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The tRNA-like structure at the 3' terminus of turnip yellow mosaic virus RNA. Differences and similarities with canonical tRNA

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ABSTRACT

The 3' terminus of TYMV RNA, which possesses tRNA-like properties, has been studied. A 3' terminal fragment of 112 nucleotides was obtained by cleavage with RNase H after hybridization of a synthetic oligodeoxynucleotide to the viral RNA. The accessibility of cytidine and adenosine residues was probed with chemical modification. Enzymatic digestion studies were performed with RNase T1, nuclease S1 and the double-strand specific RNase from the venom of the cobra *Naja naja oxiana*.

A model is proposed for the secondary structure of the 3'-terminal region of TYMV RNA comprising 86 nucleotides. The main feature of this secondary structure is the absence of a conventional acceptor stem as present in canonical tRNA. However, the terminal 42 nucleotides can be folded in a tertiary structure which bears strong resemblance with the acceptor arm of canonical tRNA. Comparison of this region of TYMV RNA with that of other RNAs from both the tymovirus group and the tobamovirus group gives support to our proposal for such a three-dimensional arrangement. The consequences for the recognition by TYMV RNA of tRNA-specific enzymes is discussed.

INTRODUCTION

An intriguing property of many plant viral and some animal viral RNAs is their capacity to esterify one specific amino acid to their 3' terminus (1,2). The first virus shown to have this property was turnip yellow mosaic virus (TYMV), whose RNA can be aminoacylated with valine (3,4). Besides aminoacyl-tRNA synthetase a variety of other tRNA-specific enzymes like CTP, ATP:tRNA nucleotidyl transferase, the peptide chain elongation factors eEF-1 and EF-T, peptidyl-tRNA hydrolase and possibly RNase P are able to recognize the 3' end of TYMV RNA (for a review see ref. 1). It is conceivable, therefore, that all viral RNAs concerned possess a 3'-terminal structure which has some features in common with the canonical tRNA. Although the nucleotide sequences of various amino acid accepting viral RNA termini have been determined (5-8) no detailed knowledge exists concerning their secondary or tertiary structures. In some cases cloverleaf models of the secondary struc-

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tures based on the nucleotide sequences have been proposed with the anti-codon in the correct position.

Figure 1 illustrates three such models for the 3' terminus of TYMV RNA (5,6). They all encompass about 105 nucleotides clearly exceeding the number present in tRNA molecules. In the present study we have sought experimental support for these proposals by chemical modification and enzymatic digestion of a 3' terminal fragment of this viral RNA. Our results point to a structure which on one hand bears a strong resemblance to that of the generally accepted structure of tRNA, and on the other deviates appreciably from the models of Figure 1.

We hypothesize that Nature has found a three-dimensional solution to accommodate for an anomalous cloverleaf structure in order to ascertain faithful recognition of this viral RNA terminus by tRNA-specific enzymes. Part of our results was presented at the 5th International Congress of Virology in Strasbourg (1981).

MATERIALS AND METHODS

Isolation of TYMV and TYMV RNA. TYMV (type strain) was grown on Chinese cabbage (*Brassica pekinensis*, var. Witkrop). Infected leaves were harvested 3-4 weeks after inoculation. The virus was isolated according to the method of Dunn and Hitchborn (9) and was finally suspended in 0.01 M Na acetate buffer pH 6.0 and 10^{-3} M Na azide and stored at 4°C. Isolation of TYMV RNA with phenol was as described elsewhere (10).

Isolation of enzymes. RNase H (E.C.3.1.26.4) was isolated from *E. coli*.

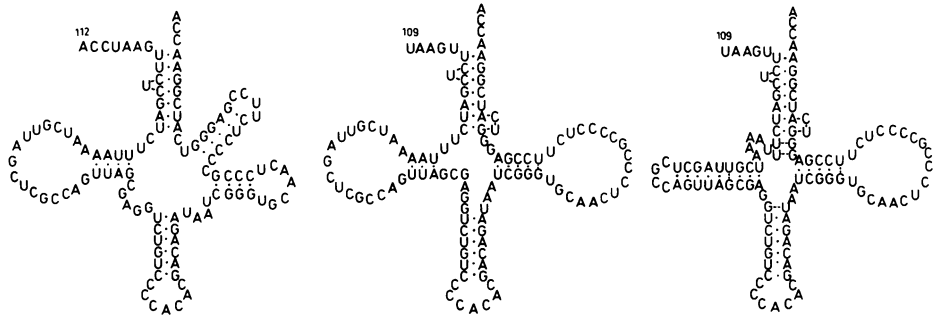

Figure 1. Proposals for the secondary structure of the 3' terminus of TYMV RNA by Silberklang *et al.* (5) and Briand *et al.* (6).

The procedure was essentially as reported by Darlix (11). CTP, ATP:tRNA nucleotidyl transferase (E.C.2.7.7.25) was isolated from yeast (*Saccharomyces cerevisiae*) according to the method described by Rether *et al.* (12). The double-strand specific ribonuclease from the venom of the cobra *Naja naja oxiana* was purified from the venom (Sigma Chemical Co.) as described by Vassilenko and Rait (13).

Synthesis of the deoxyoligonucleotide. The deoxyoligonucleotide (5'-3') d-($GGAGGT_{OH}$) was prepared according to previously published methods (14,15).

Labeling and isolation of the 3'-terminal fragment of TYMV RNA. A solution containing 800 µg of TYMV RNA, 20 µg of (5'-3')d-($GGAGGT_{OH}$) and 80 µCi (α-^{32}P)-ATP (± 40 Ci/mmol, Amersham) was lyophilized and 120 µl of two times concentrated buffer (80 mM Tris-HCl pH 7.9, 20 mM $MgCl_2$, 0.2 mM EDTA, 100 mM NaCl, 0.2 mM DTE) was added. Subsequently 10 µg of RNase H, 5 µg of CTP, ATP:tRNA nucleotidyl transferase and water was added up to a total volume of 240 µl. The resulting mixture was incubated for 16 h at 4°C, whereafter an equal volume of sample buffer (20 mM Na citrate pH 5.0, 9 M urea, 1 mM EDTA, xylene cyanol and bromophenol blue) was added. This mixture was layered on a 7.5% acrylamide, 7 M urea slab gel (40 x 20 x 0.2 cm) (16). Electrophoresis occurred until the bromophenol blue marker had reached the end of the gel. After autoradiography (see Fig. 2b), the RNA fragment of 112 nucleotides was eluted from the gel (17), precipitated three times with ethanol and dissolved in 0.01 M Na acetate pH 6.0.

