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Five pseudoknots are present at the 204 nucleotides long 3' noncoding region of tobacco mosaic virus RNA

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ABSTRACT

The 104 nucleotides long 3' terminal region of TMV RNA was shown previously to contain two pseudoknotted structures (Rietveld et al. (1984), EMBO J. 3, 2613-2619). We here present evidence for the occurrence, within the 204 nucleotides long 3' noncoding region, of another highly structured domain located immediately adjacent to the tRNA-like structure of 95 nucleotides (Joshi et al. (1985) Nucleic Acids Res. 13, 347-354). A model for the three-dimensional folding of this region, containing three more pseudoknots, is proposed on the basis of chemical modification and enzymatic digestion.

The existence of these three consecutive pseudoknots was supported by sequence comparisons with the RNA from the related tobamoviruses TMV-L, CcTMV and CGMMV. Coaxial stacking of the six double helical segments involved gives rise to the formation of a 25 basepair long quasi-continuous double helix. The results show that the three-dimensional folding of the 3' non-translated region of tobamoviral RNAs is largely maintained by the formation of five pseudoknots. The organisation of this region in the RNA of the tobamovirus CcTMV suggests that recombinational events among aminoacylatable plant viral RNAs have to be considered.

INTRODUCTION

The 3' termini of plus-stranded viral RNAs are thought to play an essential role in the replication cycle of the virus (1). In some cases a specific amino acid can be esterified enzymatically to these termini, suggesting the presence of a tRNA-like structure (2,3). Recently, we and others have proposed models of the secondary structure of the 3' termini of all aminoacylatable plant viral RNAs known (4-11). Using chemical modification, enzymatic digestion and sequence comparisons we have studied the folding of 3' terminal fragments of turnip yellow mosaic virus (TYMV) RNA, brome mosaic virus (BMV) RNA and tobacco mosaic virus (TMV) RNA (5,8,10).

An intriguing feature which emerged from these studies is the presence of so-called pseudoknotted structures in the aminoacylacceptor arm of all tRNA-like structures analysed sofar. Fig. 1 illustrates the folding

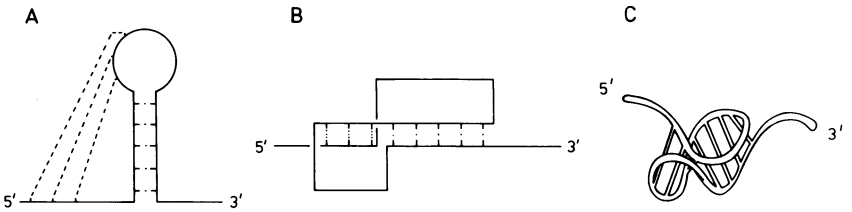

Figure 1. The formation of extended double helices by applying the principle of pseudoknotting. Nucleotides from a hairpin loop basepair with a complementary region elsewhere in the RNA chain. If these nucleotides are adjacent to the stem, a quasi-continuous double helix can be formed.
A. Conventional hairpin structure. The dashed lines represent Watson-Crick basepairing which can give rise to pseudoknot formation.
B. Schematic folding of the pseudoknot, which consists of two stem regions and two connecting loops.
C. Three-dimensional folding illustrating the coaxial stacking of the two double helical segments. The 5' proximal connecting loop crosses the major groove, the other one spans the minor groove.
A detailed description of the properties of this type of pseudoknot is given in Pleij et al. (12).

principle of these pseudoknots. Basically this implies basepairing between nucleotides of a hairpin loop and nucleotides in a complementary single stranded region located elsewhere in the RNA chain (Fig. 1A). This enables the formation of an extended quasi-continuous double helix (Fig. 1B and 1C) (12). Two examples of these pseudoknots occur in the tRNA-like structure of TMV RNA (10). In the present study we have extended our structural analysis of the 3' terminus of TMV RNA to the region upstream of the tRNA-like structure up to nucleotide 190. The results show that another highly structured domain is present comprising about 40% of the total 3' noncoding region. Surprisingly, within this domain we find three new examples of pseudoknotted structures which are arranged in tandem, thus forming a 25 basepairs long double helix-like domain. Sequence comparisons among tobamoviral RNAs show that two out of these three pseudoknots are strongly conserved. We conclude that the entire 3' noncoding region of TMV RNA contains five pseudoknots.

