris-borate pH 7, 7 M urea, 1 mM EDTA, 0.01% xylene cyanol and 0.01% bromophenol blue) and analyzed by polyacrylamide gel electrophoresis.

TYMV ^{3}H-Val-RNA fragments protected against RNase A

A solution (100 µl) containing 50 mM Na borate pH 7, 10 mM $MgCl_2$, 75 mM NH_4Cl, 1 mM DTT, 100 µM GTP and 50 µg of TYMV ^{3}H-Val-RNA fragments was incubated for 2 min at 30°C with 5 µM E. coli EF-Tu·GTP and transferred to ice for 2 min. RNase A (25 mU) was added and after 1 or 2 min at 0°C the reaction was stopped by phenol and the aqueous phases were ethanol precipitated; the dried pellets were dissolved in 50 µl of sample buffer and analyzed by polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis

Polyacrylamide (12%), bis-acrylamide (0.6%), 7 M urea gels were used at pH 7 (5,11-13). The reservoir buffers were constantly recycled and electrophoresis performed at 4°C. After a pre-run at 450 V overnight, the samples were loaded and electrophoresis performed at 700 V for 8 h. The gel was trea-

ted sequentially with 10% cold TCA, dimethylsulfoxide (DMSO), DMSO-PPO (2,5-diphenyloxazole), water (14) and finally dried (15) and exposed at -70°C using a Fuji medical X-ray film.

RESULTS

Complex formation between TYMV Val-RNA and EF-Tu·GTP

The first indication suggesting interaction between TYMV Val-RNA and E. coli EF-Tu·GTP was reported by Haenni et al. (16): as demonstrated by the nitrocellulose filter binding assay (17), the radioactivity of the binary complex EF-Tu·^{3}H-GTP retained on the filter decreased with increasing Val-RNA added, but not with uncharged RNA. To further verify that TYMV Val-RNA forms a complex with EF-Tu and GTP, the following approaches were used.

1. Beres and Lucas-Lenard (18) have reported that EF-Tu·GTP effectively protects aminoacyl-tRNAs from non-enzymatic deacylation. At pH 7.5 and 30°C, TYMV Val-RNA is deacylated with a pseudo first-order rate and a half-life of 135 min (fig. 2). When incubated with EF-Tu·GTP, the rate of deacylation of TYMV Val-RNA is significantly slower (half-life ∿13 h) thus demonstrating complex formation between TYMV Val-RNA and EF-Tu·GTP.

2. EF-Tu·GTP is known to protect aminoacyl-tRNAs from nuclease digestion (19). This is verified with Val-tRNA in fig. 3A: after 2 min at 0°C, 90% of the Val-tRNA (0.25 µM) is protected when incubated with EF-Tu·GTP (2.5 µM) as opposed to almost complete degradation in the absence of EF-Tu·GTP. Based on the curves of fig. 3A, about 0.5 µM EF-Tu·GTP protects 50% of the Val-tRNA after 1 min of incubation. TYMV Val-RNA (0.25 µM) is also protected from RNase A digestion by EF-Tu·GTP as visible in fig. 3B. However, protection is less efficient since >2.5 µM EF-Tu·GTP are necessary to obtain 50% protection of the TYMV Val-RNA after 1 min of incubation. Consequently the affinity of

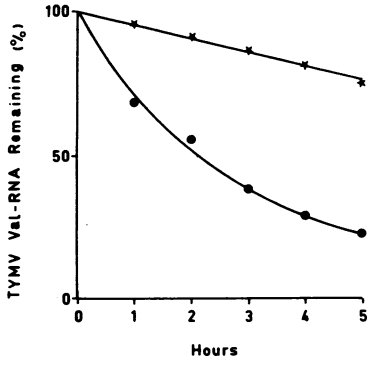

Fig. 2. Protection from non-enzymatic deacylation of TYMV ^{3}H-Val-RNA by EF-Tu·GTP. TYMV Val-RNA (0.25 µM) was incubated at 30°C and pH 7.5 in the absence (-●-) or in the presence (-✱-) of 5 µM EF-Tu·GTP. 100% correspond to 15 000 cpm.

