Interaction of RNase P from *Escherichia coli* with pseudoknotted structures in viral RNAs

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ABSTRACT

In a previous study it was shown that RNase P from *E. coli* cleaves the tRNA-like structure of turnip yellow mosaic virus (TYMV) RNA *in vitro* (Guerrier-Takada *et al.* (1988) *Cell*, 53, 267–272). Cleavage takes place at the 3′ side of the loop that crosses the deep groove of the pseudoknot structure present in the aminoacyl acceptor domain. In the present study fragments of TYMV RNA with mutations in the pseudoknot, generated by transcription *in vitro*, were tested for susceptibility to cleavage by RNase P. Changes in the specificity with respect to the site of cleavage and decreases in the rate of cleavage were observed with most of these substrates. The behaviour of various mutants in the reaction catalyzed by RNase P is in agreement with the present model of the TYMV RNA pseudoknot (Dumas *et al.* (1987), *J. Biomol. Struct. Dyn.* 263, 652–657). Base substitutions in the loop that crosses the shallow groove of the pseudoknot structure resulted, however, in an unexpected decrease in the rate of cleavage, probably due to conformational changes in the substrates. Studies on other tRNA-like structures revealed an important role in the reaction with RNase P for both the nucleotide at the 3′ side of the loop that spans the deep groove and the nucleotide at position 4, which correspond to positions −1 and 73, respectively, in tRNA precursors.

INTRODUCTION

RNase P is the enzyme responsible for the generation of mature 5′ termini of tRNAs. The enzyme from *E. coli* is a ribonucleoprotein containing an RNA subunit (M1 RNA) and a protein subunit (C5 protein, 1–3). M1 RNA alone can cleave tRNA precursors *in vitro*, provided that high concentrations of salt are present in the reaction mixture (4). The C5 protein acts as a cofactor; it enhances the rate of cleavage and obviates the need for high concentrations of salt *in vitro*. The protein also modifies the substrate specificity of the holoenzyme (5,6).

In order to elucidate the way in which M1 RNA both recognizes and processes its RNA substrates, many mutations have been made during the last few years in the M1 RNA itself (7–9) and in various tRNA precursors (10–14). Results from studies of mutations in M1 RNA have not yet led to a secondary structure, let alone a three-dimensional model, that is completely compatible with the results obtained to date. Mutations in the tRNA precursors indicate that an intact 3′ terminal CCA sequence and a correct tertiary structure of the tRNA moiety are important for efficient cleavage by RNase P.

It was found recently that RNase P cleaves the 3′ terminal tRNA-like structure of turnip yellow mosaic virus (TYMV) RNA (15,16). This tRNA-like structure differs from that of canonical tRNA in the configuration of the aminoacyl acceptor arm. In tRNA this arm has two stacked stem regions (the aminoacyl acceptor stem and the T-stem), whereas in the tRNA-like structure it has three stacked stem segments, two from the RNA pseudoknot and one being hairpin II (see Fig. 1A,B). The RNA pseudoknot itself contains, in addition to the two stacked stem structures, two single-stranded loop structures. These loops cross the deep and the shallow groove of the quasi-continuous double helix, respectively (Fig. 1B,C). The cleavage in the tRNA-like structure takes place at the border between a stem region and a single-stranded region, as it does in tRNA precursors. However, in contrast to cleavage of canonical tRNA precursors, cleavage takes place closer to the 3′ than to the 5′ end of the RNA substrate. Moreover, by end-group analysis, it has been shown, that RNase P can cleave TYMV RNA at two different sites in the tRNA-like structure ((15); Fig. 1B,C).

The tRNA-like structure appears to be a better substrate as the result of an increase in k_{cat} for M1 RNA alone than is the natural precursor to $tRNA^{Tyr}$. The presence of the RNA pseudoknot can markedly improve the efficiency of the reaction, but it has been shown not to be a prerequisite for cleavage (15).

We have now addressed the following questions. What determines or influences the specificity of the reaction with respect to the site of cleavage? What happens if mutations are introduced in the RNA pseudoknot of the tRNA-like structure of TYMV RNA? Can other viral RNA pseudoknots also be cleaved by RNase P? In order to answer these questions, we have made a number of site-directed mutations in the pseudoknotted region of the tRNA-like structure of TYMV RNA. Shortened tRNA-like substrates derived from the 3′ end of TYMV RNA and tRNA-like structures from other plant viruses, such as tobacco mosaic virus (TMV) and brome mosaic virus (BMV), have also been tested for susceptibility to cleavage by RNase P.

