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Structural Similarities among Valine-Accepting tRNA-like Structures in Tymoviral RNAs and Elongator tRNAs

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Received June 2, 1986; Revised Manuscript Received October 6, 1986

ABSTRACT: A spatial model for the tRNA-like structure at the 3′ terminus of TYMV RNA has been proposed previously on the basis of chemical and enzymatic structure mapping studies [Rietveld, K., van Poelgeest, R., Pleij, C. W. A., van Boom, J. H., & Bosch, L. (1982) *Nucleic Acids Res. 10*, 1929-1946]. In this paper we describe the determination of the primary structure at the 3′ end of a number of tymoviral RNAs. Sequence comparison shows that the RNAs from CYVV, EMV, APLV, KYMV, and OYMV can adopt secondary and tertiary structures that fit the model as proposed for TYMV RNA. The pseudoknot formation that is essential for the construction of the aminoacyl acceptor arm of TYMV RNA is conserved in all tymoviral RNAs studied. The length of the individual double-helical segments coaxially stacked in this arm can vary and is well correlated with established tymoviral relationships. When the consensus structure of the valine-accepting tRNA-like structures is compared to that of standard $tRNA^{Val}$s, some striking similarities are observed. Only a limited number of nucleotides, located at defined positions, appear to be conserved. An ACA(C) sequence in the anticodon loop probably is the major structural feature responsible for the specific recognition of the cognate valyl-tRNA synthetase.

Tymoviruses are small icosahedral plant viruses, infecting a large number of dicotyledonous plants in most parts of the world (Guy et al., 1984). Viral preparations characteristically contain two major types of particles, both with a diameter of 25-30 nm. The faster sedimenting particle (115 s) or bottom component contains the genomic RNA (M_r 2.0 × 10^6) while the slower sedimenting particle (55 S) or top component is devoid of RNA and consists of the empty protein shell. Classification of the 16 tymoviruses described to date is based on either serological relationships (Koenig, 1976), amino acid composition of the coat protein (Paul et al., 1980), or host range (Guy et al., 1984). For recent reviews on the biological and structural properties and the classification of tymoviruses, see Matthews (1981) and Koenig and Lesemann (1979, 1981).

The genomic RNA of turnip yellow mosaic virus (TYMV), the type member of the tymovirus group, was the first viral RNA shown to possess a 3′ terminus that can be specifically aminoacylated, pointing to the presence of a tRNA-like structure (Hall, 1979; Haenni et al., 1982). TYMV RNA can be charged, like some other tymoviral RNAs, with valine (Pinck et al., 1970, 1974; Yot et al., 1970).

So far, five different plant virus groups have been reported to accept a specific amino acid at the 3′ end of their genomic RNAs (Joshi et al., 1983; Kozlov et al., 1984). We have proposed spatial models of these tRNA-like structures on the basis of structure mapping studies on 3′-terminal fragments of BMV RNA, TYMV RNA, and TMV RNA [Rietveld et al. (1984) and references cited therein]. The models of the tRNA-like structure of brome mosaic virus RNA and that of tobacco mosaic virus RNA are strongly supported by sequence comparisons with related RNAs from the bromovirus and tobamovirus group, respectively. Apart from a partial sequence of the 3′ terminus of egg plant mosaic virus (EMV) RNA (Briand et al., 1976), such information was not available in the case of the tRNA-like structure of TYMV RNA (Rietveld et al., 1982).

Since a comparative analysis of related RNA sequences can furnish additional evidence for proposed secondary and tertiary structures (Fox & Woese, 1975; Noller, 1984), we have determined the primary structure at the 3′ end of a number of tymoviral RNAs. In this paper we show that the 3′ termini of andean potato latent virus (APLV) RNA, clitoria yellow vein virus (CYVV) RNA, eggplant mosaic virus (EMV) RNA, kennedya yellow mosaic virus (KYMV) RNA, and ononis yellow mosaic virus (OYMV) RNA can be folded in a secondary structure as proposed for TYMV RNA (Rietveld et al., 1982). In addition, it is evident that in the construction of the aminoacyl acceptor arm the principle of pseudoknotting plays an essential role. Interviral comparisons revealed some interesting features of the folding of this domain. Moreover, we have found some common elements in these valine-specific tRNA-like structures on one hand and in the elongator $tRNA^{Val}$s from eukaryotic and prokaryotic organisms on the other. These findings may contribute to our understanding of how tRNAs are specifically recognized by their cognate aminoacyl-tRNA synthetase.

Materials and Methods

Enzymes. An *E. coli* extract containing aminoacyl-tRNA synthetases and CTP,ATP:tRNA nucleotidyltransferase was prepared essentially as described by Joshi et al. (1982a). After the final DEAE-cellulose chromatography step, the preparation was tested for the presence of all aminoacyl-tRNA synthetases, the presence of CTP,ATP:tRNA nucleotidyltransferase, and the absence of endogenous tRNA. CTP,ATP:tRNA nucleotidyltransferase was isolated from bakers' yeast according to

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the method of Rether et al. (1974). RNase T_1 and RNase U_2 were purchased from Sankyo and Sigma, respectively.

Viruses and Extraction of Viral RNA. All tymovirus preparations used in this study, except for TYMV, were a kind gift of Dr. R. Koenig. For BdMV, two preparations were obtained from Dr. B. Verduin. The RNAs were extracted as described by Bosch et al. (1967).