MATERIALS AND METHODS

Enzymes

CTP, ATP; tRNA nucleotidyl transferase was purified from yeast according to Rether et al. (13). Double strand specific RNase from the venom of the cobra Naja Naja oxiana was a gift of Dr. A. Tara. Phosphodiesterase from Crotalus durissus was purchased from Boehringer. RNase T_1 and nuclease

S_1 were from Sankyo and Sigma, respectively.

Labeling and isolation of 3' terminal fragments of TMV RNA

Tobacco mosaic virus (vulgare strain) was a gift of Dr. B. Verduin. For each labeling experiment 20 µg of the isolated RNA in a total mixture of 500 µl containing 50 mM tris.HCl pH 8.3, 10 mM $MgCl_2$ and 2.5 µg phophodiesterase was treated at 20 °C for 15 min. After two phenol extractions and precipitation with ethanol the 3' end of the RNA was reconstituted with ATP, CTP and nucleotidyl transferase. A 10 µl mixture containing 5 µg of transferase, 10 µCi (α-^{32}P) ATP (3000 Ci/mmol, Amersham), 35 µM CTP, 50 mM tris.HCl pH 7.9 and 10 mM $MgCl_2$ was incubated at 4°C for 16 hr. The labeled RNA was subsequently electrophoresed through a 5% polyacrylamide slabgel containing 8 M urea. Radioactive fragments with a size of 180-200 nucleotides were eluted from the gel as described by Peattie and Gilbert (14). Finally the precipitated RNA fragments were dissolved in sterile doubly distilled water and stored at -20 °C.

Chemical and enzymatic probing

Chemical probing of adenosine residues using diethyl pyrocarbonate was carried out according to Peattie and Gilbert (14). Enzymatic digestions with RNase T_1 , were done in three different ways. Digestions under native (10 mM $MgCl_2$) and semi-denaturing (1 mM EDTA) conditions were performed at 37°C for 15 min. in 50 µl 50 mM sodium cacodylate buffer pH 7.0 using 0.0125 units of RNase T_1. After the reaction the RNase was removed by phenol extractions and the RNA precipitated with ethanol. Prior to electrophoresis the precipitates were dissolved in sample buffer (20 mM sodium citrate pH 5.0, 9 M urea, 1 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). Digestions of the RNA under fully denaturing conditions were carried out in 3 µl sample buffer using 0.0125 units of RNase T_1 at 50°C for 15 min. Degradation by the cobra venom RNase and S_1 nuclease was performed under native conditions only. The RNA was dissolved in 50 µl 50 mM sodium cacodylate pH 7.0, 10 mM $MgCl_2$ and digestion was done at 37 °C using 1-10 units of nuclease S_1 (5 min) or 0.001-0.0005 units of the cobra venom RNase (15 min). The RNA was recovered by precipitation with ethanol and dissolved in sample buffer. All samples were analysed on 5% polyacrylamide, 8 M urea slabgels, followed by autoradiography.

RESULTS

Previous structure mappings of TMV RNA in our laboratory were performed using specifically labeled 3' terminal fragments with a size of about 190