MATERIALS AND METHODS

Bacterial strains and viruses

E. coli BL21, harbouring the plasmid pAR1219, was used for isolation of T7 RNA polymerase. This overproducing strain was a gift from Dr. F. W. Studier and has been described by Davanloo *et al.* (17). *E. coli* BL21(DE3), carrying the plasmid pARE7, was used for isolation of C5 protein, as described by Vioque *et al.* (18). *E. coli* JM101, bacteriophage M13mp18 (19) and *E. coli* BW313 were used in the site-specific mutagenesis system described by Kunkel (20). Cucumber mosaic virus (CMV) RNA was a gift from Dr. E. J. M. Jaspars (University of Leiden). Preparations of eggplant mosaic virus (EMV) were generously donated by Dr. R. Koenig and Dr. J. Witz (Biologische Bundesanstalt, Braunschweig and University Louis Pasteur, Strasbourg, respectively).

Enzymes and reagents

AMV reverse transcriptase (Life Sciences, Inc.), restriction enzymes (Pharmacia and New England Biolabs), Klenow fragment of DNA polymerase from *E. coli* (Pharmacia), T4 DNA ligase (Pharmacia), T4 RNA ligase (Pharmacia), T4 polynucleotide kinase (Pharmacia), T4 DNA polymerase (Pharmacia), calf intestinal alkaline phosphatase (Pharmacia), ribonuclease T1 (Sankyo) and ribonuclease T2 (Bethesda Research Laboratories) were used under the conditions recommended by the manufacturers or as described by Maniatis *et al.* (21). CTP, ATP: tRNA nucleotidyl transferase was purified from baker's yeast by the method of Rether *et al.* (22). M1 RNA and C5 protein were isolated as described by Vioque *et al.* (18). T7-RNA polymerase was isolated according to the method of Van Belkum *et al.* (23). Radioactive chemicals were obtained from Amersham. All other chemicals were of reagent grade.

Construction of TYMV clones

All manipulations of DNA were performed as described by Maniatis *et al.* (21). Plasmids were isolated according to the method of Birnboim and Doly (24) and mutants were sequenced by the dideoxy method of Sanger *et al.* (25).

The synthesis of the first strand for cloning of cDNA of the 3′ end of TYMV RNA was performed as described by Maniatis *et al.* (21) with the synthetic oligonucleotide 5′ CCTGGTTC-CGAT GACCC 3′ as primer. After cloning this sequence introduces a Bst NI site at the 3′ end of the cDNA clone (digestion with Bst NI followed by transcription by T7 RNA polymerase yields the tRNA-like structure of TYMV RNA with the correct 3′ CCA end). The synthesis of the second strand performed by the method of Okayama and Berg (26). The cDNA was cloned into the Sma I site of pT7−1 (27) after treatment with the Klenow fragment of DNA polymerase from *E. coli*. From a clone with a cDNA insert of about 500 nucleotides, the 93 terminal nucleotides at the 3′ end of TYMV RNA were subcloned as a Dra I-Hind III fragment into the plasmid mp18. Site-specific mutations were introduced by application of the mutagenesis system developed by Kunkel (20). An Eco RI-Sac I fragment, containing the T7 promoter from the plasmid pQR16 (23) was cloned in front of the TYMV cDNA insert. As a consequence of this cloning procedure, a stretch of 14 nonviral nucleotides, originating from the polylinker region, preceded the TYMV sequence. Digestion with Sac I and Kpn I, followed by reactions catalyzed by T4 DNA polymerase (to generate blunt ends) and by T4 DNA ligase, reduced this stretch to 4 nucleotides. A clone with a stretch of 10 nucleotides was also found, being the result of a partial digestion with Kpn I. In this way three wild-types clones of TYMV cDNA, containing the 93 terminal nucleotides from the 3′ end of the viral genome, mpTYMV(93+4), mpTYMV(93+10) and mpTYMV(93+14), were constructed (Fig. 1A).