Aminoacylation of Viral RNAs and Analysis of Amino Acids Bound. Aminoacylation was performed in a total volume of 20 μL containing 10 μg of RNA, 2.5 mM ATP, approximately 10 mM ^{3}H-labeled amino acids (Amersham, 2–70 Ci/mmol), 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl), pH 7.5, 10 mM $MgCl_2$, 10 mM dithiotheitol (DTT), and 5 μL of the synthetase preparation. Incubation was for 1 h at 37 °C. After two phenol extractions and gel filtration on Sephadex G-25 in 10% acetic acid/1% pyridine, pH 4.0, the fractions containing the aminoacyl-RNA were pooled, freeze-dried, and dissolved in 50 μL of 5% triethylamine. Aminoacyl ester bonds were subsequently hydrolyzed during a 30-min incubation at 37 °C. After lyophilization, the amino acids were analyzed with a Chromaspek amino acid analyzer according to the method of Duisterwinkel (1981). Fractions (80) were collected, mixed with Rialuma scintillation liquid, and counted in a liquid scintillation counter.

3′ End Labeling of Viral RNAs and Sequence Analysis. Tymoviral RNAs were 3′ end labeled in a total volume of 20 μL containing 10 μg of RNA, 0.1 mM [α-^{32}P]ATP (Amersham, 3000 Ci/mmol), 2.5 μg of CTP,ATP:tRNA nucleotidyltransferase, 50 mM glycine–NaOH, pH 9.3, and 12.5 mM $MgCl_2$. After 16 h of incubation at 4 °C, the mixture was added to an equal volume of sample buffer [20 mM sodium citrate, pH 5.0, 9 M urea, 1 mM ethylenediaminetetraacetic acid (EDTA), xylene cyanol, and bromophenol blue] and electrophoresed through a 10% polyacrylamide 8 M urea slab gel until the bromophenol blue marker had reached the bottom of the gel. Detection of labeled RNA fragments was by autoradiography. For sequence analysis, fragments ranging in size from 100 to 150 nucleotides were eluted from the gel as described by Peattie and Gilbert (1980). Sequencing with RNases T_1 and U_2 was performed as described by Donis-Keller et al. (1977). Chemical sequencing reactions were done according to the method of Peattie (1979). Analysis of the resulting RNA fragments was on 10 or 20% polyacrylamide/8 M urea slab gels.

Results

Aminoacylation. Tymoviral RNAs were isolated from the viral preparations (kindly donated by Dr. R. Koenig) by extraction with phenol and incubated with a mixture of 15 ^{3}H-labeled amino acids, ATP, and an extract from *Escherichia coli* containing aminoacyl-tRNA synthetases and CTP,-ATP:tRNA nucleotidyltransferase (see also Materials and Methods). In control experiments we established that all amino acids could be bound to a tRNA mixture from yeast (Figure 1A,B) and that the enzyme preparation was free from contaminating tRNAs (result not shown). The variability in charging may be due to the availability of the respective tRNAs and aminoacyl-tRNA synthetases. No attempts were made to obtain 100% charging.

Aminoacylated viral RNAs were purified from free ^{3}H-labeled amino acids by chromatography on Sephadex G-25; the bound amino acids were released by alkaline hydrolysis and identified with an amino acid analyzer.

The results obtained for TYMV, OYMV, and KYMV are shown in panels C–E of Figure 1. All tymoviral RNA preparations tested were able to esterify valine (see Table I).. In some cases [CYVV, KYMV, and belladonna mottle virus (BdMV) RNA], it was the sole amino acid detected. Other viral RNAs bound additional amino acids, although to a lower extent (OYMV, APLV, and TYMV RNA). This probably is due to the presence of contaminating (host cell) tRNAs, although a reduced specificity of the aminoacylation of these viral RNAs might also be the case. The presence of contaminating tRNAs has been reported for BdMV and EMV RNA. $tRNA^{Lys}$ was found to be firmly bound to the latter RNA (Pinck et al., 1974). However, we could not detect this tRNA species in our viral RNA preparation. In contrast to the report of Pinck et al. (1972), we found that BdMV accepts valine and not alanine (see Table I).