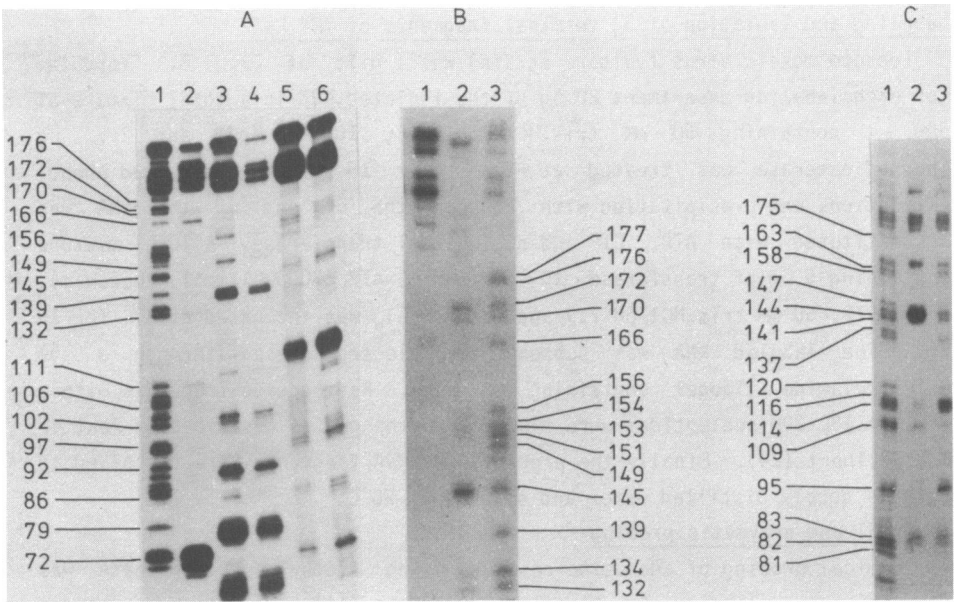

Figure 2. Structure mapping of end-labeled 3' terminal fragments of TMV vulgare RNA by chemical modification and enzymatic digestion.

A. Digestion with RNase T_1 under denaturing (lane 1) and native conditions (lane 2), with cobra venom RNase (0.001 U, lane 3; 0.0005 U, lane 4) and with nuclease S_1 (1 U, lane 5; 10 U, lane 6). Numbers on the left indicate the position of some of the G residues, numbered from the 3' end.

B. Digestion with RNase T_1 under native (lane 1), semi-denaturing (lane 2) and denaturing conditions (lane 3). The position of the residues is indicated on the right.

C. Probing of adenosine residues with diethyl pyrocarbonate (DEP) under denaturing (lane 1), native (lane 2) and semi-denaturing conditions. The position of some of the A residues is given on the right.

Electrophoresis was for 2-2,5 h at 2000 V on 5% polyacrylamide, 8 M urea slabgels. For further details see Materials and Methods.

nucleotides. These fragments resulted from spontaneous or RNase induced degradation within an A,U-rich sequence at position 179-189 from the 3'end. The same fragments were used here for an analysis of the higher order structure upstream of the tRNA-like structure using the approach as described before (10).

The results obtained from the chemical modification of adenosine residues and from enzymatic digestions with nuclease S_1, ribonuclease T_1 and the double strand specific RNase from the venom of the cobra Naja naja oxiana

Table 1.
Summary of data derived from chemical modification and enzymatic digestion of a 3' terminal fragment of TMV RNA.

	T_1(n)	T_1(sd)	S_1	CVase	DEP (n)	DEP (sd)		T_1(n)	T_1(sd)	S_1	CVase	DEP (n)	DEP (sd)
G 106		±					A 147						±
G 107		±					G 149				+		
G 108		±					G 151						
A 109							G 153						
A 110			+		+	+	G 154		±				
G 111	±	±					G 156		±				
A 114					+	+	C 157				+		
A 115					+	+	A 158						+
A 116					+	+	C 159				+		
U 117			+				A 160						+
A 120						+	A 163			+		+	+
U 123				+			A 164			+		+	+
C 124				+			U 165			+			
G 132		±					G 166	+		+			
G 134		±					C 167				+		
A 135						+	U 169				+		
A 137				+			G 170		+		+		
C 138				+			G 172		+				
G 139				+			U 174			+			
A 141							A 175						
A 142					+	+	G 176						
A 144			+		+	+	G 177						
G 145		+	+				A 179					+	

Residues are numbered from the 3' end and only nucleotides accessible to the modifying agent or the enzymes are included. DEP stands for diethyl pyrocarbonate, S_1 and T_1 for nuclease S_1 and RNase T_1 respectively. The cobra venom RNase is abbreviated as CVase; (n) and (sd) represent native and semi-denaturing conditions; + means strong degradation, ± indicates the presence of a weak band in the autoradiographs. For further details see Materials and Methods.

are shown in Fig. 2 and summarised in Table 1.