Site-directed mutagenesis

Site-directed mutagenesis was performed according to the method of Kunkel (20). The DNA oligonucleotides were synthesized in a DNA synthesizer from Pharmacia. An additional mutation (insertion of A) had to be included for some of the mutants and the wild-type constructs of TYMV cDNA clones because sequence analysis showed that the A in the Bst NI site (CCAGG) was missing. This loss of A was probably due to the 5′-3′ exonucleolytic activity of the DNA polymerase from *E. coli* that was used in the synthesis of the second strand of the cDNA by digestion of the 5′ CCT single-stranded overhang of the deoxyoligonucleotide, which was used as the primer in the synthesis of the first strand of the cDNA.

Plasmids and cloning procedures

The (42+G)mer clone was constructed with the use of DNA oligonucleotides. These oligonucleotides, corresponding to the 3′ terminal 42 nucleotides of TYMV RNA, were synthesized on a DNA synthesizer (Applied Biosystems 380 B) and purified by preparative gel electrophoresis on a 12% polyacrylamide gel prepared into 7 M urea. The single-stranded oligonucleotides were annealed and ligated to an oligonucleotide that corresponds to a synthetic promoter from bacteriophage T7. The double-stranded fragments of DNA were cloned between the Eco RI and Bam HI sites of pUC19.

The plasmid mpSPB3HT, linearized with Tth III, was a gift from Dr. T. Dreher (Oregon State University). Transcription with SP6 RNA polymerase yields an RNA transcript that contains the 5′ GAAUAC sequence followed by a stretch of 200 nucleotides from the 3′ end of brome mosaic virus (BMV) RNA (28).

The 3′ end of the genome of the tobacco rattle virus (TRV, PLB strain) was cloned as a Hind III-Ava I fragment from the plasmid pX34 ((29); donated by Dr. G. C. Angenent, University of Leiden) into the vector pGEM2. The resulting construct yields, after digestion with Sma I and transcription with T7 RNA polymerase, a 111-nucleotide-long RNA transcript from TRV preceded by 10 nucleotides from the polylinker region, which was designated as pTRV(111+10).

The 195 nucleotides from the 3′ end of the tobacco mosaic virus genome (TMV-L strain) were cloned as a Pst I-Bam HI fragment of cDNA from the plasmid ptL3NC-A7 (Takamatsu, N. and Okada, Y., unpublished data) into the vector pT7-2 to generate the plasmid pTMV(195+25). A shorter TMV sequence was made by digesting pTMV(195+25) with Hind III and Bss HII. The sticky ends were filled in with the Klenow fragment of DNA polymerase from *E. coli*. Blunt ligation was subsequently performed with T4 DNA ligase. The resulting plasmid was designated pTMV(94+14). An even shorter sequence from TMV, containing the cDNA of the aminoacyl acceptor arm of TMV RNA, was cloned as an Eco RI-Bam HI fragment from the plasmid pTMV(94+14) into pT7-1. This plasmid was called pTMV(35+9). All cDNA clones from TMV were digested with Mlu I before transcription *in vitro* by T7 RNA polymerase. The TMV transcripts consequently contain a 3′ CGCG sequence downstream from the 3′ CCA sequence. The RNA transcripts also contain 191, 90 or 31 nucleotides from the 3′ end of TMV

RNA, preceded by 25, 14 and 9 nucleotides respectively from the polylinker region, as indicated between the parentheses in the name of each plasmid. The RNA transcript from the plasmid pTMV(35+9) has a 5′ end that differs slightly from that of the wild-type TMV-L RNA (see Fig. 2F,G).

Transcription of mutant and wild-type RNAs

Plasmids were digested with the appropriate restriction enzyme before transcription with T7 RNA polymerase or SP6 RNA polymerase. Transcription reactions were carried out as described by Davanloo *et al.* (17).

RNase P assays and analysis of end groups of the products

RNase P assays were performed as described by McClain *et al.* (13). The nature of the end groups of the products after cleavage by RNase P were determined as previously described by Guerrier-Takada *et al.* (15).

Labelling and isolation of the 3′ terminal fragments of some plant viral RNAs

Viral RNAs containing tRNA-like structures, for which no cDNA was available, were 3′- labelled with [5′-^{32}P]pCp and T4 RNA ligase or with [α-^{32}P]ATP and CTP, ATP:tRNA nucleotidyl transferase. The labelling with pCp was carried out with 10 μg of viral RNA in 30 mM HEPES at pH 8.3, 10 mM $MgCl_2$, 5 mM ATP, 7 mM β-mercaptoethanol, 10% (v/v) dimethylsulfoxide, 8 units of T4 RNA ligase and 20 μCi [5′-^{32}P]pCp in a volume of 20 μl for 16 hr at 4°C. After electrophoresis on 5% denaturing polyacrylamide slab gels, labelled fragments were localized by autoradiography and eluted with 0.3 M sodium acetate, pH 5.5. The RNA was precipitated with ethanol and dried.