Chemical modification studies. Chemical mocification of the cytidine and adenosine residues of the 3'-terminal fragment of TYMV RNA was performed essentially as described by Peattie and Gilbert (18). For reactions under non-denaturing conditions a buffer was used containing 50 mM Na cacodylate pH 7.0, 10 mM $MgCl_2$. Modifications under semi-denaturing and under denaturing conditions were carried out in a buffer containing 50 mM Na cacodylate pH 7.0, 1 mM EDTA. The reaction products were analyzed on a 15% acrylamide, 7 M urea slab gel (40 x 20 x 0.03 cm) and detection was by autoradiography.

Enzymatic digestions. In all cases, the lyophilized fragment of 112 nucleotides (approximately 50,000 Cerenkov cpm) was dissolved in the appropriate buffer and preincubated at the desired temperature for 10-15 min, whereafter the enzymes (1 µl) were added (RNase T1 (Sankyo): 0.05 units, nuclease S1 (Sigma Chemical Co.): 1 unit, cobra venom ribonuclease: 0.05 units). Digestion by RNase T1 under non-denaturing conditions was at 37°C for 15 min in a buffer containing 50 mM Na cacodylate pH 7.0, 10 mM $MgCl_2$. Digestion under denaturing conditions was in sample buffer (20 mM Na citrate

pH 5.0, 9 M urea, 1 mM EDTA, xylene cyanol, bromophenol blue). Digestion by nuclease S1 was performed in 40 mM Na acetate pH 4.5, 200 mM NaCl, 10 mM $ZnCl_2$. Incubation was at 37°C for 5 or 30 min. Digestion by the cobra venom ribonuclease occurred at 0°C for 30 sec in a buffer containing 50 mM HEPES pH 7.0, 10 mM $MgCl_2$, 50 mM NH_4Cl, 50 mM KCl. After digestion under non-denaturing conditions, 2 volumes of ethanol were added to precipitate the RNA fragments. After centrifugation, the pellet was dissolved in sample buffer. The RNA fragments were analyzed by electrophoresis on a 15% acrylamide, 7 M urea slab gel (40 x 20 x 0.03 cm) and detection was by autoradiography.

RESULTS

ISOLATION OF A 3'-TERMINAL FRAGMENT OF TYMV RNA

The strategy followed in specifically cleaving TYMV RNA close to its 3' terminus is as follows. First the viral RNA is end-labeled by CTP, ATP:tRNA nucleotidyl transferase (E.C.2.7.7.25) and (α-^{32}P)-ATP making use of the fact that the 3' terminus is -C-C_{OH} (6). The labeled RNA is then hybridized (Figure 2a) to the deoxyhexanucleotide (5'-3')d-(GGAGGT) which is complementary to the nucleotides 113-118. Cleavage of the hybrid by RNase H (19,20) yields a 3'-terminal fragment of 112 nucleotides which is purified by electrophoresis on a 7.5% polyacrylamide gel containing 7 M urea. After autoradiography (Figure 2b) the desired fragment is eluted from the gel according to Peattie (17). The length of the deoxyhexanucleotide and the specificity of its nucleotide sequence do not allow to isolate an unique fragment but combined with the end-labeling technique this isolation procedure is adequate for the purpose of the present investigation.

STRUCTURAL STUDIES ON THE tRNA-LIKE FRAGMENT

Chemical Modification

Secondary interactions in the isolated fragment were first studied with the chemical methods described by Peattie and Gilbert (18). The chemical reactions probing the accessibility of certain parts of the nucleotides have the advantage that they can be used in a variety of environments. They give direct information on the conformation of the RNA fragment. Basepairing interactions of cytidine residues can thus be probed with the alkylating agent dimethyl sulphate (DMS) under mild conditions with and without Mg^{++} and under heavily denaturing conditions. Striking differences in accessibility of the cytidine residues become apparent under the different conditions. The data are summarized in Table 1. In the native state only a few cytidine residues (C53, C55, C57) are clearly exposed to methylation by DMS (Figure 3,

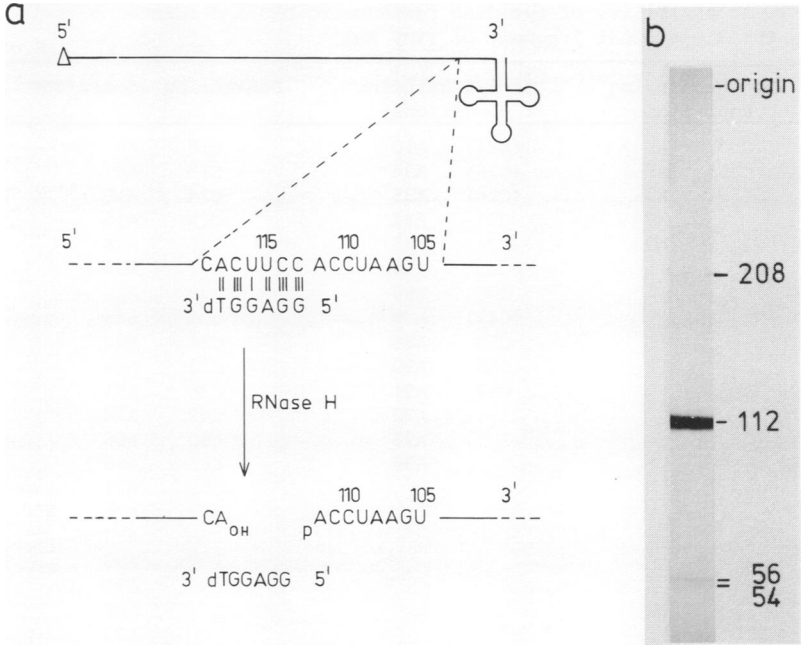

Figure 2. (a) Strategy for the specific cleavage of TYMV RNA by RNase H. (b) Isolation of a labeled 3' terminal fragment of TYMV RNA by preparative slab gel electrophoresis as described under Materials and Methods. A part of the autoradiogram is shown. The faint band of 208 nucleotides arises also from cleavage by RNase H, those of 54 and 56 nucleotides are due to breakdown in the anticodon loop.

