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Three-dimensional models of the tRNA-like 3′ termini of some plant viral RNAs

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Various plant viral RNAs possess a 3′ terminus with tRNA-like properties. These viral RNAs are charged with an amino acid upon incubation with the cognate aminoacyl-tRNA synthetase and ATP. We have studied the structure of end-labelled 3′-terminal fragments of turnip yellow mosaic virus RNA and brome mosaic virus RNA 2 with chemical modifications of the adenosine and cytidine residues and with enzymatic digestions using RNase T1, nuclease S1 and the double-strand-specific ribonuclease from cobra venom. The data indicate that the 3′ termini of these plant viral RNAs lack a cloverleaf structure as found in classical tRNA. The three-dimensional folding, however, reveals a striking resemblance with classical tRNA. The models proposed are supported by phylogenetic data. Apparently distinct three-dimensional solutions have evolved to meet the requirements for faithful recognition by tRNA-specific enzymes. The way in which the aminoacyl acceptor arms of these tRNA-like structures are constructed reveal novel features in RNA folding which may have a bearing on the secondary and tertiary structures of RNA in general. The dynamic behaviour of brome mosaic virus RNA 2 in solution presumably is illustrative of conformational transitions, which RNAs generally undergo on changing the ionic conditions.
Key words: plant viral RNAs/tRNA-like structures/3-D RNA folding/dynamic behaviour of RNA

Introduction

Our knowledge of the three-dimensional structure of single-stranded RNA molecules is very limited. To date the spatial folding of a few species of tRNA has been elucidated by X-ray diffraction of crystallized tRNAs (Robertus *et al.*, 1974; Kim *et al.*, 1974; Woo *et al.*, 1980; Moras *et al.*, 1980). N.m.r. measurements, chemical modification and enzymatic digestion of tRNA in solution are in agreement with these structures (Rich and RajBhandary, 1976; Schimmel *et al.*, 1979; Peattie and Gilbert, 1980; Wrede *et al.*, 1979; Auron *et al.*, 1982). Although some notable differences exist, the major features of these tRNA structures are the same: the overall molecular shape is an extended L consisting of an aminoacyl acceptor arm and an anticodon arm. This spatial arrangement, placing the aminoacyl moiety and the anticodon at the two extremities of the tRNA molecule, presumably plays an essential role in the recognition by tRNA-specific enzymes and in the interaction with the ribosomes. A deeper insight into these recognition problems may be gained when additional information becomes available on the tertiary structures of other tRNA species.

In this respect, it is of great interest that various plant viral RNAs possess a 3′ terminus with tRNA-like properties. These termini are recognized by a variety of tRNA-specific enzymes. They become esterified to a specific amino acid residue in the presence of the cognate aminoacyl-tRNA synthetase and ATP. Under these conditions, turnip yellow mosaic virus (TYMV) RNA accepts valine, tobacco mosaic virus (TMV) RNA histidine and the brome mosaic virus (BMV) RNAs tyrosine (for reviews, see Haenni *et al.*, 1982; Hall, 1979).

Surprisingly the secondary structure at the 3′ terminus of these viral RNAs deviates considerably from the cloverleaf structure, characteristic for tRNA (Rietveld *et al.*, 1982; Florentz *et al.*, 1982; Ahlquist *et al.*, 1981; and this paper). It is therefore of great interst to know how the three-dimensional folding of these RNA molecules meets the requirements for faithful recognition by tRNA-specific enzymes despite this anomaly of the secondary structure.

Results

The three-dimensional structure of the 3′ terminus of TYMV RNA

We have previously isolated a 3′-terminal fragment of 112 nucleotides of TYMV RNA by site-specific cleavage using RNase H and a deoxyoligonucleotide complementary to the sequence at the cleavage site (Rietveld *et al.*, 1982). Structure mapping of this fragment by chemical modifications and digestions with specific nucleases led us to propose the secondary structure illustrated in Figure 1a. Phylogenetic comparisons (Rietveld *et al.*, 1982) and recently reported enzymatic digestions of a larger fragment (Florentz *et al.*, 1982) lent further support to this proposal. A striking feature of Figure 1a is the apparent absence of a conventional aminoacyl acceptor stem. The experimental data indicated, however, that G13, G14 and G15 are involved in interactions with other parts of the molecule. The same holds true for C25, C26 and C27. The possibility was therefore envisaged that the three guanosines base pair with the three cytidines in the order mentioned above. Very strong evidence in favour of this base pairing is derived from the conservation of the triple C and triple G regions in a number of other amino acid accepting plant viral RNAs, both from the tymovirus and the tobamovirus group (Rietveld *et al.*, 1982). When the $(G)_3$:$(C)_3$ helix thus formed is stacked co-axially between the helices of stem I and II, an aminoacyl acceptor arm emerges with the characteristic 12 bp, continuously stacked (Figure 1b) as in canonical tRNA. Previously, shortcomings of our separation technique following chemical modifications of the cytidines with dimethyl sulphate (DMS) prevented us from presenting strong evidence for stem and loop IV of Figure 1a. Recently the accessibility of all cytidines of the 112 nucleotide fragment could be probed, however. The results (not shown) are in full agreement with the arrangement of stems and loops III and IV of Figure 1a. An anticodon arm can thus be constructed by stacking stems III and IV co-axially to form a helix containing 12 bp (assuming that U46 pairs with G66). The anticodon CAC is in the expected position. Model building