Labelling with CTP, ATP:tRNA nucleotidyl transferase was carried out with 10 μg of viral RNA in 50 mM Tris-HCl at pH 7.9, 10 mM $MgCl_2$, 10 μCi [α-^{32}P]ATP and 5 μg of nucleotidyl transferase in a volume of 10 μl for 16 hr at 4°C. Gel electrophoresis and recovery of RNA were the same as in the case of the labelling with pCp.

Digestions with RNase T1

TYMV RNA substrates obtained from transcription *in vitro* by T7 RNA polymerase were dephosphorylated with calf intestinal alkaline phosphatase and subsequently 5′-labelled with T4 polynucleotide kinase and [γ-^{32}P] ATP (21). Labelled RNA was purified on a 8% polyacrylamide slab gel prepared in 8 M urea as described in the case of the labelling with pCp. Partial digestions with RNase T1 and 5′-labelled RNA were performed as described for 3′-labelled RNA (30).

RESULTS

Rates of cleavage of RNA substrates derived from the 3′ end of TYMV RNA

The wild-type tRNA-like structure of TYMV RNA, as present in the (82+6)mer (which contains the 3′ terminal 82 nucleotides of the TYMV genome and 6 nucleotides at the 5′ side of the transcript derived from the polylinker region) is known to be a better substrate for cleavage by M1 RNA than the natural precursor to tRNA^{Tyr} (15). Addition of C5 protein decreases the rate of cleavage of TYMV RNA, whereas it increases that of the tRNA precursor. The decrease is due to a lower k_{cat} value (see also Table 1, upper four lines). In our study of the effect of mutations in the pseudoknot domain of TYMV RNA using constructs derived from the (93+4) and (93+10)mer (cf. Fig. 1A), it was necessary first to compare the kinetic behaviour of