lanes 1 and 4). Probably C2 and C3 are also exposed, as well as some cytidines beyond C60, cut our electrophoretic separation does not permit conclusions regarding these residues and also regarding C8 and C11. Under fully denaturing conditions all cytidines in the region from C18 to C60 can be visualized with C22 as a notable exception (lanes 3 and 6). So far our degradation procedures except alkaline degradation and the uridine specific chemical reaction failed to detect this nucleotide in position 22. Perhaps we are dealing with a uridine or a modified cytidine residue.

Residues which are only exposed under heavily denaturing conditions are probably included in tight stem regions. From our results (cf. Table 1) this seems to be the case with C18, C19, C29, C30, C31, C42 and C50. Semi-denaturation upon elimination of Mg^{++} from native RNA, results in the exposure of C27, C33 and C36 and in partial exposure of C24, C25, C26, C31 and C42. These residues may be involved in tertiary interactions or may form part of

Table 1. Accessibility of cytidine residues to DMS and adenosine residues to DEP in the 3'-terminal fragment of TYMV RNA

Non-denaturing conditions		Semi-denaturing conditions		Denaturing conditions	
C53	(A54)	(C24)	A10	C18	A4
C55	(A56)	(C25)	A34	C19	A5
C57	A76	(C26)	A35	C24	A10
	A84	C27	A44	C25	A16
	(A93)	(C31)	A45	C26	A34
		C33	A54	C27	A35
		C36	A56	C29	A44
		(C42)	A76	C30	A45
		C53	A84	C31	A47
		C55	A90	C33	A49
		C57	A91	C36	A51
			A92	C42	A54
			A93	C50	A56
			A99	C53	A68
				C55	A72
				C57	A76
				C58	A84
				C59	A90
					A91
					A92
					A93
					A99

Residues between brackets are attacked to a lesser extent.

a stem region which is more readily opened (cf. Discussion).

The accessibility of the adenosine residues was probed with diethyl pyrocarbonate (DEP). The reaction with DEP is sensitive to stacking of adenine bases which occurs always in double-stranded helices but can occur also in single-stranded regions. Residues of the former type will become destacked under heavily denaturing conditions only: A4, A5, A16, A47, A49, A51, A68 and possibly A72 are examples of this type (cf. Figure 4). From the same figure it can be seen that A76, A84 and A54, A56 and A93 are exposed to the modifying agent under all conditions although the latter three are slightly protected under non-denaturing conditions. The remaining residues A10, A34, A35, A44 and A45 which are stacked under non-denaturing conditions but become accessible upon removal of Mg^{++} may be involved in some tertiary interaction.

Enzymatic Digestions

The availability of specific nucleases which cleave the RNA chain only in single or in double-stranded regions enabled a further probing of secondary and tertiary interactions. This experimental approach has the limitation

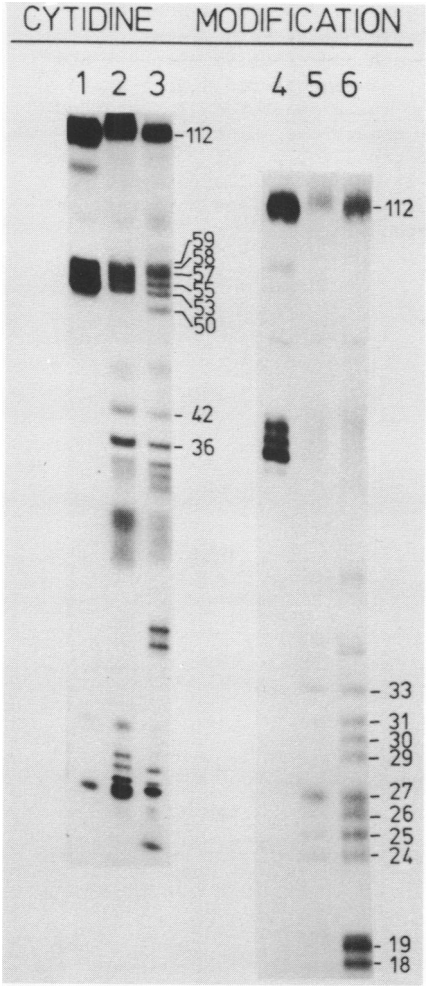

Figure 3. Probing of cytidine accessibility with dimethyl sulphate under native (lanes 1 and 4), semi-denatured (lanes 2 and 5) and fully denatured (lanes 3 and 6) conditions. Electrophoresis occurred for 2 h at 2,000 V (lanes 1-3) or for 3 h (lanes 4-6). For further details see Materials and Methods.

that certain parts of the three-dimensional RNA structure may be inaccessible to enzymes for steric reasons which calls for caution in the interpretation of the results.

The results of the digestion with RNase T1 (Table 2) show that under non-denaturing conditions only G28, G37, G69, G71, G75 and G87 are accessible to the enzyme (Figure 5). It can be concluded that probably the other guanosine residues are part of double-stranded regions or are buried in the inner part of the tRNA-like structure.