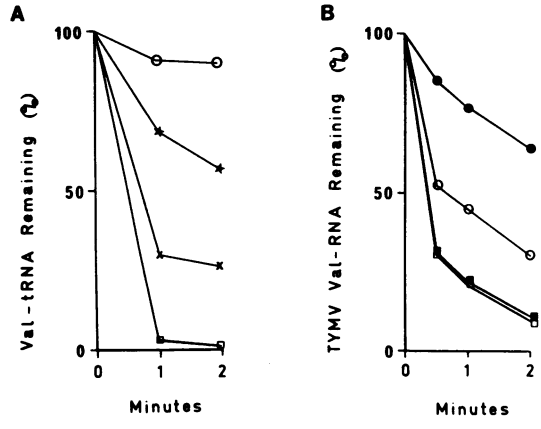

Fig. 3. Protection from RNase A of ^{3}H-Val-tRNA or of TYMV ^{3}H-Val-RNA by EF-Tu·GTP. (A) Val-tRNA or (B) TYMV Val-RNA (0.25 µM) was digested by RNase A in the absence (-□-) or in the presence of EF-Tu·GTP (-●- 5 µM, -o- 2.5 µM, -✱- 0.75 µM, -x- 0.375 µM). -■-: TYMV Val-RNA digested by RNase A in the presence of 5 µM EF-Tu·GDP. 100% correspond to 15 000 cpm.

TYMV Val-RNA for EF-Tu·GTP is about 5 times lower than that of Val-tRNA. Since, when in 20 fold excess, EF-Tu·GTP but not EF-Tu·GDP protects TYMV Val-RNA from RNase digestion, the conformation adopted by EF-Tu upon GTP binding is a prerequisite for complex formation.

Binding of TYMV Val-RNA 3'-terminal fragments to immobilized EF-Tu·GTP

To establish the length of the shortest TYMV Val-RNA 3'-terminal fragment that can still form a complex with EF-Tu·GTP, the following strategy was adopted. TYMV RNA was aminoacylated with ^{3}H-valine and the ^{3}H-Val-RNA was partially digested by RNase T_1. The resulting Val-RNA fragments were chromatographed on a column of immobilized EF-Tu·GTP. This column was recently used successfully for the selective purification of aminoacylated tRNAs (10). The Val-RNA fragments appearing in the effluent (buffer II) as well as those eluting at increased ionic strengths (buffers III and IV) were pooled as indicated in fig. 4 and analyzed by polyacrylamide gel electrophoresis. On the fluorogram of fig. 5, the Val-RNA fragments that appeared in the effluent (pool 1 of fig. 4) are presented in lane 3 and those that appeared in the eluate (pool 2 of fig. 4) are shown in lane 4. A sample of the total Val-RNA fragments applied to the EF-Tu·GTP column is presented in lane 2, whereas lane 1 shows the profile of TYMV Val-RNA prior to RNase T_1 digestion. A comparison of lanes 3 and 4 with lane 2 shows that valylated fragments of 47 nucleotides or longer are retained on the EF-Tu·GTP column. In this experi-

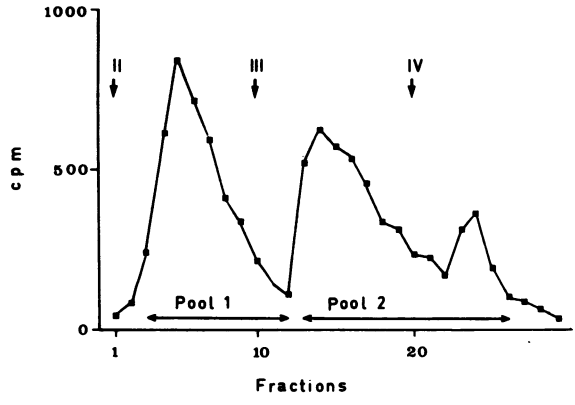

Fig. 4. Elution profile of the RNase T_1 fragments of TYMV ^{3}H-Val-RNA from the EF-Tu·GTP column. II, III and IV refer to the buffers applied to the column. Fractions were pooled as indicated.

ment elution of the EF-Tu·GTP-complexed valylated RNA fragments with buffer III was started before all the short (<47 nucleotides) fragments interacting only very weakly with immobilized EF-Tu·GTP had been removed from the column. In another experiment, after loading of the Val-RNA fragments and washing with buffer II, buffer II' was applied to the column; lane 5 presents the profile of the EF-Tu·GTP-complexed Val-RNA fragments that eluted with buffers III and IV. Only valylated fragments equal to or longer than 47 nucleotides in length were detected by polyacrylamide gel electrophoresis under these conditions.