In Fig. 3A a model of the secondary structure for the region upstream of nucleotide 105, as determined under semi-denaturing conditions (1 mM EDTA) is presented. In the presence of Mg^{2+} (10 mM) additional basepairs are observed (Fig. 3B, dashed lines). This can be concluded from the decreased accessibility of A120, G132, G134, A135, G145, A147, G154, G156, G170 and G172 under native conditions. These additional basepairs give rise to double helical segments numbered II, IV and V which can be stacked on top of stem I, III and VI respectively, according to the pseudoknot folding principle (see Fig. 1). A quasi-continuous double helix, comprising 25 basepairs and consisting of three consecutive pseudoknots (nucleotides 106-135, 136-157 and 158-177, respectively), can thus be formed (see Fig. 3C). Stem region VI which consists of only three basepairs under

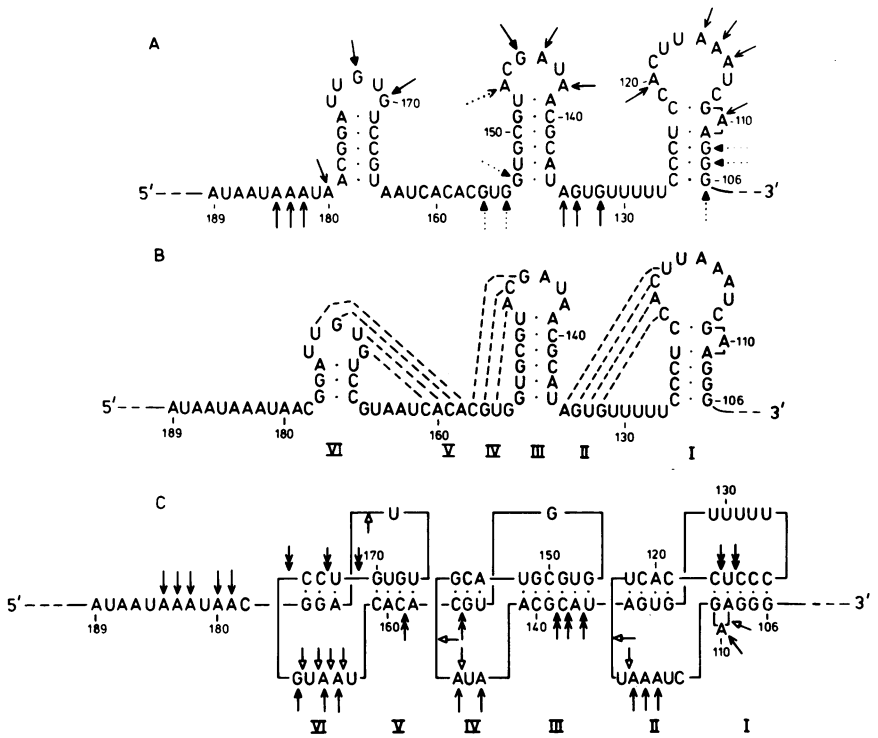

Figure 3. Models of the structure of the region located upstream of the tRNA-like structure of TMV RNA. The partial sequence is taken from the complete primary structure as reported by Goelet et al. (15).

A. The secondary structure under semi-denaturing conditions (1 mM EDTA).

B. Model showing the interactions (dashed lines) which give rise to the formation of three consecutive pseudoknots when Mg^{2+} is added. Roman numbers indicate the six double helical segments involved (stem region I, III and VI are also present in A.).

C. The structure under native conditions (10 mM $MgCl_2$). This schematic folding is obtained by coaxial stacking of helices I to VI (compare Figure 1).

The arrows indicate the residues or phosphodiester bonds which are accessible to the various probes (⟶, RNase T_1 ; ⟿, nuclease S_1 ; ⟾, cobra venom RNase; ⟶, diethyl pyrocarbonate). Weak bands in the autoradiographs are indicated by dotted arrows (see also Table 1 and Figure 2).