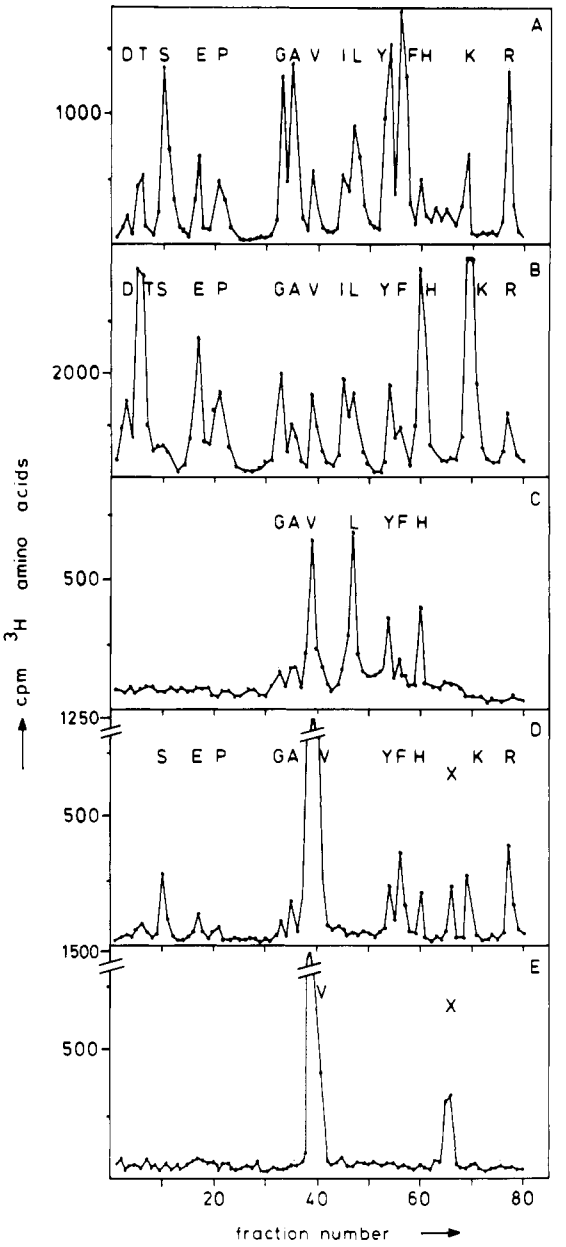

FIGURE 1: Identification of amino acids esterified to tymoviral RNAs. The position of the amino acids in the elution pattern is indicated by the one-letter code. "X" represents an unidentified radioactive compound (see legend to Table I). (A) Composition of the ^{3}H-labeled amino acid mixture used for charging of the viral RNA. (B) Amino acids esterified to a mixture of tRNAs from bakers' yeast by the *E. coli* extract (see also Materials and Methods). (C) Amino acids esterified to an RNA preparation of TYMV. (D) Amino acids esterified to an RNA preparation of OYMV. (E) Amino acids esterified to an RNA preparation of KYMV.

Table I: Molar Ratios of Amino Acids Bound to Preparations of Tymoviral RNAs by an Aminoacyl-tRNA Synthetase Preparation from *E. coli*[a]

tymoviral RNA	amino acids bound							
	V	Y	H	F	L	K	R	X
APLV-Ay	0.36	0.21	0.14			0.07	0.22	+
BdMV	1.00							+
CYVV	1.00							-
EMV	0.50	0.50						+
KYMV	1.00							+
OYMV	0.39	0.03	0.03	0.21		0.11	0.23	+
TYMV	0.38	0.15	0.08	0.08	0.31			-

[a] Calculation of the ratios of amino acids bound to the individual tymoviral RNA preparations was based on the radioactivity found in the various fractions emerging from the amino acid analyzer (see Figure 1) and the specific activity of the tritiated amino acids. Compound X, of which the structure is unknown, is only found upon incubation of *E. coli* synthetases and some viral RNAs. It is not present in the original amino acid mixture and is not formed upon the incubation with the tRNA as acceptor or when wheat germ extracts are used. Therefore, in the calculation of the molar ratios, compound X was not taken into account. For abbreviations used for the viruses, see the text.

Adenylation. Adenylation of the 3′ terminus of viral RNAs may provide an indication for the presence of a tRNA-like structure. Moreover, it enables specific 3′-terminal labeling of RNA fragments, which then can be submitted to nucleotide sequencing. Effective adenylation has been achieved by incubating TYMV RNA (lacking the 3′-terminal A residue) with CTP,ATP:tRNA nucleotidyltransferase and [α-^{32}P]ATP (Briand et al., 1977; Rietveld et al., 1982). We have applied this procedure to the labeling of other tymoviral RNAs. Electrophoretic separation of the reaction mixtures on a 10% polyacrylamide/8 M urea slab gel, followed by autoradiography, revealed one or more labeled RNA fragments for each tymoviral RNA tested (Figure 2). These fragments correspond to degradation products of the viral RNAs and possibly to tRNAs from the host cells. All tymoviral RNAs tested gave rise to labeled fragments that were larger than tRNA, except erysimum latent virus (ELV) RNA. In the latter case, only labeled RNA smaller than or equal to the size of tRNA was found. We have not investigated this RNA further, nor did we try to identify the putative contaminating tRNA(s). It may be noted here that ELV is only distantly related to the other tymoviruses (Koenig & Lesemann, 1981). A possible lack of a tRNA-like structure would be consistent with this observation. Adenylation of BdMV RNA gave variable results depending on the source of the virus (Figure 2).

Sequencing. For sequence analysis ^{32}P-labeled 3′-terminal fragments of 100–150 nucleotides in length were eluted from gels as shown in Figure 2. This size was selected because contamination with host tRNAs can be excluded. For sequencing, they were submitted to chemical degradation as described by Peattie (1979) and to limited digestions with RNase T_1, RNase U_2, and alkali according to Donis-Keller (1977) (see Materials and Methods). A representative experiment with OYMV RNA is shown in Figure 3.

Probably because of the stable secondary structure of the 3′-termini of various viral RNAs, band compression often occurred, as could be deduced from the alkali ladder. Several experiments had to be carried out, therefore, before the sequence could be determined unambiguously. Regions difficult to unravel were located between nucleotides 1 and 20 from the 3′ end and around positions 70–80 in the D stem and loop.

Table II gives a summary of the sequence at the 3′ terminus of KYMV, OYMV, CYVV, EMV, and APLV RNA. For reasons of comparison, we have included the 3′-terminal sequences of CcTMV and TYMV RNA as reported earlier (Meshi et al., 1981; Briand et al., 1977; Silberklang et al., 1977).

Discussion

The experiments described in this paper represent the first systematic study on the primary structure of tymoviral RNAs.