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(Figure 2b) indicates that such a three-dimensional folding is sterically feasible. In fact the resemblance with the well-established tertiary structure of $tRNA^{Phe}$ (Figure 2a) is striking.

Obviously it remains for biophysical studies such as high-frequency n.m.r. and X-ray diffraction to locate the various elements in more detail and to prove the co-axial arrangement of stems I and II on one hand and stems III and IV on the other.

The tRNA-like structures at the 3' terminus of RNAs from bromoviruses and cucumber mosaic virus

Recently Ahlquist *et al.* (1981) determined the 3'-terminal sequences of RNAs 1, 2, 3/4 from each of the three bromoviruses [brome mosaic virus (BMV), cowpea chlorotic mottle virus (CCMV), and broad bean mottle virus (BBMV)]. These authors proposed models for the secondary structures of the 3'-terminal fragments of these RNAs and those of the cucumber virus (CMV) RNAs, sequenced by Symons (1979). A representative model is illustrated in Figure 3. These fragments appear to have extensively base paired regions with strong intra- and interviral similarities. Although quite different from the structure of the TYMV RNA terminus (cf. Figure 1) these models also lack an apparent conventional aminoacyl acceptor stem. The question therefore arises whether they also can adopt a tertiary structure with some of the characteristics of an amino acid acceptor arm like that of canonical tRNA. Below we show that this is the case (cf. Figure 2c).

Structure mapping of the BMV RNA 2 terminus

For structural studies, BMV RNA 2 was end-labelled with [α-^{32}P]ATP and CTP, ATP:tRNA nucleotidyltransferase (EC. 2.7.7.25) as described for TYMV RNA (Rietveld *et al.*, 1982). In contrast to the latter RNA, BMV RNA 2 has a complete CCA-end. A small fraction lacking the terminal adenosine enables labelling, albeit to a lower specific activity than TYMV RNA. After labelling, an RNA fragment of ~160 nucleotides, derived from BMV RNA 2 cleavage by an RNase presumably contaminating the CTP, ATP:tRNA nucleotidyltransferase, could be isolated by electrophoresis on a 10% polyacrylamide gel. This fragment possesses tRNA-like properties (Bastin and Hall, 1976).

Structure mapping of the fragment was carried out by chemical modifications (Peattie and Gilbert, 1980) and by digestions with specific nucleases as described above for TYMV RNA (Rietveld *et al.*, 1982). The chemical methods allow the probing of the accessibilities of nucleotides under mild conditions in the presence or absence of Mg^{2+} and under heavily denaturing conditions. Table I summarizes the results obtained after modification of the adenosines with diethylpyrocarbonate (DEP) and of the cytidines with DMS for the BMV RNA 2 terminal fragment in the native (N) (at 10 mM Mg^{2+} and 37°C), in the semi-denatured (SD) (at 1 mM EDTA and 37°C), and in the fully denatured (D) (at 1 mM EDTA and 90°C) states (Peattie and Gilbert, 1980). This table also includes the cleavage data obtained with T1 RNase under non-denaturing (N) and denaturing (D) conditions and with cobra venom RNase. An important conclusion which emerges from Table I is that, in the presence of 10 mM Mg^{2+}, A8-A10 are not or only very weakly accessible to DEP, whereas A11 is freely accessible under all experimental conditions. This is illustrated in detail in Figure 4b which represents a part of one of the original autoradiograms. Furthermore, the cobra venom RNase appears to cut behind A8 and A9. This indicates that A8-A10 are involved in an interaction with a complementary region elsewhere in the molecule. That such a complementarity exists in all RNAs of the bromovirus group and in those of CMV had already been pointed out by Ahlquist *et al.* (1981). They reported that the extreme 3' end of each RNA can be folded into two different configurations, one locally base paired to form hairpin a, the other with the overlined complement in the loop of hairpin e (Figure 3). Accordingly they found that the single-strand specific nuclease S1 does not cut in the sequences 7–10 and 111–114 of CCMV RNA 3/4. Our data mentioned above concerning A8-A10 and the inaccessibility of C114 and C116 to DMS (Table I and Figure 4a) are in full accord with such a base pairing between G5-A10 and U111-C116. They also show that this interaction is stable in the presence of Mg^{2+} (10 mM) but is disrupted upon elimination of Mg^{2+}: in the