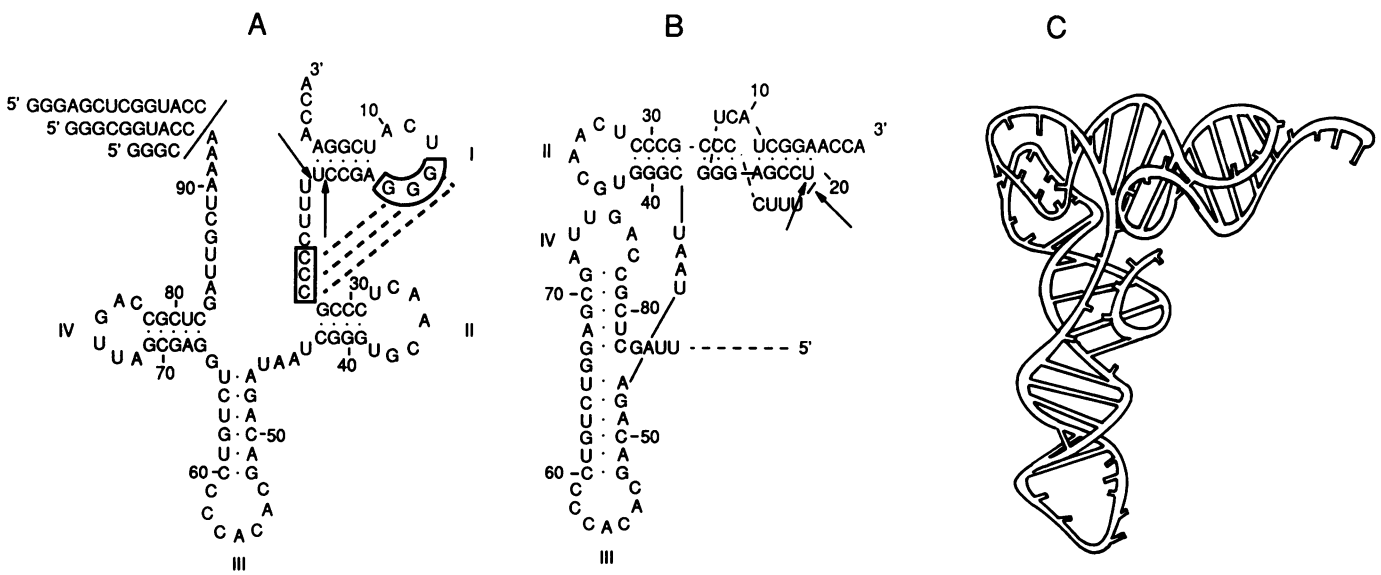

Figure 1. Structural models of the 3′ end of TYMV RNA. Numbering of the nucleotides and the hairpins is from the 3′ end. Numbering of the hairpins is indicated by Roman numbers. The sites of cleavage by RNase P are indicated by an arrow. (A) Secondary structure of the wild-type constructs of TYMV RNA used in this study. Sequences upstream from the slash are the different 5′ polylinker sequences. Pseudoknotting is indicated by dashed lines between boxed regions. Numbering of the nucleotides is from the 3′ end.(B) The L-arrangement of the tRNA-like structure of TYMV RNA. (C) Artist's rendering of the three-dimensional tRNA-like structure of TYMV RNA derived from data from chemical and enzymatic analyses (36).

the wild-type (93+4) and (93+10)mers with that of the (82+6)mer in a cleavage reaction catalyzed by RNase P. As can be concluded from Table 1, the same kinetic parameters were found in reactions with M1 RNA as with the holoenzyme, but an increase in the k_{cat} value was observed in reactions with the holoenzyme.

The 3′ terminal CCA sequence plays an important role in the reaction catalyzed by RNase P as was demonstrated earlier for tRNA precursors (11,13,14). This is also the case with fragments of TYMV RNA that contain only the aminoacyl acceptor domain. Elimination of the 3′ CA or CCA sequence results in a drastic reduction in the rate of cleavage (results not shown).

Specificity of cleavage in RNA substrates derived from the 3′ end of TYMV RNA

RNase P was found to cleave the tRNA-like structure of TYMV RNA at two sites: C19/U20 and U20/U21 (see Fig. 1). The presence or absence of the anticodon domain influences this choice of sites in the case of reactions catalyzed both by M1 RNA and the holoenzyme. The number of base pairs in the quasi-continuous double helix of the aminoacyl acceptor arm also has an effect on the specificity, but this effect is difficult to interpret (15). The (44+G)mer (Fig. 2A), which has 12 base pairs, is cleaved at the two abovementioned sites, both in a reaction with M1 RNA and with the holoenzyme. The 44-$mer_{synthetic}$ (Fig. 2B)