native conditions (Fig. 3B) is extended by two basepairs (G166-C178 and U165-A179) upon disruption of stem V under semi-denaturing conditions (Fig. 3A). The absence of an RNase T_1 cut after G166 upon addition of EDTA supports the formation of a five basepair long stem under semi-denaturing conditions (see also Fig. 2B). All cuts from the cobra venom RNase are in agreement with the model in Fig. 3C and are found in five of the six

proposed double helical segments. Further support for this model can be derived from strong nuclease S_1 cuts at positions 116-118, 142-145 and 162-166. The single stranded nature of these regions is further confirmed by the increased DEP modification of the adenosines present (A114, A115, A116, A142, A144, A163 and A164). The absence of enzymatic degradations at positions 127-131, 154 and 174 can be explained by the steric properties of the pseudoknots involved. Nucleotide regions crossing the minor groove of the RNA double helix turn out to be potentially more vulnerable than nucleotides spanning the major groove (see Discussion and Pleij et al. (12)). Upon removal of Mg^{2+}, not only basepairing patterns change. For instance, the conformation of the region including A114-A116 is seen to change significantly although the nucleotides involved are part of a single stranded region in both native and semi-denatured conformations. These adenosine residues apparently become unstacked upon disruption of stem II. The reverse is true for residues A142 and A144 which become unstacked when stem IV is formed.

DISCUSSION

Basepaired regions

The major outcome of our experimental analysis of the secondary and tertiary structure of the 3' noncoding region of TMV RNA is the presence of five so-called pseudoknots in a stretch of 180 nucleotides. Evidence for two of these pseudoknots in the tRNA-like structure was presented before (10). Comparisons with other tobamoviral RNAs yielded strong support for their presence. It is therefore gratifying to see (Fig. 4) that the three pseudoknots described in this paper are also present in the same region of three related tobamoviral RNAs of which the sequence at the 3' terminus is known (16-18). Stem region I (see also Fig. 3C) can be constructed in an identical manner in all four sequences considered: five basepairs, including a bulging A residue. The many compensating base changes in this stem in the case of CGMMV and CcTMV are striking. The top of stem I in CGMMV consists of a G·U pair (G111·U122), although this pair could easily be replaced by a more obvious A·U pair involving A110, eventually followed by a sixth basepair (G111·C121). The G·U pair is preferred however, because it enables the formation of a single bulged A residue at the same position as in the other three RNAs. The location of this bulge was deduced from the experimental data obtained for the RNA from the vulgare strain of TMV. The presence of two successive A residues however, offers the possibility of