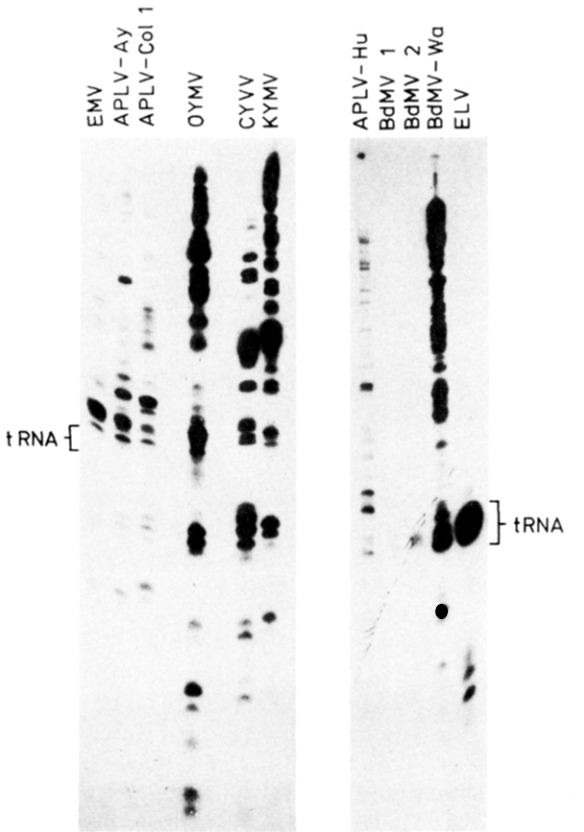

FIGURE 2: Detection of tRNA-like fragments in tymoviral RNA preparations. The RNAs were labeled with [α-^{32}P]ATP and CTP,ATP:tRNA nucleotidyltransferase and electrophoresed on a 10% polyacrylamide/8 M urea slab gel. Degradation products of the viral RNAs containing the 3′ terminus and contaminating tRNAs were detected by autoradiography. In both panels, the position of tRNA is indicated. The gel on the left was run for 2 h at 30 W and the one on the right for 2.5 h. For abbreviations used for the viruses, see the text.

The 3′-terminal sequences established provide further support for the model of the secondary and tertiary structure of the 3′ terminus of TYMV RNA (Rietveld et al., 1982; Florentz et al., 1982). Furthermore, a comparison of the generalized structure of the valine-accepting tRNA-like structure with that of elongator tRNA^{Val} reveals a few common structural features that may function as determinants for recognition of the cognate valyl-tRNA synthetase.

Aminoacylation Specificity. The first question we had to address was which amino acid can be esterified to the various tymoviral RNAs. Charging with an amino acid other than valine may point to a secondary structure at the 3′ terminus that differs completely from that proposed for TYMV RNA. One example of the latter phenomenon is offered by a member

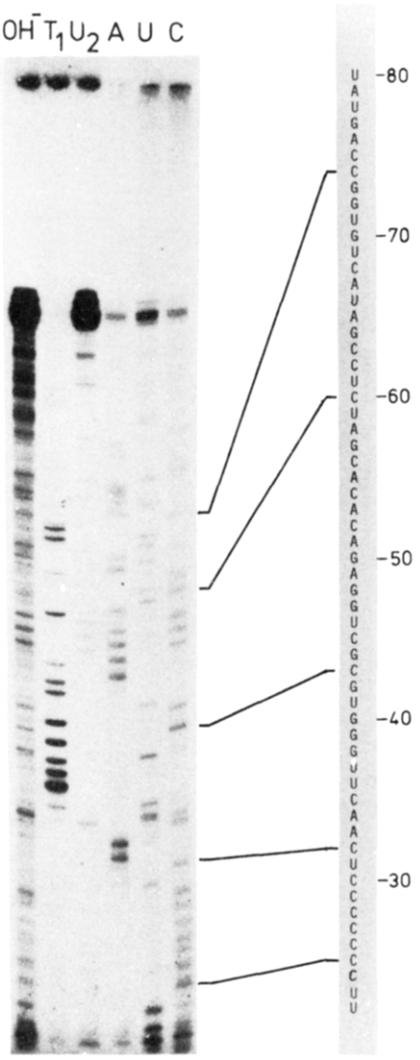

FIGURE 3: Sequence analysis of a 3′-terminal fragment of OYMV RNA. We have used alkali digestion (OH^-), RNase T_1 digestion (T_1), RNase U_2 digestion (U_2), and the chemical reactions specific for A, U, and C residues. The deduced sequence is shown alongside the gel. Numbering is from the 3′ end.

of the tobamovirus group, i.e., the cowpea strain of TMV (CcTMV or sunhemp mosaic virus), the RNA of which accepts valine instead of histidine. The 3′-terminal structure of this viral RNA resembles that of TYMV RNA rather than that of the tobamoviral RNAs (Rietveld et al., 1984; Beachy et al., 1976; van Belkum et al., 1985).

It can be concluded from Table I that all tymoviral RNAs tested accept valine as the major amino acid. Some viral RNAs, however, can be esterified to other amino acids as well. It has been suggested by Pinck et al. (1972, 1974) that this is due to the presence of contaminating host tRNAs, encapsidated in or associated with the virions. We have not further examined whether this was true in our viral RNA preparations but the fact that the primary structures as established in this paper are readily folded into a tRNA-like structure as proposed for TYMV RNA almost certainly identifies the RNAs as valine-accepting species.

The tRNA-like Structure of Tymoviral RNAs. All tymoviral 3′ termini sequenced permit the adoption of a secondary structure that is largely identical with that of TYMV RNA (see Figure 4A) proposed earlier (Rietveld et al., 1982; Florentz et al., 1982). The valine-accepting 3′ terminus of CcTMV RNA is also included because its 3′-terminal 80 nucleotides show a 60% homology with TYMV RNA and can

Table II: Summary of Sequence Data of Some Tymoviral RNAs[a]