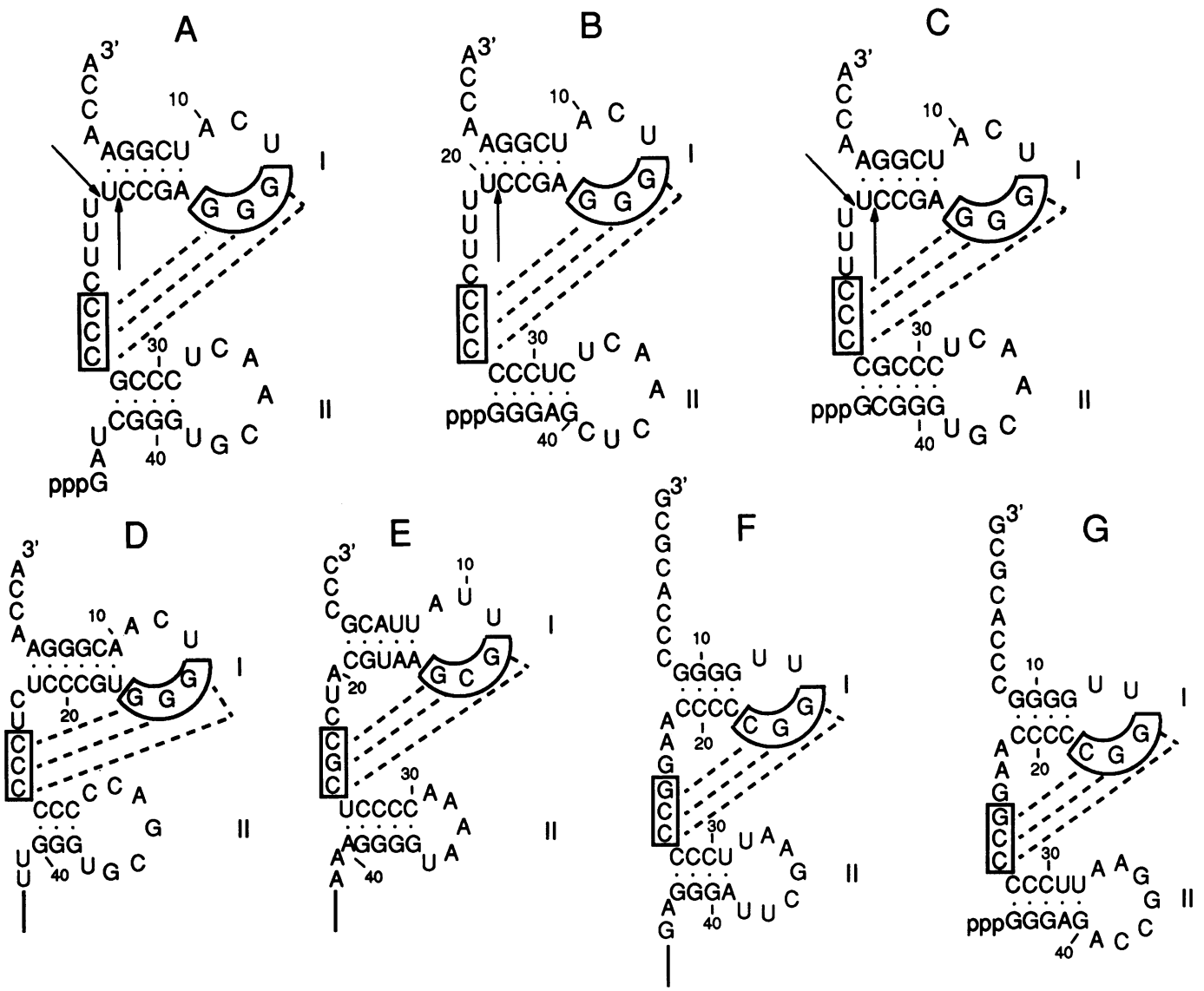

Figure 2. Secondary structure of 3′ termini of several plant viral RNAs. Numbering of the nucleotides and the hairpins is from the 3′ end. Numbering of the hairpins is indicated by Roman numbers. Pseudoknotting is indicated by dashed lines between boxed regions. The short TYMV substrates contain the 44 (A) or 42 (C) 3′ terminal nucleotides of the TYMV genome and an extra G at the 5′ end. The 44$mer_{synthetic}$ (B, see Ref. 23) has a different sequence in hairpin II from the one in the (44+G)mer (A). The sites of cleavage by RNase P in are indicated by arrows. The aminoacyl acceptor domain of the tRNA-like structures of EMV, TRV and TMV RNA are shown in (D), (E) and (F), respectively. The TMV(35+9)mer, with a sequence in hairpin II that differs from that of wild-type TMV RNA is shown in (G). The TMV RNAs have an extra 3′ terminal CGCG sequence.

with 13 base pairs is, however, only cleaved at the C19/U20 site, irrespective of the presence of the C5 protein. In the present study it was found that the (42+G)mer (Fig. 2C), with 13 base pairs and resembling the 44-mer$_{synthetic}$, has a unique site of cleavage in the reaction with the holoenzyme, but the cleavage also occurs at the other site (U20/U21) when M1 RNA alone is used (results not shown).

Deletions in the loop that crosses the deep groove of the RNA pseudoknot in the tRNA-like structure of TYMV RNA

The two sites for cleavage by RNase P in the tRNA-like structure of TYMV RNA are located at the 3′ side of the loop that crosses the deep groove, U21-C24 (Fig. 1). Increasing the size of deletions in this connecting loop results in substrates that are increasingly less susceptible to RNase P, as reflected by decreases in the k_{cat} value for these substrates. Deletion of the whole loop yields a fragment of RNA that cannot be cleaved by RNase P (Table 1). It appears that the pseudoknot is still present in the ΔU_{22}-U_{23} and ΔU_{21}-U_{23} mutants, but not in the ΔU_{21}-C_{24} mutant, from preliminary results of partial digestions with RNase T1 (Mans *et al*, manuscript in preparation). The fact that this connecting loop can be shortened to two nucleotides or even one without affecting the pseudoknot structure is in agreement with the proposed conformation of this loop (31). The loss of the cleavage site at U20/U21 in the substrates with deletions in this loop is also consistent with the proposed conformation (see Discussion).