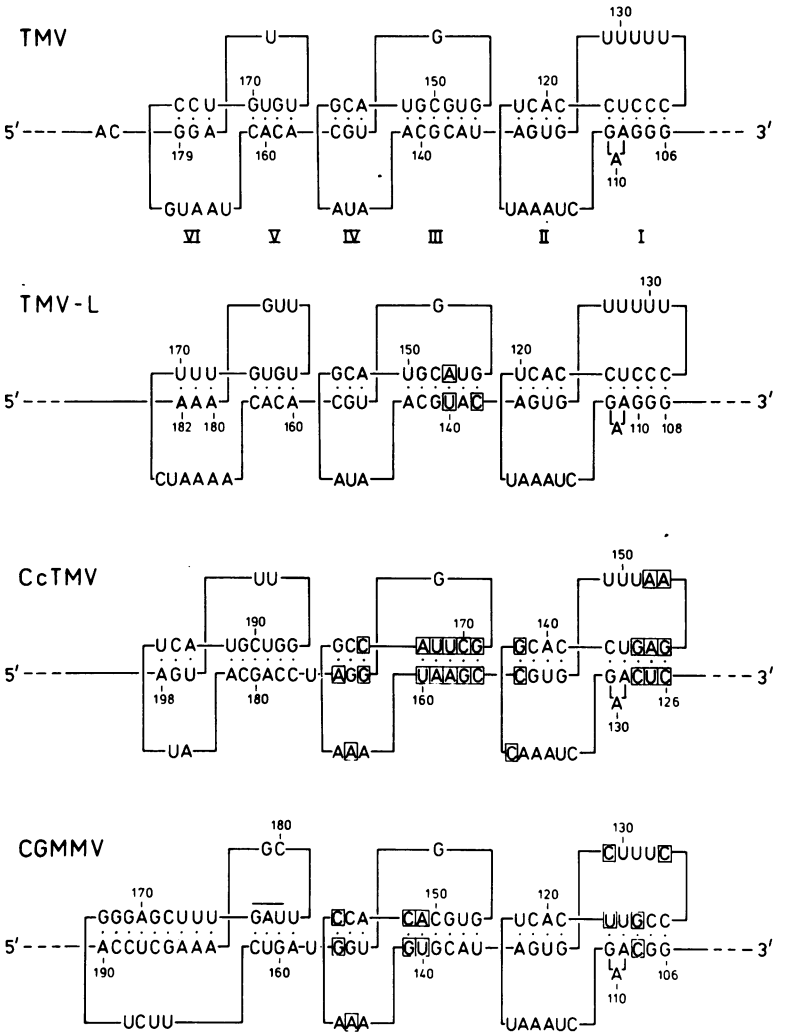

Figure 4. Sequence comparisons of a part of the 3' noncoding region of some tobamoviral RNAs. The numbering is from the 3' end. The schematic presentation of sections of the RNA from TMV vulgare (common strain), TMV-L (tomato strain), CcTMV (cowpea strain or sunn-hemp mosaic virus) and CGMMV (cucumber green mottle mosaic virus or watermelon strain) is according to Figs. 1B and 3C. Substitutions, in comparison to TMV vulgare, within the two 3' proximal pseudoknots are boxed. The overlined sequence in CGMMV RNA represents the stopcodon.

structural variability: the bulge can occur at two adjacent positions. Such an ambiguous basepairing in a bulged double helix can give rise to structural switches which may be functionally important (19). Stem region

II is nearly fully conserved. Four basepairs, of which three are invariant, are spanned by a six nucleotides long, nearly strictly conserved, connecting loop. Stem region III is built up from six basepairs except in the case of CcTMV RNA. The heavily substituted stem in the latter RNA consists of five basepairs only. In all RNAs stem III is bridged by a single G residue (see below). Stem region IV of CcTMV RNA, which consists of three basepairs like in the vulgare and L strain, contains a non-Watson-Crick basepair. Such a G·A pair is feasible however at the end of a double helical region. For this reason the possible A163·U176 basepair is left unpaired. In analogy to CcTMV, segment IV in CGMMV is also proposed to consist of three basepairs although a fourth pair (A144·U158) would restore the quasi-continuous double helix. Both options should be kept open until experimental data for these two RNAs are at hand.

In contrast to segments I to IV, segments V and VI show little homology. Some similarities are seen only between the vulgare and the closely related tomato strain. One might say that the only conserved feature of the third, 5' proximal pseudoknot is its position relative to the first two.

Connecting loops

The double helical segments I to VI are connected by single stranded pieces of RNA. These so-called connecting loops (see Fig. 1) span either the major or the minor groove. We concluded earlier that bridging of the wide groove is possible with two nucleotides only (12). This is for instance the case in the aminoacylacceptor arm of BMV RNA (8). Of interest in the data presented here is that even one single nucleotide (G154 and U174 of TMV RNA) seems to be sufficient to bring about this connection. Because the minimal distance between two phosphates on opposite strands across the wide groove in a regular RNA-A helix is about 11 Å (20) (thereby bridging seven basepairs) and because a single nucleotide can span at most a distance of about 8 Å (21) we assume that the conformation of the quasi-continuous double helix must deviate from a regular A helix. Such a deviation may be realised in a number of ways: for instance by bending of the double helix resulting in a narrowing of the wide groove (22), the presence of special base sequences like in DNA (23) or by binding ligands like Mg^{2+} or polyamines (24). In this connection it should be recalled that bridging of six or seven basepairs by G154 poses less sterical problems than spanning four basepairs by a single nucleotide (e.g. U174), due to the geometry of the RNA double helix (12). It is interesting to note that nucleotides spanning the wide groove (e.g. U127-U131, G154 and U174 in TMV RNA) appear