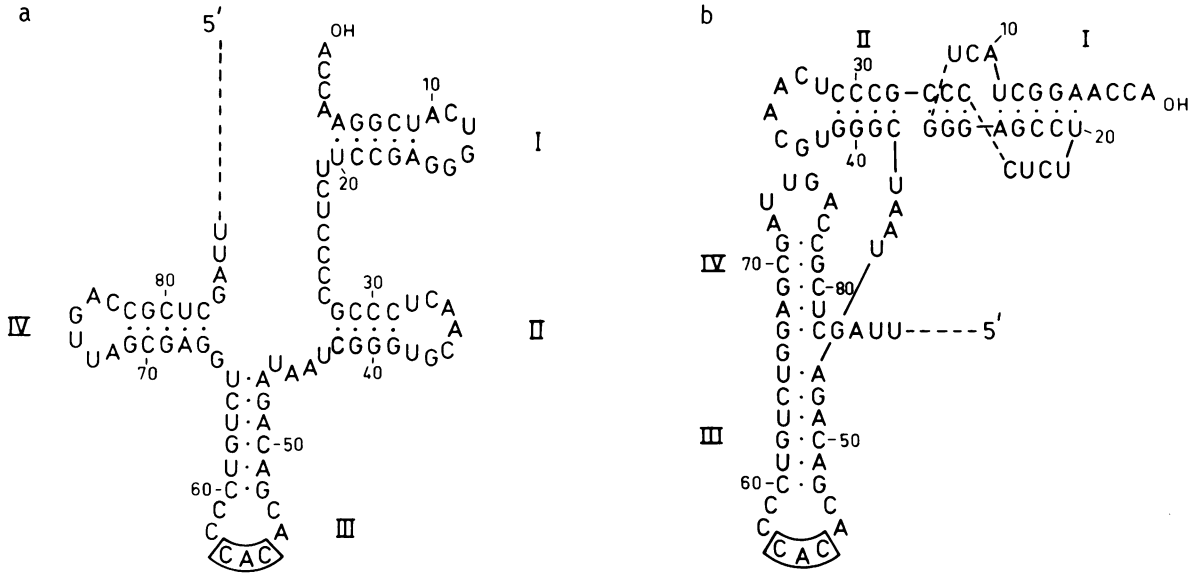

Fig. 1. The structure at the 3' end of TYMV RNA. Secondary structure **(a)**, and L arrangement **(b)** derived from chemical modification and enzymatic digestion data [for experimental details compared Rietveld *et al.* (1982)]. The boxed region indicates the anticodon.

semi-denatured state A8-A10 are accessible to DEP, while C114 and C116 are accessible to DMS.

Three-dimensional folding of the 3' terminus of BMV RNA 2

In the case of TYMV RNA we have seen that base pairing between complementary regions distantly located plays a dominant role in a three-dimensional folding yielding an aminoacyl acceptor arm and an anticodon arm resembling those of canonical tRNA. That this is also true for BMV RNA 2 when G5-G10 base pair with U111-C116, is illustrated in Figure 5a.