		130	120	110	100	90	80	70	60	50	40	30	20	10
TYMV RNA	5′			A CCUAAGUUCU	CGAUCUUUAA	AAUCGUUAGC	UCCCCAGUUA	GCCAGGUCUG	UCCCCACACG	ACAGAUAAUC	GGGUGCAACU	CCCGCCCCUU	UUCCGAGGGU	CAUCGGAACC
CcTMV RNA	5′			C AGUCAUGGUU	UCCAUCCCGU	AAAGUUCAUA	ACCCCGAACU	CGCCGCGCCG	UCAAGACACG	ACGGUCAGUG	CGGAGCAUUA	CCCCCCCAAA	ACCCUGGGGA	UACAGGCCCC(A)
APLV Col I RNA	5′				AGGYYAA	AAUCA GYAA	UGAAGGCACU	CCUUCUCCCU	UUAGCACACA	AAGGUCAAAU	GGGUGCGCACU	CCCCCCCUUU	UCCGAGGGUC	AUCCGAAACC(A)
CYVV RNA	5′		GU UGAUUCCCCC	UUCUCUCCUC	UUCCUCCUGA	GAAUAGUUUC	UCUCG??AGU	GCCAGGCCCA	UUCACACACA	AUGGGUAUUG	CGUGCAACCC	CCCCCGUCCAU	CUCGAACGCU	CAUCCAGACC(A)
EMV RNA	5′					GAAACUACA	GUUCCGCUCC	UCUAGCACAC	ACAGGUCAAU	UGGGUCGCAC	CCCCCCCUCU	CCCGUGGGUC	AACGGGAACC(A)
KYMV RNA	5′					UUU	AGUUGCCAGU	GCGACACCCG	UUCCCACACA	ACGGGCAUGG	GUGCAACUCC	CCCGUCCCAC	UCUGGACGGU	CACCAGGACC(A)
OYMV RNA	5′	A UAGGAUCCGU	CCGUAGUUCG	GGGAUUGGUU	GCCCUAAGCA	ACCCGCUUAA	AUAUGACCGC	UCUAGCACAC	ACAGGUCCCG	UGGGUUCAAC	UCCCCCCCUU	UUCCCAGGGU	AUCGGAAACC(A)

[a] The 3′-terminal sequences of CcTMV RNA, TYMV RNA, and part of EMV RNA were published before (Meshi et al., 1981; Briand et al., 1976a, 1977; Silberklang et al., 1977). In TYMV RNA the position of the stop codon of the coat protein cistron is indicated. In CYVV and OYMV RNA, possible stop codons are underlined (see also Discussion). Unidentifiable pyrimidines are abbreviated "Y"; nondeterminable residues are marked "?".