A comparison of lanes 4 and 5 indicates that the 47 nucleotide-long fragment is the shortest RNase T_1 fragment from the 3' end of TYMV Val-RNA capable of interaction with EF-Tu·GTP in the conditions defined by buffer II' and at ambient temperature. The interaction however, is improved when the chain length is increased: maximal efficiency is achieved at a chain length of 65 nucleotides.

Some degradation of Val-RNA fragments has occurred during chromatography: in particular the high molecular weight TYMV Val-RNA fragments present in lane 2 are absent from lanes 3-5.

Shortest TYMV Val-RNA fragment protected from RNase A by EF-Tu·GTP

Since the valylated aminoacyl RNA domain of TYMV RNA is recognized by EF-Tu·GTP, one could expect it to be protected against RNase A. To verify this possibility, the RNase T_1 fragments of TYMV ^{3}H-Val-RNA were further incubated in the presence of EF-Tu·GTP and RNase A. The RNase A-resistant material recovered after 1 or 2 min was analyzed by polyacrylamide gel elec-

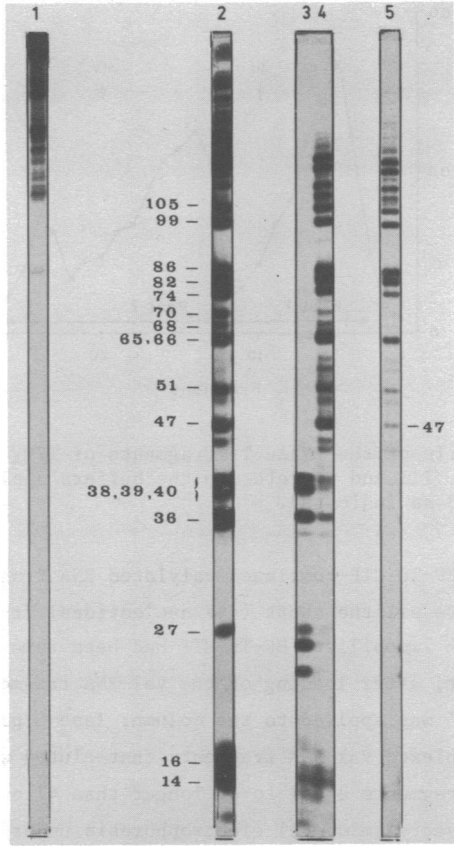

Fig. 5. Gel electrophoretic determination of the RNase T_1 fragments of TYMV ^{3}H-Val-RNA bound to immobilized EF-Tu·GTP. Lane 1: TYMV Val-RNA (30 µg); lane 2: Val-RNA fragments (50 µg); lanes 3 and 4: unbound (effluent, pool 1 of fig. 4) and bound (eluate, pool 2 of fig. 4) Val-RNA fragments (100 µg) respectively; lane 5: bound Val-RNA fragments from a separate experiment in which the column with immobilized EF-Tu·GTP was washed following buffer II with 5 ml of buffer II'. The specific activity of TYMV Val-RNA was ∿10 000 cpm/µg. Exposure time: lanes 1, 3-5: 4 days and lane 2: 6 days. All the samples presented were analyzed on the same gel. Numbering refers to the length of the TYMV Val-RNA fragments obtained by partial RNase T_1 digestion.

trophoresis (fig. 6). All the large Val-RNA fragments complexed to EF-Tu·GTP tend to be shortened to oligonucleotides between ∿112 and 55 (or 56), the latter resulting from cuts within the anticodon loop. Consequently the anticodon RNA domain is accessible to RNase A digestion whereas the aminoacyl RNA domain is not. This is in accordance with the 'footprinting' experiments performed using aminoacyl-tRNA and EF-Tu·GTP (20,21).