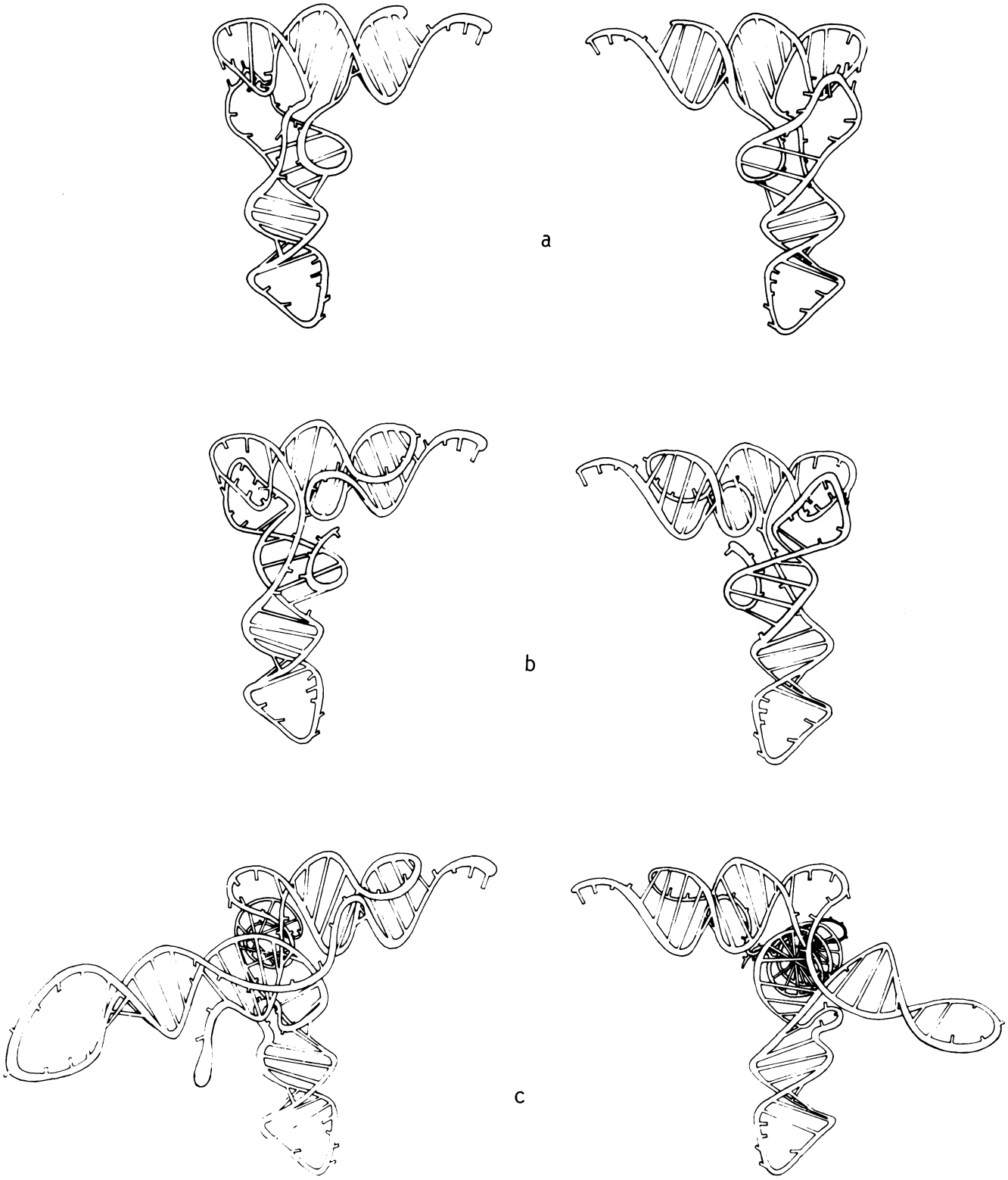

Fig. 2. The three-dimensional folding of $tRNA^{Phe}$ from yeast **(a)**, the 3' end of TYMV RNA **(b)** and the 3' end of BMV RNA 2 **(c)**. FIgure 2a is a drawing derived from X-ray diffraction data (Robertus *et al.*, 1974; Kim *et al.*, 1974). Wire models were built based on the data obtained from chemical modification and enzymatic digestion of the 3' ends of TYMV RNA and BMV RNA 2, described in Rietveld *et al.* (1982) and this paper. Artist's views of these models are presented in **b** and **c**, respectively.

The aminoacyl acceptor arm contains 12 bp continuously stacked, the anticodon arm shows a continuous stack of 11 bp and a loop containing the anticodon AUA in the expected position.

Although the model of Figure 5a seems to differ substantially from that proposed by Ahlquist *et al.* (1981) (Figure 3), the difference is more apparent than real. Stems b, c, d and e (using the notation of Ahlquist *et al.*) are essentially identical in both models and differ in their mutual orientation only. A clear distinction is the absence of hairpin a and loop e in Figure 5a. It may be assumed that a significant amount of free energy is gained by the stacking of stem e and stem (G5-A10):(C116-U111).

One of the connecting links between the two double-helical segments of the aminoacyl acceptor arm is the loop A117-U118. Model building (cf. Figure 2c) establishes that this two-nucleotide connection is sterically feasible. Modification with DEP in the native state of BMV RNA 2 (Table I) shows that A117 is part of a single-stranded region.

The aminoacyl acceptor arm is connected with a single-stranded region of seven nucleotides, the first of which have the sequence UUCG, reminiscent of the TψCG sequence of classical tRNA. An important difference with the latter RNA and the viral RNAs from the tymo- and the tobamovirus group is that this seven-nucleotide stretch does not form a closed Tψ loop but is connected with hairpin d instead.

It is conceivable that stem c, extended with the four base pairs (C49-A52):(G79-U76), forms the anticodon arm with four nucleotides bulging out in the middle. The number of stacked base pairs and the high accessibility of the loop of hairpin c are comparable with the analogous elements of the anticodon arm of the $tRNA^{Phe}$, although it must be noted that a proper D stem and loop is absent in our model of BMV RNA 2.