The specificity of cleavage by the single-strand specific nuclease S1 is

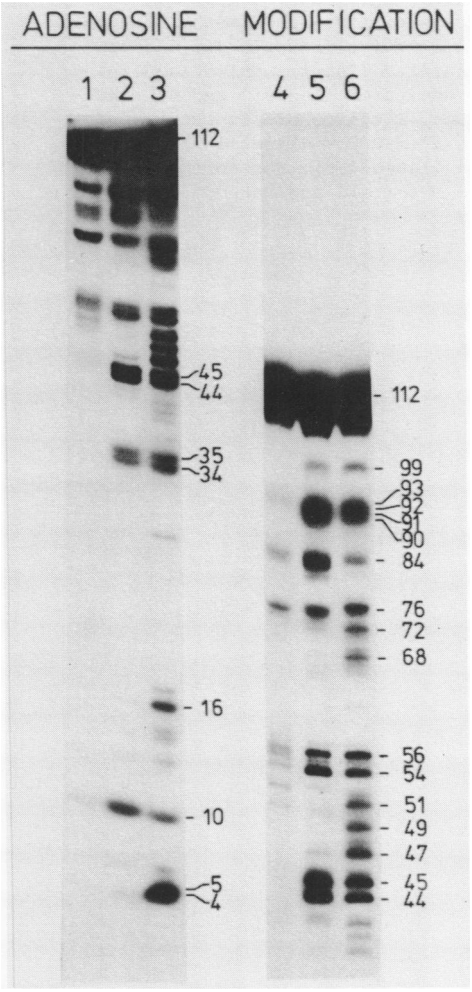

Figure 4. Probing of adenosine accessibility with diethyl pyrocarbonate under native (lanes 1 and 4), semi-denatured (lanes 2 and 5) and fully denatured (lanes 3 and 6) conditions. Electrophoresis occurred for 2 h at 2,000 V (lanes 1-3) or for 4 h (lanes 4-6). For further details see Materials and Methods.

dependent on the time of incubation as can be seen in Figure 5. Within the first 5 min of incubation the enzyme attacks only residues: A54, C55, A56, C57, U74, G75 and to a lesser extent G87 and C88, but when the incubation is prolonged up to 30 min also A44, A45, U46, A47, G48, C50, G52, C53 and to a lesser extent A34, A35, C36, G37, A49 and A51 are attacked. As will be pointed out in the Discussion we think that residues 47-52 are part of a stable stem structure. The seeming discrepancy that they are attacked upon prolonged incubation with nuclease S1 will be discussed below (cf. Discussion).

Finally, the tRNA-like fragment was incubated with the cobra venom ribo-

Table 2. Cleavage of the 3'-terminal RNA fragment of TYMV RNA by various nucleases under non-denaturing conditions

Cleavage after residue:			
RNase T1	Nuclease S1		Cobra venom
	5 min	30 min	ribonuclease
G28	A54	(A34-G37)	A5 -U9
G37	C55	(A49)	U12-U20
G69	A56	(A51)	C25-C26
G71	C57	A44-C57	G39
G75	U74	U74	G41
G87	G75	G75	A45-C53
	(G87)	G87	C64-G66
	(C88)	C88	

Residues or regions of residues between brackets are only slightly attacked.

nuclease, specific for double-stranded RNA (13,21,22); the result is shown in Figure 5. The enzyme cuts in the regions of nucleotides 5-9, 12-20, 25-26, 39, 41, 45-53 and 64-66, implying that these nucleotides are involved in double-stranded structures.
The results of the various enzymatic digestions are compiled in Table 2.

DISCUSSION

From the data obtained by chemical modification (Table 1) and by enzymatic digestion with specific nucleases (Table 2) a model for the secondary structure of the 3'-terminal fragment of TYMV RNA can be deduced (Figure 6). Here we first discuss the various structural elements of this model and the experimental basis whereupon they rest. Secondly we address the question of how this tRNA-like structure can be faithfully recognized by a variety of tRNA-specific enzymes and propose a three-dimensional arrangement for the aminoacyl arm of the fragment. We then show that the sequences involved in the stabilization of this arrangement are conserved in some other plant viral RNAs, both from the tymovirus and from the tobamovirus group. We further deal with a prediction of our model concerning the minimum size of the 3'-terminal fragment sufficient for recognition by the valyl-tRNA synthetase. We finally discuss the merits of the model to avoid steric hindrance at the 3' terminus of TYMV RNA.

The Secondary Structure of the tRNA-like 3' Terminus of TYMV RNA

A major feature of the model of Figure 6 is a stem and loop structure (I) which is not found in the secondary structure of tRNA. Strong evidence for such a stem is derived from the cleavages in this region by the cobra venom ribonuclease (cf. Table 2), which is reported (21,22) to cut specifi-