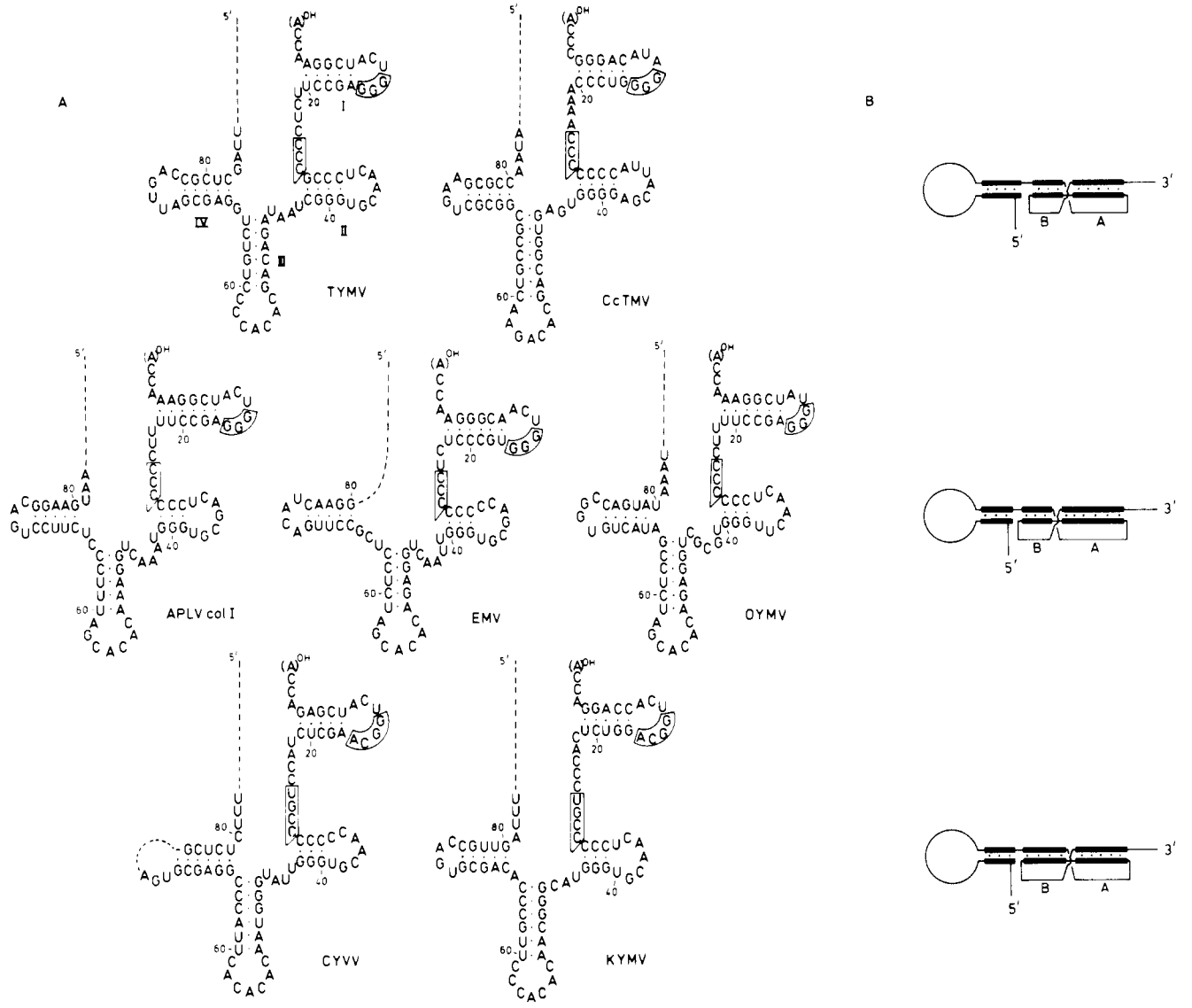

FIGURE 4: Secondary structure at the 3′ terminus of six tymoviral RNAs and one tobamoviral RNA (CcTMV). (A) The sequences determined in this study are folded according to the model of the secondary structure of the tRNA-like region of TYMV RNA (reported earlier; Rietveld et al., 1982). Numbering is from the 3′ end. For abbreviations of the viruses, see the text. Boxed regions indicate complementarities involved in pseudoknot formation. (B) Schematic representation of the architecture of the aminoacyl acceptor arm, showing the coaxial stacking of the three double-helical segments. Loop A spans the deep groove, and loop B crosses the shallow groove [for further details, see Pleij et al. (1985)].

be folded in a similar secondary structure as that of the latter RNA (Meshi et al., 1981).

In all cases the complementarity of a region in hairpin loop I (see TYMV RNA) with the single-stranded region at the 3′ side of stem II is strictly conserved. This complementarity plays an essential role in the construction of the aminoacyl acceptor arm of the tRNA-like structure of TYMV RNA. Likewise, the aminoacyl acceptor arm of all other tymoviral RNAs can adopt a structure that consists of three coaxially stacked double-helical segments (see Figure 4B). Interestingly, the total number of base pairs in this stack is always 12, despite variations in the number of base pairs in each individual segment. We earlier noted that the size of stem I and II can vary in length (Rietveld et al., 1982). Our data, as shown in Figure 4B, indicate that also the middle segment, which results from a special type of tertiary interaction, can vary and consists of three or four base pairs. In fact, three classes of valine-accepting tRNA-like structures can be discerned, depending on the way the aminoacyl acceptor domain is built. In the case of APLV, EMV, and OYMV, a stack of six, three, and three base pairs is found; in that of CYVV and KYMV a stack of five, four, and three and in that of TYMV and CcTMV a stack of five, three, and four base pairs is found. It is interesting to see that a classification of tymoviruses based on these structural aspects is in agreement with the one based on serological relationships as proposed by Koenig (1976) (see below).

An intriguing question is whether an aminoacyl acceptor arm consisting of three segments containing four base pairs each has evolved in one of the other valine-accepting tRNA-like structures. The size of connecting loop A (see Figure 4B) bridging the deep groove of the RNA duplex varies from two to five nucleotides, whereas loop B, bridging the minor groove, consists of two or three nucleotides. This is feasible in view of the geometric properties of pseudoknots of this type [for a more detailed description of this folding principle, see Pleij et al. (1985)].

Common Features with tRNA. Figure 5C shows the consensus structure derived from the seven valine-accepting tRNA-like structures as given in Figure 4. The coaxial stacking of helices I and II and the tertiary interaction on the one hand and the stacking of helices III and IV on the other,

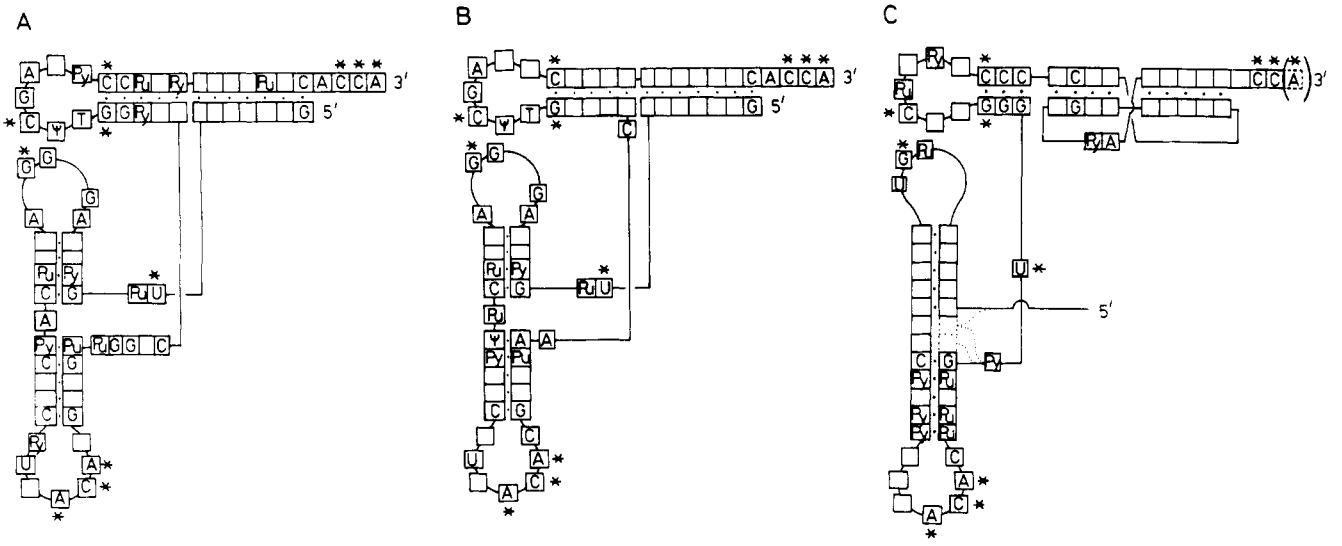

FIGURE 5: Consensus structures for prokaryotic (A) and eukarytoic (B) $tRNA^{Val}$ [derived from Sprinzl et al. (1985)] and plant viral valine-specific tRNA-like structures (C). Invariable residues are indicated; nucleotides common to all three valine-accepting species are indicated by an asterisk. The construction of the aminoacyl acceptor arm in the tRNA-like consensus structure was arbitrarely chosen to be five plus four plus three base pairs. Dotted lines indicate the various alternative structures found. Pu and Py stand for purine and pyrimidine, respectively.