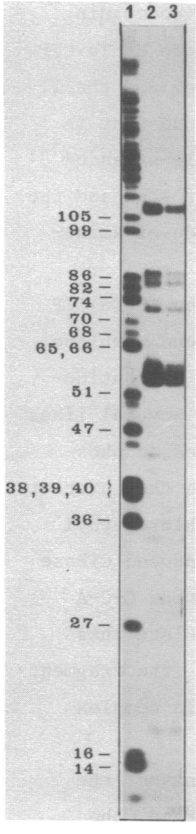

Fig. 6. Gel electrophoretic determination of the length of the shortest RNase T_1 fragment of TYMV ^{3}H-Val-RNA protected by EF-Tu·GTP from RNase A. Lane 1: Val-RNA fragments (30 µg); lanes 2 and 3: Val-RNA fragments (100 µg) incubated with EF-Tu·GTP, then digested by RNase A for 1 and 2 min respectively. Numbering is as in fig. 5. The specific activity of the Val-RNA was ∿4000 cpm/µg. Exposure time was 9 days.

DISCUSSION

It is now established that the aminoacyl residue and the backbone of the single-stranded 3' end in aminoacyl-tRNA are essential for recognition by EF-Tu·GTP (reviewed in ref. 21); however the possible requirement of other structural features of tRNA is not well documented.

The present study of the interaction of TYMV Val-RNA fragments with immobilized EF-Tu·GTP desmonstrates that the shortest Val-RNA fragment able to form a sufficiently stable complex with EF-Tu·GTP is 47 nucleotides long. Interestingly, in the tRNA-like structure of TYMV RNA (fig. 1), the aminoacyl RNA domain is formed within the 47 nucleotides from the 3' terminus. Consequently, the aminoacylated aminoacyl RNA domain fulfills the requirements for complex formation with EF-Tu·GTP and the anticodon RNA domain is not essential. This conclusion is further supported by the observation that EF-Tu·GTP protects the valylated aminoacyl RNA domain from digestion by RNase A.

The studies of Boutorin et al. (20) and Wikman et al. (21) concerning the protection from RNase digestion of aminoacyl-tRNA complexed to EF-Tu·GTP have shown that the C-C-A end, the remainder of the acceptor stem and the T stem are protected. In light of these and our results, we conclude that in the aminoacylated aminoacyl RNA domain, in addition to the single-stranded 3' end with the aminoacyl residue, the helix formed by the acceptor stem and the T stem constitutes an essential feature for EF-Tu·GTP recognition of aminoacyl-tRNAs.

It can be speculated that the aminoacyl-tRNA binding site on EF-Tu·GTP comprises a site for the positioning of the single-stranded 3' end with the aminoacyl residue, as well as a site for the positioning of the 12 continuously stacked base pairs that compose the aminoacyl RNA domain. Several lines of evidence support this model: (i) 2'-(3')-O-aminoacyl-dinucleoside phosphates such as C-A-Phe can interact with E. coli EF-Tu·GTP since they protect cysteine 81 from modification by L-1-tosylamido-2-phenylethyl chloromethyl ketone (22,23); (ii) aminoacyl-tRNAs stimulate the kirromycin-induced GTPase activity of EF-Tu; in the presence of kirromycin, the trinucleotide C-C-A bearing an aminoacyl residue, or tRNA devoid of C-C-A, can stimulate this GTPase activity (24); (iii) the 3'-half molecule of Val-tRNA^{Val}, the fragment U-C-C-A-C-C-A-Ala, or denatured Leu-tRNA^{Leu} fail to form a stable complex with EF-Tu·GTP (25-27).

A similar situation occurs with another tRNA-recognizing enzyme, the CCA enzyme. Again two regions in the tRNA molecule can be discerned, the reacting 3' end, and the non-reacting portion which improves the efficiency of catalysis (28). Each part of the substrate functions independently since covalent linkage between the two parts of the tRNA is not necessary (for a review see ref. 29).

By determining the length of the shortest 3'-terminal viral RNA fragment that can be adenylated by the CCA enzyme, it was previously concluded that the aminoacyl RNA domain fulfills the requirements for adenylation (5,6); these results suggest that the non-reacting portion of tRNA molecules must consist of the continuous stacking of the 12 base pairs of the aminoacyl RNA domain. Since a continuous stacking of 12 base pairs is recognized by the bacterial elongation factor Tu and by the CCA enzyme, we would like to propose the existence of structural similarities between these two proteins.

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