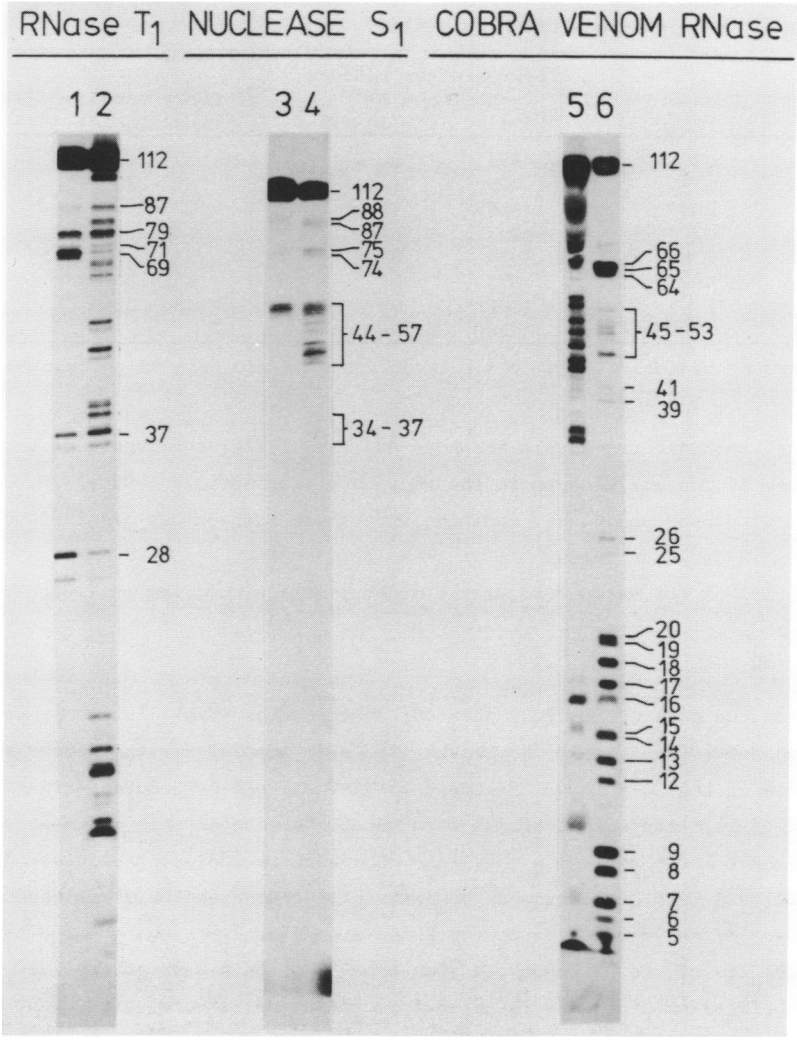

Figure 5. Enzymatic digestion of the tRNA-like fragment with RNase T1 (lanes 1 and 2), nuclease S1 (lanes 3 and 4) and cobra venom RNase (lane 6). Digestion occurred under native conditions (lanes 1,3,4 and 6) or under fully denatured conditions (lane 2). Digestion with nuclease S1 was for 5 min (lane 3) and for 30 min (lane 4). Lane 5 shows the results of adenosine modification with diethyl pyrocarbonate under fully denatured conditions and serves as a reference. Upon incubation without nucleases the tRNA-like fragment migrated with the same rate during electrophoresis showing essentially no degradation. Electrophoresis was run for 2.5 h at 2,000 V. The lower weak band seen in the doublets in lanes 1 and 2 (e.g. G37 and G28) probably originates from a cleavage of the linkage between A1 and C2, leaving behind a labeled fragment shortened by one nucleoside. For further details see Materials and Methods.

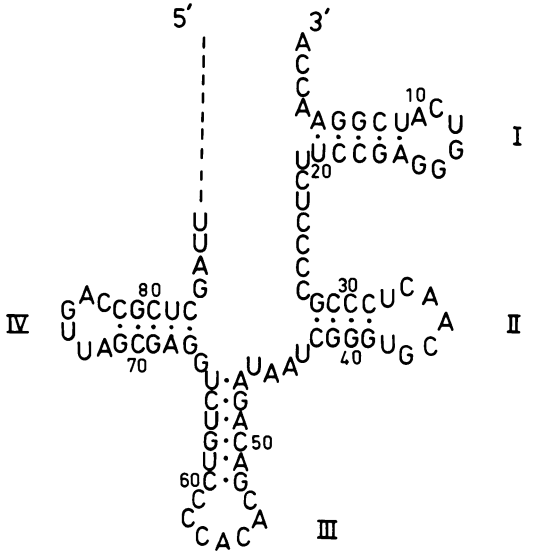

Figure 6. Model for the secondary structure of the tRNA-like fragment of TYMV RNA, based on chemical modification and enzymatic digestions (for details compare the text).

cally in double-stranded regions. RNase T1 and nuclease S1 do not cleave this part of the molecule under non-denaturing conditions. In itself the latter finding does not prove double-strandedness since steric hindrance could prevent cleavage by the enzyme. However, the chemical modification data (Table 1) are also in line with the existence of this novel stem: in the native state A5 and A16 are inaccessible to DEP and C18 and C19 to DMS. The finding that reaction of A4 with DEP requires complete denaturation of the RNA may indicate that this adenine is stacked on top of the helix. Stacking of A10 at the other end of the stem is suggested by the limited exposure of this nucleotide to DEP even after semi-denaturation. Other nucleotides of loop I like G13, G14 and G15 seem to be involved in interactions with distantly located nucleotides. G13, G14 and G15 are also recognized by the cobra venom ribonuclease (Table 2). We shall come back to the behaviour of this triple G sequence below.

The stems and loops numbered II, III and IV in Figure 6 bear resemblance to the TΨC, the anticodon and the D stems and loops of tRNA, respectively. Both chemical modification and enzymatic digestion are in agreement with the existence of stem II. C29, C30, C31 and C42 are only exposed to DMS

under heavily denaturing conditions (Table 1) and G39 and G41 are recognized in the native state by the cobra venom ribonuclease. Under the latter conditions none of the internucleotide linkages of stem II are cleaved by RNase T1 or nuclease S1 (Table 2).

The existence of loop II can be deduced from the accessibility for chemical modification of nucleotides C33, A34, A35 and C36 under semi-denaturing conditions. Under non-denaturing conditions, however, these 4 nucleotides cannot be modified, which points to the possibility of tertiary interactions with other parts of the fragment.

Stem and loop III are also included in the models proposed by other investigators (cf. Figure 1). Loop III contains the valine anticodon CAC in a position analogous to that in tRNA. Experimental support for stem III is derived from the inaccessibility under non-denaturing conditions to DEP of A47, A49 and A51, and to DMS of C50, and also from the finding (Table 2) that the cobra venom ribonuclease cleaves the RNA chain at the position of A47, G48, A49, C50, A51, G52, U63, C64 and U65, while RNase T1 does not cleave after G48, G52 and G62 under native conditions. At variance with the inclusion of residues 47-52 in a stable stem structure are the cleavages by nuclease S1 which occur, however, upon prolonged incubation with the enzyme (Table 2). We suggest that these are due to separation of the chain fragments U46-G52 and C60-G66 during short breathing of the stem, as a result of cuts in the connecting loop. This would implicate that the results with nuclease S1 are only reliable for defining single-stranded regions if obtained after incubations for short periods of time.