taken together with the perpendicular orientation of the resulting two quasi-continuous helices, give rise to the familiar L arrangement as known for standard tRNA. The aminoacyl acceptor arm invariably contains 12 base pairs, just as the other limb of the L shape that comprises the anticodon domain. In some cases however, unpaired nucleotides occur between the two helix segments III and IV. EMV RNA is the only exception in having 13 bases in this stack. Hairpin loops II and III resembling the TΨC loop and the anticodon loop of normal tRNA, respectively, always contain seven nucleotides like in tRNA, whereas loop I (absent in tRNA) and loop IV, corresponding to the D loop in tRNA, vary in size. The same is true for the variable loop.

It is interesting to note that some residues found in all tRNAs appear to be conserved in the tymoviral tRNA-like structures as well. The most obvious one is that of the CC(A) end. The only other residues are the G·C base pair at the end of the T stem and the possible G·C pair between residues from the T and D loop (see Figure 5). However, no direct proof is available as yet for the presence of the latter Watson–Crick base pairing. It may also be noted that a conserved residue is found in the loop connecting the aminoacyl acceptor arm and the anticodon stem of the tymoviral RNAs (e.g., U-43 of TYMV RNA). This uridine might correspond to the conserved uridine residue at position 8 in elongator tRNAs, which is thought to play a crucial role in aminoacylation (see below). On the other hand, residues being invariant in standard tRNAs are clearly not so at identical sites in the viral tRNA-like structures, e.g., the universal U-33 at the 5′ side of the anticodon, A-14 in the dihydrouridine loop, or the TΨ sequence in the T loop. The absence of a number of conserved residues, together with the fact that the 3′ termini of tymoviral RNAs do not contain modified nucleotides, suggests that these tRNA-like structures do not mimic standard tRNAs in every respect and therefore cannot participate in the elongation cycle on the ribosome.

Interaction with Valyl-tRNA Synthetase. An intriguing question is how tRNA-like structures succeed so well in recognizing the aminoacyl-tRNA synthetases. For instance, the kinetic parameters of the valylation of TYMV RNA are quite similar to those for $tRNA^{Val}$ from yeast (Giegé et al., 1978). A comparison of the 3′-terminal structures of tymoviral RNAs with that of $tRNA^{Val}$ may reveal which features are important for the recognition of the valyl-tRNA synthetase.

In the past considerable effort has been directed at locating regions on the tRNA that are important for binding of synthetase, by use of approaches like tRNA modification, tRNA–synthetase cross-linking, site-directed mutagenesis, etc. (Schimmel & Söll, 1979). The only generalization that could be made was that the synthetase appears to bind along and around the inside of the L shape of tRNA, contacting regions like the anticodon, the 5′ side of the dihydrouridine stem, and the acceptor terminus (Chambers et al., 1973).

When the consensus structures of all prokaryotic (Figure 5A) and eukaryotic (Figure 5B) tRNAs specific for valine are compared to that of the valine-accepting tRNA-like structure (Figure 5C), some interesting features emerge. In addition to the conservations already mentioned above, which are common to all tRNAs, we note the strict conservation in all consensus structures of an ACA sequence at the 3′ side of the anticodon loop, partially overlapping the anticodon. We therefore propose that this region is largely responsible for the specific recoginition of the valyl-tRNA synthetase. This sequence homology was noted by others (Haenni & Chapeville, 1980) when one member of the tymoviral family (TYMV) was compared with $tRNA^{Val}$ from yeast. In addition, studies of phosphate modification with (ethylnitroso)urea of $tRNA^{Val}$ complexed with valyl-tRNA synthetase from yeast showed that the tRNA-like structure of TYMV RNA and $tRNA^{Val}$ were equally accessible at the 3′ side of the anticodon stem and loop (R. Giegé, personal communication). Moreover, the role of the anticodon nucleotides in recognition of tRNAs by aminoacyl-tRNA synthetases has been well documented [for a recent review, see Kisselev, (1985)]. This is especially true for $tRNA^{Val}$. The crucial role of the A-35 and C-36 residues from the anticodon of $tRNA^{Val}$ became clear from a number of studies [Kisselev (1985) and references cited therein]. Similar observations on the importance of the anticodon as the major determinant for synthetase recognition were reported for $tRNA^{fMet}$ and $tRNA^{Trp}$ (Kisselev, 1985; Pelka & Schulman, 1986; Garret et al., 1984).

It is doubtful whether any residue in the aminoacyl acceptor arm is involved in the specific recognition of the valyl-tRNA synthetase. The C-5·G-76 base pair shared by all $tRNA^{Val}$ structures is not found in the tRNA-like consensus structure. Also, the residue at position 4 in the tRNA-like structure

(corresponding to position 73 in tRNA) is a G in the case of CcTMV RNA instead of the more common adenine. This means that this residue cannot function as a discriminator (Crothers et al., 1972). Moreover, the presence of the two so-called connecting loops in the aminoacyl acceptor arm of the tymoviral RNAs suggests that this complete region cannot be a major determinant for synthetase recognition.

As far as the corner of the L shape of the tRNA-like structure is concerned, we have mentioned above the conservation of a uridine residue adjacent to the aminoacyl acceptor arm (e.g., U-43 of TYMV RNA). It is possible that this uridine, which in the three-dimensional structure occurs at a similar position as U-8 in tRNA, is involved in the formation of a transient covalent bond with the synthetase (Starzyk et al., 1985). Site-directed mutagenesis of this particular base may shed light on its possible involvement in the aminoacylation reaction.