The RNAs from BMV, CCMV and CMV contain an extra stem d of extreme stability (cf. Ahlquist *et al.*, 1981 and Table I). It is absent in the RNAs of BBMV and is thus dispensable for the tRNA-like functions of the viral RNAs.

A striking structural feature of all RNAs from the bromovirus group and from CMV is the extra arm (designated the

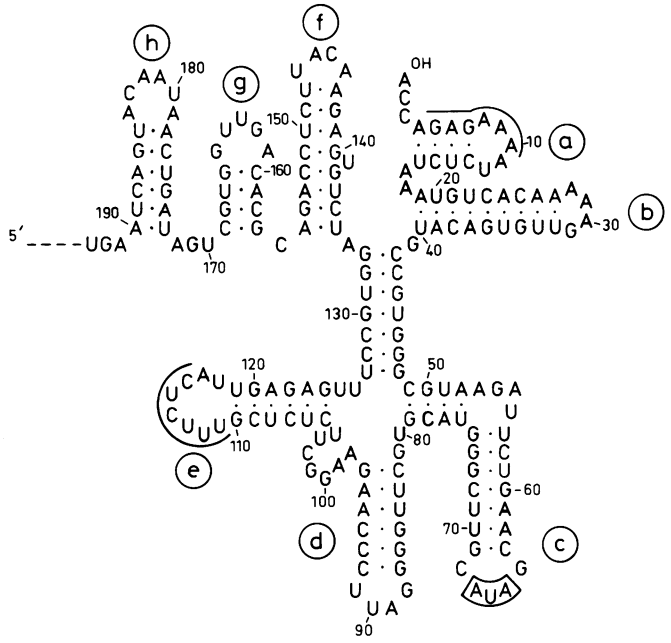

Fig. 3. Secondary structure at the 3′ terminus of BMV RNA 2 as proposed by Ahlquist *et al.* (1981). Overlining represents complementarity. The boxed region indicates the anticodon.

Table I. Compilation of data from chemical modifications and enzymatic digestions of a 3′-terminal fragment of BMV RNA 2

Chemical modification								Enzymatic digestion			
DEP				DMS				RNase T1			Cobra venom
	N	SD	D		N	SD	D		N	D	RNase
A1				C2				G5			
A4				C3				G7			A8, A9
A6	−	−	+	C13	+	−	+	G21	+	+	U16, A17, A18
A8	±	++	+	C15	+	±	+	G31	++	++	U22, C23, A24
A9	±	++	+	C23	−	−	+	G34	−	+	U35, G36, A37
A10	±	++	+	C25	−	−	+	G36	−	+	G46, G47, G48
A11	+	+	+	C38	−	−	+	G41	+	+	G50, U51
A17	+	+	+	C42	−	+	+	G44	−	+	U57, C58
A18	+	+	+	C43	−	+	+	G46	+	+	A61, A62
A19	−	±	+	C49	−	+	+	G47	−	+	C68
A24	−	−	+	C58	−	−	+	G48	−	+	U71, C72
A26	−	−	+	C63	−	−	+	G50	−	+	U76, A77
A27	+	+	+	C68	−	−	+	G54	+	+	U83-G85[a]
A28	+	+	+	C72	−	−	+	G60	−	+	C92-A96[a]
A29	+	±	±	C78	±	±	+	G64	+	+	C105-U108[a]
A30	+	+	+	C82	−	−	+	G69	−	+	C122-A123[a]
A37	−	−	+	C92	−	−	+	G73	−	+	G130-G133[a]
A39	±	+	+	C93	−	−	+	G74	−	+	
A52	−	+	+	C94	−	−	+	G75	−	+	
A53	±	+	+	C102	−	+	+	G81	+	+	
A55	+	+	+	C105	−	−	+	G85	−	−	
A61	−	−	+	C107	−	−	+	G86	−	−	
A62	−	−	+	C109	−	−	+	G87	−	−	
A65	++	+	+	C114	−	+	+	G88	−	−	
A67	++	+	+	C116	−	+	+	G97			
A77	±	+	+	C128	−	+	+	G100	+	+	
A89				C129	−	+	+	G101	±	+	
A95								G110	−	+	
A96								G120	−	+	
A98								G122	−	+	
A99								G124	−	+	
A117	+	±	+					G130	−	+	
A121	−	−	+					G132	−	+	
A123	−	−	+					G133	−	+	

[a]Multiple cuts are observed in this region.
Numbering of the nucleosides (A,C,G,U) starts at the 3′ end. Further symbols are N (native), SD (semi denatured), D (denatured), − (not accessible), + (accessible), + + (extremely accessible), ± (weakly accessible). Cutting by the cobra venom RNase at for instance A8 disrupts the 3′ phosphoester bond and yields a fragment comprising nucleotides 1−7 according to the present numbering. For further experimental details see Materials and methods.