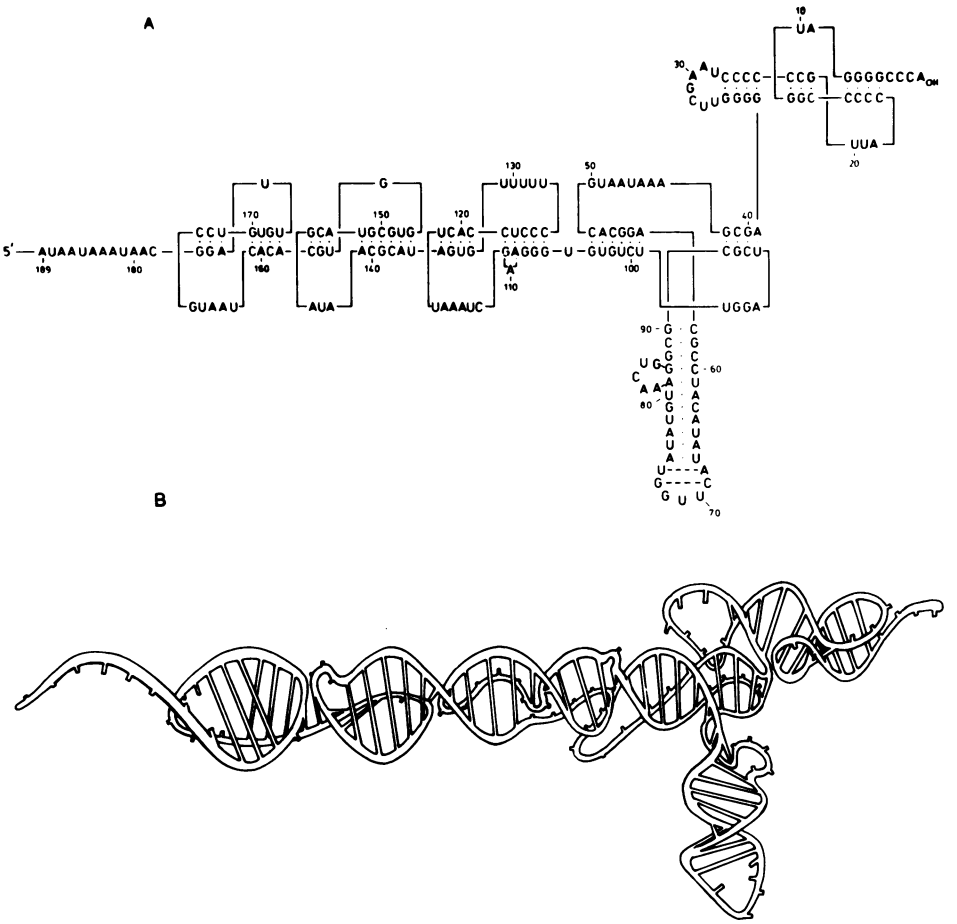

Figure 5. Models of the three-dimensional folding of the 3' terminal 189 nucleotides of TMV RNA. The tRNA-like structure was described earlier (10).
A. Schematic presentation showing the L-arrangement in the tRNA-like domain (nucleotide 1-105) and the stalk region described in this paper (nucleotide 106-179).
B. Artist's view of the spatial folding.

to be more protected against degradation by S_1 nuclease and RNase T_1 (see Fig. 3). This might be explained by the fact that these residues are located within the outer envelope of the double helical cylinder. This could make these single stranded regions less vulnerable to enzymatic attack for steric reasons. As was clarified by model building studies the connecting loop crossing the minor groove runs outside this cylinder, with the base residues pointing outwards into solution. This will render these

single stranded RNA stretches more sensitive to enzymatic degradation and chemical modification.

Stacking interactions

The coaxial stacking of segments I to VI is basically similar to that found in the aminoacylacceptor arm of TMV (10) and TYMV RNA (5). In the latter, three stacked double stranded segments are present, whereas in the RNA structure described here six such segments are found. The total length of the quasi-continuous helix is comparable in all four tobamoviral RNAs, but in CGMMV 31 or even 32 basepairs (including A144·U158) are present. In this case the double helix penetrates the coding part of the RNA.

In Fig. 5A is illustrated that the three pseudoknots can be stacked directly on one of the pseudoknots in the tRNA-like structure assuming that U105 is bulging out. In doing so one obtains an RNA domain consisting of 35 basepairs having a total length of about 100 Å. An attempt to visualise the three-dimensional folding of the entire 3' noncoding region of TMV RNA is given in Fig. 5B.

The 3' noncoding region of CcTMV RNA

The 3' noncoding region of the RNA from the vulgare strain of TMV can be divided into a number of structural regions or domains. These are the aminoacylacceptor arm comprising nucleotides 1-40 from the 3' end, the anticodon arm (41-105) and the newly described "stalk" (106-179) which ends at an A,U-rich sequence (180-189) close to the coding region (see Fig. 5A). CGMMV and TMV-L RNA show an identical organisation.