Like the anticodon loop in tRNA, loop III seems rather exposed to the environment: in the native RNA molecule C53, C55 and C57 readily react with DMS while C55 is recognized by nuclease S1. Under these conditions A54 and A56 are partially protected against DEP but they become fully exposed after semi-denaturation. Interactions due to stacking may be responsible for this behaviour since both nucleotides are readily attacked by nuclease S1. Similar results were obtained for the anticodon loop of $tRNA^{Phe}$ from yeast (18, 23). So far we have not been able to probe the accessibility of C58, C59 and C60 satisfactorily. This may be due, as mentioned above, to shortcomings of our separation technique following DMS treatment or to protection under native conditions. In the anticodon loop of $tRNA^{Phe}$ the corresponding bases of C58 and C59 form part of the coordination pocket site for Mg^{2+} and are also protected (18,24). These and other experimental limitations likewise prevent us from presenting strong evidence for stem and loop IV. Reaction

with DEP shows that A68 and possibly A72 are stacked in the native RNA molecule whereas A76 and A84 are fully exposed under these conditions, which is in agreement with our model. No cleavages by the cobra venom ribonuclease have been observed in this region. Although Lockard and Kumar (22) were able to cleave the D stem of 3 different tRNAs with an analogous enzyme, Favorova *et al.* (25) and Boutorin *et al.* (26) failed to do so. Possibly this discrepancy is due to differences in enzyme preparation, or in ionic conditions for hydrolysis, or both. A justification for the proposal of stem and loop IV might be the fact that this structure is the most stable hairpin possible in this region according to the rules of Tinoco *et al.* (27). It may also be noted that the sequence CUCGAU (position 80-85) is reminiscent of a region in tRNA supposed to be recognized by tRNA synthetase (28-31).

A Three-Dimensional Model for the Acceptor Arm

The most intriguing part of our model is the arrangement of the first 42 nucleotides, suggesting that the tRNA-like fragment of TYMV RNA lacks the conventional aminoacyl stem. As mentioned in the Introduction, however, TYMV RNA is recognized by at least 5 tRNA-specific enzymes, some of which are reported to interact with the aminoacyl stem (21,25). This strongly suggests that an aminoacyl stem should exist and stem I therefore is a candidate to be considered. So far it has remained unexplained why C24, C25, C26 and C27 become only partially reactive with DMS after semi-denaturation and require full denaturation for complete reaction with the modifying agent (cf. Table 1). Indications that G13, G14 and G15 are involved in base-pairing with distantly located nucleotides have been discussed above on the basis of the cuts introduced at the position of these three guanosines by the cobra venom enzyme (Table 2). The possibility may be envisaged therefore, that G13, G14 and G15 basepair with C27, C26 and C25, respectively (or with C26, C25 and C24, respectively) in such a way that stems I and II become coaxial. If such a structure (visualized in Figure 7) possesses sufficient stability, it would provide our tRNA-like fragment with an acceptor arm which might be recognizable by tRNA-specific enzymes. Obviously further biochemical and biophysical investigations are necessary to prove the existence of such a coaxial arrangement of stems I and II.

The 3' Termini of EMV, TMV and CcTMV RNA

It is most striking that the triple C and triple G regions involved in the stabilization of the coaxial arrangement of stem I and II appear to be conserved in some other amino acid accepting plant viral RNAs. Figure 8 shows secondary structures at the 3' terminus of one other viral RNA from

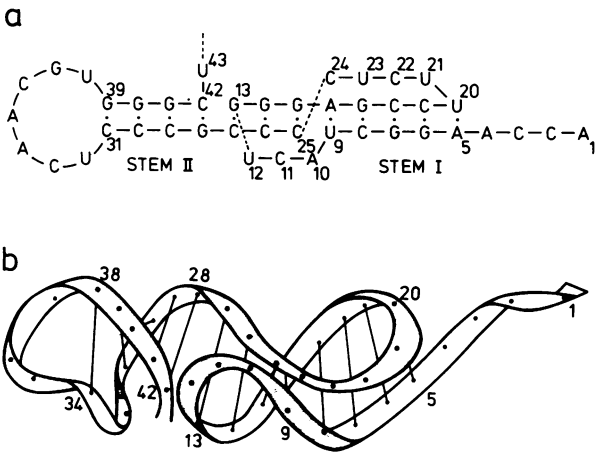

Figure 7. Model for the three-dimensional structure of the aminoacyl arm comprising the last 42 nucleotides at the 3' terminus of TYMV RNA. (a) Schematic representation of the continuous stacking of 12 basepairs, consisting of stem I, the interaction between G13, G14, G15 and C25, C26, C27, and stem II. (b) Speculative folding of the aminoacyl arm of the tRNA-like structure as based on the three-dimensional structure of $tRNA^{Phe}$ from yeast (32,33). The putative interaction between A34 and U38 is also included. For further details see text.

the tymovirus group (eggplant mosaic virus) and two from the tobamovirus group (tobacco mosaic virus and the cowpea strain of tobacco mosaic virus). The RNA of the cowpea strain of TMV accepts valine (34) like TYMV RNA and EMV RNA, in contrast to other members of the tobamoviruses which bind histidine (1). The sequence of about 1,000 nucleotides at the 3' end of CcTMV RNA was reported recently and, surprisingly, it turned out that the 3' terminal 80 nucleotides show a strong homology (60%) with TYMV RNA (35). We found that this region of CcTMV RNA can be folded in an almost identical fashion as we propose for TYMV RNA. In Figure 8 the region of the last 42 nucleotides is shown.