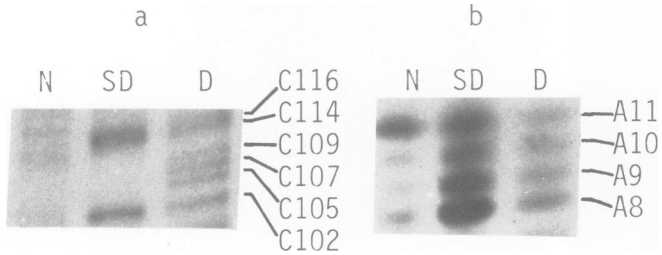

Fig. 4. Probing of cytidine and adenosine accessibility with chemical modification. The terminally labelled fragment of BMV RNA 2 was studied as described in Materials and methods. Parts of the autoradiogram are presented in each case. Cytidines are probed under **(a)** and adenosines in **(b)**. For further details see Table I.

extended b stem) comprising two double-helical segments: (U127-G133):(G48-C42) and hairpin b. The configuration in the centre of the extended b stem is somewhat complicated, however, as can be concluded from the cleavage by the cobra venom RNase after U16, A17 and A18 (Table I) and the insensitivity to nuclease S1 (Ahlquist *et al.*, 1981). The latter three nucleotides are part of the link between hairpin b and the aminoacyl acceptor arm. This link plays an important role in a highly interesting conformational transition which the BMV RNA 2 fragment undergoes upon removal of Mg^{2+} (see below).

Model building (Figure 2c) indicates that the extended b stem of 16 bp most likely is located at the side of the L shaped molecule, where in classical tRNA the variable loop is exposed (Brennan and Sundaralingam, 1976). A position at the opposite side is sterically less likely, since the single-strand connection of seven nucleotides (A11-A17) would then have to cross over or under the aminoacyl acceptor arm.

Finally it is noteworthy that in the model of Figure 2c elements like (A117-U118), the extended b stem and the bulge of four nucleotides in the anticodon arm, all are located at the side of the molecule, where in tRNA the variable loop is exposed.

Dynamic behaviour of BMV RNA 2 in solution

An interesting outcome of the chemical modification studies on BMV RNA 2 is the disappearance of a number of double-helical regions in the absence of divalent cations and presence of low salt conditions (semi-denaturation; cf. Table I). Under these conditions, hairpins b, c, d and e survive and interestingly the last 15 nucleotides fold into a new hairpin (hairpin a of Figure 5b). Apparently lowering of Mg^{2+} results in melting out of the 3′-terminal half of the aminoacyl acceptor arm (cf. Figure 5b) under reformation of base pairs between G7 and C13, A6 and U14, G5 and C15, and possibly A4 and U16 (cf. Figure 5b). The latter four base pairs were already proposed by Ahlquist *et al.* (1981) but the dependence on Mg^{2+} of the specific long range interaction between loop e and the sequence in hairpin a (cf. Figure 5b) has not been reported as yet.

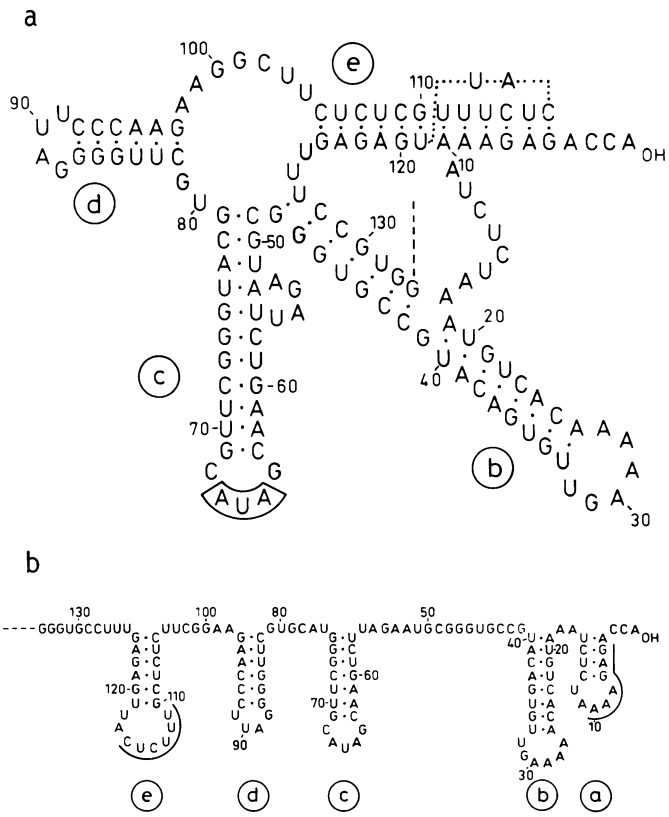

Fig. 5. The structure at the 3′ end of BMV RNA 2. L arrangement (10 mM Mg^{2+}) **(a)** and the secondary structure (1 mM EDTA) **(b)**. The overlined sequences are complementary. The boxed region indicates the anticodon.