Interestingly, CcTMV deviates from the other tobamoviral RNAs by a 3' end accepting valine instead of histidine (25). The 3' terminal 80 nucleotides appear to have a 60% homology with the nucleotides in the tRNA-like structure of TYMV RNA (17). On the other hand the structure of the helix-like domain or stalk of CcTMV RNA strongly resembles those found in the other tobamoviral RNAs (see Fig. 4). The same holds true for their coding sequences (18). There are about 45 nucleotides left between the putative tRNA-like structure (1-80) and the stalk (126-198) (see Fig. 6) which so far do not show any sequence or structural homology with the 3' noncoding region of either TYMV or TMV RNA. Assuming that CcTMV and TMV derive from a common ancestor one could suppose that CcTMV acquired a valine accepting tRNA-like structure by an RNA recombination event involving a tymoviral RNA. Recombination at the RNA level has been indicated for plant viruses (26) and has been demonstrated for some animal RNA viruses (27-29). A more extensive study on the differences and resemblances in the 3'

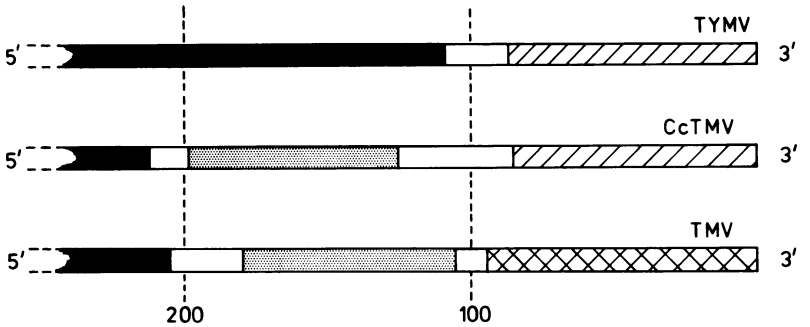

Figure 6. Structural organisation of the 3' noncoding region of RNAs from a tymovirus (TYMV) and two tobamoviruses (TMV and CcTMV). The black regions represent the various coding parts. The hatched region indicates a valine specific tRNA-like structure, whereas the cross-hatched region represents a histidine accepting structure. The dotted regions represent the stalk-like domain. White areas are stretches of RNA of unspecified structure except in the case of TMV RNA for nucleotides 96-105 (10). Numbering is from the 3' end.

noncoding regions of all aminoacylatable plant viral RNAs will be published elsewhere.

Computer-aided secondary structure predictions

Prediction of the secondary structure of the 3' noncoding regions of the tobamoviral RNAs using the program of Zuker and Stiegler (30) in most cases led to structures completely different from that shown in Fig. 3A. If no interior and bulge loops and bifurcations were allowed to form (Zuker 2 program) and if the analysis was restricted to the 5' half of the 3' noncoding region, the hairpins corresponding to stem region I, III and VI were regularly found. This again stresses the need for introduction of pseudoknotted structures in the algorithms used for RNA secondary structure predictions (12).

Statistics

What is the chance of finding three consecutive pseudoknots having 25 basepairs in tandem in an RNA chain like that of TMV? This is in fact comparable with the chance of finding a single stem region of 25 basepairs, taking into account the number of nucleotides occurring in the connecting loops, the single bulging A residue and the single G·U basepair as in the case of TMV vulgare. The probability of finding three such pseudoknots in a 6500 nucleotides long RNA molecule is of the order of 10^{-4}, which is quite low indeed. This already in itself indicates that it can be almost excluded

that the presence of these three conserved pseudoknots in the 3' noncoding region of TMV RNA is simply fortuitous.

CONCLUDING REMARKS

Although the evidence for the presence of the pseudoknots located outside the tRNA-like structure is strong, we can only speculate about its functional meaning. Being about 100 Å in length it might serve to keep the tRNA-like structure apart from the remainder of the molecule, thereby rendering the 3' end well accessible to enzymes. The strong conservation among the tobamoviral RNAs of certain features of the pseudoknots described here (e.g. the AAAUCGAA sequence around the bulging A residue) suggests an important though unknown biological role. This notion is further corroborated by the observation that some of these features appear to be conserved in other aminoacylatable plant viral RNAs as well (to be published).

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