All these observations lead to the same conclusion as formulated by Kisselev (1985): "... there is no reason to look for a recognition site other than the anticodon in all $tRNAs^{Val}$ of eukaryotes." We only want to add here that the A residue at the 3′ side of the anticodon (A-37 in $tRNA^{Val}$) might contribute further to this specificity.

This strong conservation of the ACA stretch in the anticodon loop necessary for valylation may point to the fact that valylation itself or the binding of valyl-tRNA synthetase is obligatory in viral RNA replication. It may be recalled that Joshi et al. (1982b) have demonstrated valylation of TYMV RNA in Chinese cabbage leaves and *Xenopus laevis* oocytes. A similar in vivo aminoacylation has been reported for the tyrosine-specific BMV RNA (Loesch-Fries & Hall, 1982). Site-directed mutagenesis studies on cDNA clones of BMV RNA (Dreher et al., 1984; Bujarski et al., 1985, 1986) have shown that modifications in the putative anticodon loop and stem (Rietveld et al., 1983) did not affect tyrosylation but rather replication. This makes the orientation of this particular stem (stem C) in the tRNA-like structure and its assignment as the anticodon stem questionable. This is further supported by the observation made by Kozlov et al. (1984) in barley stripe mosaic virus RNA: in this case stem C is very short. It remains to be seen where exactly the determinants of synthetase recognition are located in the 3′ terminus of BMV RNA.

Relationships among Tymoviruses. In general, nucleotide sequences provide the best source of information to establish evolutionary relationships among different organisms (or viruses). The sequence data available for the tymoviruses are relatively scarce compared to those of many other (plant) virus groups. So far, only the sequences of the coat protein messenger RNA (Guilley & Briand, 1978) and the 5′-terminal leader of TYMV RNA (Briand et al., 1976a) are known, as is a short 3′-terminal stretch of EMV RNA (Briand et al., 1976b). From these data it can be derived that the 3′ noncoding region of TYMV RNA is 109 nucleotides in length. In the case of OYMV and CYVV RNA, sequences exceeding this length were determined. Unfortunately, no coat protein sequences are known for these viruses. In CYVV RNA a potential stop codon is found at position 92 (from the 3′ end) just downstream of a 26 nucleotide long stretch of pyrimidines only. Such a pyrimidine-rich region is also present immediately upstream of the stop codon of the TYMV coat protein cistron. It may also be present at an analogous position in EMV RNA as can be deduced from the recently determined amino acid sequence of the coat protein of this virus (Dupin et al., 1984).

The partial sequences of a limited set of tymoviral RNAs certainly do not allow a classification within this group. Nevertheless, it is interesting to see that the way in which the aminoacyl acceptor arm of the otherwise strongly conserved tRNA-like structure is folded independently supports earlier proposed classifications as based on serology (Koenig, 1976), amino acid compositions (Paul et al., 1980), and host range (Guy et al., 1984).

As already mentioned above, our data suggested a close relationship between KYMV and CYVV on one hand and between APLV, EMV, and OYMV on the other (see Figure 4B). The latter virus was classified on basis of host range (Guy et al., 1984) as belonging to the APLV cluster, which is in agreement with our findings. Serological methods, however, indicate that OYMV has a somewhat more distant relationship with the other members of this group. EMV was classified earlier as a member of the CYVV cluster, but our data confirm its classification in the APLV cluster as proposed by Koenig (1976).

The construction of the aminoacyl acceptor arm of TYMV RNA so far is unique among the tymoviruses. In this respect it resembles CcTMV, which in fact is a tobamovirus. The tRNA-like strucure of CcTMV RNA in itself is rather peculiar in that it has a C residue at position 4 instead of an A residue, the stretch of four A residues (A-21–A-24), which spans the deep groove in the pseudoknot, and the GAC anticodon, which is different in the wobble position from all other tymoviral RNAs. If recombination on the RNA level has occurred in the past, leading to an exchange of a histidine-accepting 3′ terminus for a valine-accepting tymoviral one (Van Belkum et al., 1985), it probably was an early event in the evolution of the tymoviruses.

We finally want to point out that, apart from the compensating base changes in the stem regions, the mutational rates in these tRNA-like structures seem to be highest in the two connecting loops in the pseudoknot and in the variable loop. This is exemplified below for a few viruses. We earlier noted a difference in the sequence of the type strain of TYMV we have studied for a long period in our laboratory with that published by others (Rietveld et al., 1982; Briand et al., 1977; Silberklang et al., 1977). We have evidence that residue C-22 is replaced by a U residue, which is just in the four-nucleotide stretch crossing the deep groove in the pseudoknot. Partial sequences available for the APLV strains Ay and Hu revealed a sequence identical with that of the Col I strain, except for one substitution in the variable loop (Rietveld, 1984). The same is true for EMV because the sequence given in Figure 4 does not completely overlap the previously published partial sequence (Briand et al., 1976). The two sequences again differ in the variable loop even in a rather drastic way. We therefore believe that the function of this variable loop is largely to connect the two separate domains in the tRNA-like structure, except for the possibly important role of the conserved U residue already mentioned above.

ACKNOWLEDGMENTS

We thank Francis Mac Donald for typing the manuscript, Pieter Jan Wiersema for his contribution in some of the experiments, and Barend Kraal for helpful suggestions. We acknowledge Dr. B. Verduin for the gift of two strains of BdMV. We are indebted to Dr. R. Koenig for the generous gift of the tymoviruses, helpful discussion, and reading of the manuscript.

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