The secondary structures for the 3' terminus of EMV RNA and TMV RNA as shown in Figure 8 are based on the reported nucleotide sequences (36,7). All four sequences can be folded in such a way that the same coaxial arrangement is obtained as presented for TYMV RNA, resulting in a continuous stack of 12 basepairs in the case of the valine accepting RNAs and 11 basepairs in the case of TMV RNA. The structure proposed for TMV RNA further deviates slightly from the other three viral RNAs in having only 5 nucleotides in loop I and 6 nucleotides in the region connecting stem I and II, instead of

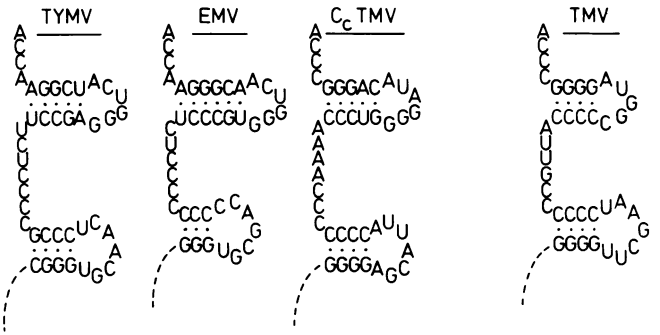

Figure 8. Proposed secondary structures for the 3' termini of TYMV RNA, EMV RNA, CcTMV RNA and TMV RNA.

6 and 7, respectively. Note that in the triple G sequence of loop I of TMV RNA a guanosine residue is replaced by a cytidine which is compensated for by the substitution of a cytidine in the triple C region by a guanosine. The same number of 12 stacked basepairs is found in the aminoacyl arm of $tRNA^{Phe}$ from yeast (32,33) suggesting that the tertiary structure proposed in Figure 7 corresponds with the aminoacyl arm of $tRNA^{Phe}$. The resemblance goes even further because the interaction between A58 and T54 in the TΨC loop of $tRNA^{Phe}$ appears to be conserved in all four viral RNAs. A U-A or A-U pair is in principle possible at exactly the same position in our loop II, adding a thirteenth basepair to the above mentioned stack of 12 basepairs. Also the number of 7 nucleotides in loop II is the same as in all tRNAs. Furthermore we assume that in all 4 viral RNAs the 3' terminal 4 nucleotides do not take part in basepairing as is usual for tRNAs.

A third group of plant viruses whose RNAs can be aminoacylated are the bromoviruses (1,2). These RNAs all accept tyrosine. Ahlquist *et al.* have proposed models for the secondary structures of nine different 3' termini of these RNAs and the 3' termini of CMV RNA (8). These structures appear to have extensively basepaired regions with strong intra- and interviral similarities. The only common feature with the tymoviral and tobamovirus RNAs discussed here is the apparent absence of a conventional aminoacyl stem. Attempts to fold the region comprising the first two stems and loops according to the same principles as shown in Figure 7 failed, suggesting that the three-dimensional solution for the building of a tRNA-like structure must differ from the one proposed here for TYMV RNA.

Another remarkable feature of the tertiary structure of TYMV RNA as

shown in Figure 7 is the occurrence of two short stretches of single-stranded RNA having 3 (A10-U12) and 4 (U21-C24) nucleotides, respectively. These single-stranded pieces of RNA have to bridge distances of almost half a turn and a quarter of a turn of an A helix, respectively. This gives rise to some sort of triple-stranded structure encompassing 8 basepairs and 7 nucleotides in single-stranded regions in the case of TYMV RNA. Preliminary model building revealed that these 3 and 4 nucleotides are sufficient indeed for this bridging, even though rather sharp turns in the sugar phosphate backbone are necessary. It also turned out that the two single-stranded pieces are both located at one side of the helix which might be relevant in conjunction with the recognition of these molecules by the various tRNA-specific enzymes. Our experimental data indicate that these short regions are really single-stranded, although a tertiary interaction of some of the bases with the adjacent double-stranded helix should be envisaged, thereby contributing to the stability of this part of the molecule.

Although we have no experimental support for the arrangement of our stem and loop III in relation to the aminoacyl arm we are rather confident that it is part of the anticodon arm in much the same way as in classical tRNA. The properties of this stem and loop in respect to chemical modification and enzymatic digestion are strongly reminiscent to what has been found for the anticodon loop of $tRNA^{Phe}$ from yeast (18,22,23). Fitting our stem and loop IV in the canonical tertiary structure is less easy to perform for two reasons. First our experimental data do not give strong evidence for the proposed secondary structure. Secondly, the dihydrouridine stem and loop of the canonical tRNA are involved in complex tertiary interactions with the rest of the molecule. The latter might be one of the reasons for our poor results concerning stem and loop IV. We believe therefore that statements about the tertiary structure in the corner of the putative L-form of the tRNA-like fragment of TYMV RNA, if it exists at all, are premature at this stage. On the other hand, we expect that by analogy to tRNA, tertiary interactions will certainly be present in order to interlock the two arms of this fragment into a rigid structure.

Minimum Size for Aminoacylation

An important difference of our model with previous models for the 3' end of TYMV RNA (see Figure 1) is that in fact the 5' end of our fragment of 112 nucleotides is not needed for the formation of the aminoacyl stem. The question therefore can be raised as to whether the size of the 3' terminal fragment which still can be aminoacylated could be smaller than 112 nucleo-

tides. Our model predicts that a fragment of 80-85 nucleotides would be sufficient for recognition by the valyl tRNA-synthetase. In the accompanying paper Joshi *et al.* (37) show that the size of the smallest fragment in a T1 RNase digest of TYMV RNA which can accept valine, is 86 nucleotides. Fragments of 82 or fewer nucleotides appear to have lost this capacity. This observation shows that the last 27 nucleotides at the 5' end of the fragment of 112 nucleotides are dispensable for recognition by the synthetase. The results of Joshi *et al.* are also in agreement with the observation of Meshi *et al.* (35) that the valine accepting CcTMV RNA only shows a strong homology with TYMV RNA up to 80 nucleotides from the 3' end. Furthermore the number of nucleotides in the fragment with tRNA-like properties now fits with the number of nucleotides present in classical tRNAs.

If our model for the aminoacyl arm is correct, then Nature has found an interesting way to avoid steric hindrance at the 3' terminus of TYMV RNA by the rest of the molecule. The accessibility of the CCA end for one or more tRNA-recognizing proteins in the infected cell (viral replicase?) might be reduced significantly when the tRNA part of TYMV RNA would be connected to the main part of the molecule as indicated in the models of Figure 1. In our model this connection is in the corner of the putative L-form instead of at the end of the aminoacyl stem. The presence of six or seven nucleotides in single-stranded regions immediately adjacent to the double helix of the aminoacyl stem might be the most economical solution for a piece of RNA which should have tRNA-like properties and which at the same time is part of a larger molecule.