Discussion

The tRNA-like 3′ termini of various plant viral RNAs, although lacking a cloverleaf structure, can be folded into a three-dimensional structure, with a striking resemblance to classical tRNA. This conclusion holds true for representatives of different groups of viruses like tymoviruses, the tobamoviruses, the bromoviruses and for the cucumoviruses. Although a detailed elucidation of the tertiary structures of these viral RNA termini has to await biophysical studies, such as high-frequency n.m.r. and X-ray diffraction, the chemical modifications and enzymatic digestions reported here are in surprisingly good agreement with the foldings depicted in Figure 2b and 2c.

Our study also reveals novel features in RNA folding, which may have wider implications. We believe that the building principles underlying the construction of the aminoacyl acceptor arms of the viral RNA termini, may be of significance for understanding the secondary and tertiary structures of RNAs in general. The dynamic behaviour of BMV RNA 2 in solution is also presumably illustrative of conformational transitions, which RNAs generally undergo, on changing the ionic conditions.

Thirdly, the models for the tRNA-like structures permit predictions concerning the minimum sizes of the 3′-terminal fragments required for recognition by aminoacyl-tRNA synthetases.

Conservation of secondary and tertiary structures

Previously (Rietveld *et al.*, 1982) we pointed out that the stabilization of the co-axial arrangement of stems I and II of the TYMV RNA fragment (Figure 1b) is conserved in eggplant mosaic virus (EMV), the cowpea strain of TMV (CcTMV), and tobacco mosaic virus (TMV). Recently we found that this is also the case in green tomato aspermy mosaic virus (GTAMV).

With regard to the bromoviruses it may be recalled that Ahlquist *et al.* (1981) noted that, although the 3′-terminal nucleotide sequences of RNAs 1, 2, 3/4 from BMV, CCMV, CMV and BBMV show a striking homology, the similarity in secondary structure is even more dramatic. The complementarity between the sequences, which are overlined in Figure 3, is conserved in all viral RNAs mentioned above, notwithstanding intraviral and interviral sequence differences. This argues strongly in favour of the base pairing between these sequences which plays such a crucial role in the folding shown in Figure 5a and in our proposal for the tertiary structure illustrated in Figure 2c. This proposal, therefore, like that of the tertiary structure of the 3′-terminal fragment of TYMV RNA, has a sound phylogenetic basis.

Novel features in RNA folding

When the structures of the aminoacyl acceptor arm of TYMV RNA and TMV RNA [Figure 8 of Rietveld *et al.* (1982)] and that of BMV RNA proposed here (Figure 5a) are

analyzed further, interesting features of the higher order folding of RNA become apparent. Both in TYMV RNA and BMV RNA we see that the remainder of the genomic RNA, which is covalently attached to the 5′ side of the tRNA-like structures, does not leave this RNA moiety near its CCA end, as one would expect on the basis of the classical cloverleaf, but does so near the corner of the L shaped molecule, in contrast to earlier proposals (Silberklang *et al.*, 1977; Briand *et al.*, 1977). In our models the 5′ side of the RNA chain arriving at the CCA end submerges again into the aminoacyl acceptor arm itself which means a relatively sharp turn comprising two nucleotides in BMV RNA, four in TYMV RNA, and three in TMV RNA. These short single-stranded stretches span distances in a regular helix of A-RNA which model building (Labquip) showed to be quite acceptable. It is noteworthy that the loops A10-U12 and U21-C24 of TYMV RNA (Figure 1a and b) are located at one side of the molecule which may be advantageous for recognition by tRNA-specific enzymes. Another advantage of this arrangement may be that steric hindrance near the 3′ end is avoided as much as possible allowing free access to proteins.

In Figure 6 we show schematically how the different aminoacyl acceptor arms are constructed, including that for the classical tRNA. In all three examples shown the polarity of the chains in the 12 bp helices remains the same regardless of the arm being constituted of two or three structural elements. The advantage of the TYMV and TMV RNA solution may be that a closed Tψ loop is made possible, in contrast to BMV RNA.

A common feature in the models of the plant viral RNAs is that a part of a loop in a classical hairpin becomes involved in a tertiary interaction. In BMV RNA six nucleotides out of eight (hairpin e, Figure 5b) are base paired to a single-strand region near the 3′-terminus, whereas a more complicated RNA folding occurs in TYMV RNA. Here (cf. Figure 1a and b) in a single-strand stretch, three nucleotides immediately bordering on a double helical stem (here stem II) fold towards three complementary nucleotides located in a loop immediately next to another neighbouring stem (stem I). The final result is a long continuous stack of base pairs (here 12) in which the 3 bp arising from the above tertiary interaction are stacked co-axially between helical stems I and II. The way these plant viruses construct an aminoacyl acceptor arm which is freely accessible is a novel feature in the folding of RNA. The type of interactions discussed here are not taken into account in current computer programs predicting secondary structures of RNA (Zuker and Stiegler, 1981; Roberts and Söll, 1982). The possibility, however, that intra-loop nucleotides next to stem regions are involved in tertiary interactions and so form longer stacks of basepairs, should be considered seriously.

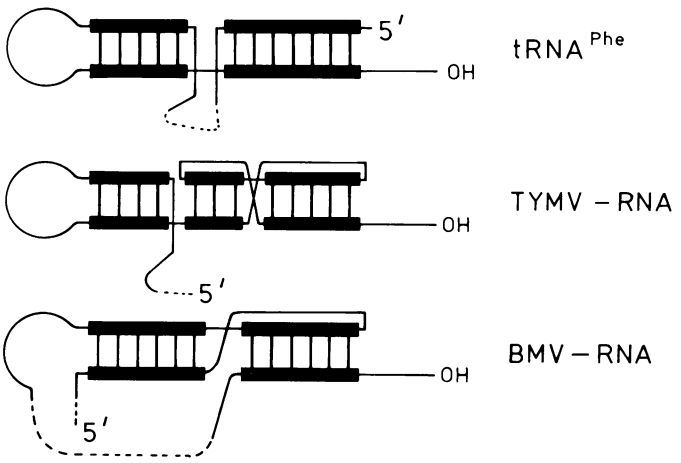

Fig. 6. Schematic representation of the aminoacyl acceptor arms of $tRNA^{Phe}$, TYMV RNA and BMV RNA 2 showing building principles.

Predictions concerning minimum sizes for aminoacylation

The models presented here also allow us to predict the minimum number of nucleotides required for recognizing aminoacyl-tRNA synthetase and nucleotidyl transferase. In the case of TYMV RNA (Figure 2b) for instance the models predict that approximately 85 nucleotides are minimally required for recognizing valyl-tRNA synthetase. Accordingly Joshi *et al.* (1982) reported that the minimum size for aminoacylation is 86 nucleotides and not more than 100 as previous models predicted (Silberklang *et al.*, 1977; Briand *et al.*, 1977). Analogously, Figure 2c suggests that the minimum size of the BMV RNA 2 terminus is ~133 nucleotides and that of the BBMV RNA terminus, which lacks hairpin d (Ahlquist *et al.*, 1981), ~114 nucelotides. These predictions are now open to experimental verfication.

The enigmatic function of the tRNA-like plant viral RNA termini

Although it is not the aim of the present investigation to study the function of these tRNA-like viral structures, a brief mention is necessary. The apparent absence of an anticodon in the expected position of BBMV and CMV RNAs suggests a role in transcription rather than in translation of these viral RNAs. In this context, the possibility may be envisaged that a tRNA recognizing host- or virus-donated protein subunit of the viral RNA replicase mediates the binding of the latter to the 3′ end of the viral RNAs. Obviously other functions, even at the translational level, are not excluded (compare reviews Haenni *et al.*, 1982; Hall, 1979).

Materials and methods

The isolation of TYMV, and the preparation of TYMV RNA and end-labelled 3′-terminal 112 nucleotide fragment were as described by Rietveld *et al.* (1982).

BMV RNA 2 was a gift of Dr. L.van Vloten-Doting. It was end-labelled by incubating 25 μg of RNA with 2.5 μg. CTP, ATP:tRNA nucleotidyltransferase and 20 Ci [α-^{32}P]ATP (sp. act. 3000 Ci/mmol; Amersham) in a total volume of 20 μl containing 50 mM glycine/NaOH pH 9.3 and 12.5 mM $MgCl_2$. The reaction mixture was diluted with an equal volume of sample buffer (20 mM Na citrate pH 5.0, 9 M urea, 1 mM EDTA, xylene cyanol and bromophenol blue) and submitted to electrophoresis on a 10% polyacrylamide gel containing 8 M urea at 1400–1600 V (27.5 W) for 2 h. The 3′-terminally labelled fragment of ~160 nucleotides was eluted from the gel with 0.3 M Na-acetate pH 6.0 and precipitated twice with ethanol and dissolved in redistilled water. Lyophilized samples were submitted to chemical modification of the cytidine and adenosine residues with DMS and DEP, respectively, according to Peattie and Gilbert (1980), as described earlier (Rietveld *et al.*, 1982). Details of the enzymatic digestions can also be found in Rietveld *et al.* (1982).

Acknowledgements

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