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REFERENCES

1. Haenni, A.L., Joshi, S. and Chapeville, F. (1982) in *"Progress in Nucleic Acids Research and Molecular Biology"* (Davidson, I.N. and Cohn, W.E., eds.), in press.
2. Hall, T.C. (1979) *Int.Review of Cytology 60*, 1-26.
3. Pinck, M., Yot, P., Chapeville, F. and Duranton, H.M. (1970) *Nature 226*, 954-956.
4. Yot, P., Pinck, M., Haenni, A.-L., Duranton, H.M. and Chapeville, F. (1970) *Proc.Natl.Acad.Sci. U.S.A. 67*, 1345-1352.
5. Silberklang, M., Prochiantz, A., Haenni, A.-L. and RajBhandary, U.L.

(1977) *Eur.J.Biochem. 72*, 465-478.
6. Briand, J.P., Jonard, G., Guilley, H., Richards, K. and Hirth, L. (1977) *Eur.J.Biochem. 72*, 453-463.
7. Guilley, H., Jonard, G., Kukla, B. and Richards, K.E. (1979) *Nucleic Acids Res. 6*, 1287-1308.
8. Ahlquist, P., Dasgupta, R. and Kaesberg, P. (1981) *Cell 23*, 183-189.
9. Dunn, D.B. and Hitchborn, J.H. (1965) *Virology 25*, 171-192.
10. Bosch, L., Bonnet-Smits, E.M. and Van Duin, J. (1967) *Virology 31*, 453-460.
11. Darlix, J.-L. (1975) *Eur.J.Biochem. 51*, 369-376.
12. Rether, B., Bonnet, J. and Ebel, J.-P. (1974) *Eur.J.Biochem. 50*, 281-288.
13. Vassilenko, S.K. and Ryte, V.K. (1975) *Biokhimiya 40*, 468-483.
14. Arentzen, R., Van Boeckel, C.A.A., Van der Marel, G. and Van Boom, J.H. (1979) *Synthesis*, 137-138.
15. Van Boom, J.H., Burgers, P.M.J., Verdegaal, C.H.M. and Wille, G. (1978) *Tetrahedron 34*, 1999-2007.
16. Donis-Keller, H., Maxam, A. and Gilbert, W. (1977) *Nucleic Acids Res. 4*, 2527-2538.
17. Peattie, D.A. (1979) *Proc.Natl.Acad.Sci. U.S.A. 76*, 1760-1764.
18. Peattie, D.A. and Gilbert, W. (1980) *Proc.Natl.Acad.Sci. U.S.A. 77*, 4679-4682.
19. Donis-Keller, H. (1979) *Nucleic Acids Res. 7*, 179-192.
20. Stepanova, O.B., Metelev, V.G., Chichkova, N.V., Smirnov, V.D., Rodionova, N.P., Atabekov, J.G., Bogdanov, A.A. and Shabarova, Z.A. (1979) *FEBS Lett. 103*, 197-199.
21. Boutorin, A.S., Clark, B.F.C., Ebel, J.-P., Kruse, T.A., Petersen, H.V., Remy, P. and Vassilenko, S. (1981) *J.Mol.Biol. 152*, 593-608.
22. Lockard, R.E. and Kumar, A. (1981) *Nucleic Acids Res. 9*, 5125-5140.
23. Wrede, P., Wurst, R., Vournakis, J. and Rich, A. (1979) *J.Biol.Chem. 254*, 9608-9616.
24. Teeter, M.M., Quigley, G.J. and Rich, A. (1980) in *"Nucleic Acid-Metal Ion Interactions"* (Spiro, T.G., ed.), Wiley Interscience, New York, pp. 145-177.
25. Favorova, O.O., Fasiolo, F., Keith, G., Vassilenko, S.K. and Ebel, J.-P. (1981) *Biochemistry 20*, 1006-1011.
26. Boutorin, A.S., Remy, P. and Ebel, J.-P. (1982) *Eur.J.Biochem. 121*, 587-595.
27. Tinoco, I., Borer, P.N., Dengler, B., Levine, M.D., Uhlenbeck, O.C., Crothers, D.M. and Gralla, J. (1973) *Nature New Biol. 246*, 40-41.
28. Igloi, G.L. and Cramer, F. (1978) in *"Transfer RNA"* (Altman, S., ed.), MIT Press, Cambridge.
29. Rosa, J.J., Rosa, M.D. and Sigler, P.B. (1979) *Biochemistry 18*, 637-647.
30. Renaud, M., Dietrich, A., Giegé, R., Remy, P. and Ebel, J.-P. (1979) *Eur.J.Biochem. 101*, 475-483.
31. Renaud, M., Bocha, H., Dietrich, A., Remy, P. and Ebel, J.-P. (1981) *Biochim.Biophys.Acta 653*, 145-159.
32. Kim, S.H., Suddath, F.L., Quigley, G.J., McPherson, A., Sussman, J.L., Wang, A.H.J., Seeman, N.C. and Rich, A. (1974) *Science 185*, 435-440.
33. Ladner, J.E., Jack, A., Robertus, I.D., Brown, R., Rhodes, D., Clark, B.F.C. and Klug, A. (1975) *Proc.Natl.Acad.Sci. U.S.A. 72*, 4414-4418.
34. Beachy, R.N., Zaitlin, M., Bruening, G. and Israel, H.W. (1976) *Virology 73*, 498-507.
35. Meshi, T., Ohno, T., Iba, H. and Okada, Y. (1981) *Mol.Gen.Genet. 184*, 20-25.
36. Briand, J.P., Richards, K.E., Bouley, J.P., Witz, J. and Hirth, L. (1976) *Proc.Natl.Acad.Sci. U.S.A. 73*, 737-741.
37. Joshi, S., Chapeville, F. and Haenni, A.-L. (1982), accompanying paper.