Note that the cleavage of the deletion mutants by M1 RNA is more efficient than that by the holoenzyme, in contrast to the cleavage of the (93+10)mer from which these deletion mutants are derived (Table 1).

Mutations in the loop that crosses the shallow groove of the RNA pseudoknot in the tRNA-like structure of TYMV RNA

Although the connecting loop, A10-U12 (Fig. 1), which spans the shallow groove, does not contain sites susceptible to cleavage by RNase P, mutations in this loop can still, in principle, affect the rate of cleavage by the enzyme. Such mutations may preclude putative interactions between nucleotides in this loop and M1 RNA or they may alter the conformation of the aminoacyl acceptor arm of the tRNA-like structure. Alterations of this type may be considered to be represented by two mutations studied here: A10→C and U12→G. In the A10→C mutant C10 can basepair with G15. As a possible consequence, the sequence of three G-C base pairs and the pseudoknot may be disrupted. However, such a change in basepairing is not supported by results of partial digestions with RNase T1 (Fig. 3). G13 and G14 of the mutant are, however, more accessible to RNase T1 than the corresponding nucleotides of the wild-type substrate. In the U12→G mutant, G12-G15 can basepair with C24-C27 to generate an aminoacyl acceptor arm of 13 base pairs. In that case, the extra G12-C27 base pair shortens the loop that spans the shallow groove to two nucleotides and the loop that spans the deep groove to three nucleotides. The presence of this type of pseudoknot is supported by results of a partial digestion with RNase T1 (Fig. 3). As can be seen in Table 2, a decrease is observed in the initial velocity for these latter two mutants. Other mutations in this loop also reduce the initial velocity, even though conformational changes were not anticipated. Note that the ΔC_{11} deletion mutant, in which two nucleotides remain in the loop over the shallow groove, as in the U12→G mutant, is cleaved with the same low efficiency (Table 2). Shortening of this loop, therefore, seems to have a dramatic effect on the cleavage by RNase P.

Cleavage of the deletion mutant $\Delta C_{11}U_{12}$ could only be detected when five-fold higher than usual concentrations of M1 RNA were used (results not shown). This mutant has only one nucleotide left in the loop that crosses the shallow groove, if the RNA pseudoknot exists at all. Two nucleotides are sufficient to span the shallow groove without a distortion of the double helix (31). A single nucleotide might, however, destabilize the conformation to such an extent that formation of the pseudoknot cannot occur. In fact, preliminary probing of the structure suggests the absence of a pseudoknot (Mans *et al.*, manuscript in preparation).

In conclusion, all mutations in the loop that crosses the shallow groove decrease to some extent the initial rate of cleavage in reactions with M1 RNA. The rate of cleavage by the holoenzyme is increased for all these mutated RNAs, but the relative rate (the rate compared to the wild-type) is decreased (Table 2). Conformational changes in the tRNA-like structure, which can explain the reduction in the initial velocity, are inferred from results of partial digestions with RNase T1 (Fig. 3).

Most mutations in the loop that crosses the shallow groove lead to a very slight preference, in terms of cleavage for U20/U21 in reactions with M1 RNA alone (Table 2).

Substitutions in the sequence of three G-C base pairs in the RNA pseudoknot of the tRNA-like structure of TYMV RNA

Introduction of a mismatch mutation in the sequence of three G-C base pairs results in a destabilization of the RNA pseudoknot.

Table 1. Kinetic parameters and sites of cleavage in reactions of M1 RNA and RNase P with the tRNA^{Tyr} precursor and tRNA-like substrates from TYMV.

RNA substrate[a]	Enzyme	K_m[d]	k_{cat}[e]	Sites of cleavage[f] C19/U20	U20/U21
pTyr[b]	M1	59	0.2	–	–
	M1+C5	33	6.6	–	–
wt(82+6)mer[b]	M1	68	1.5	24	76
	M1+C5	59	0.7	74	26
wt(93+4)mer	M1	62	1.2	53	47
	M1+C5	69	75	–	–
wt(93+10)mer	M1	64	1.4	52	48
	M1+C5	67	58	–	–
$\Delta U_{22}U_{23}$[c]	M1	63	1.0	100	0
	M1+C5	75	0.15	–	–
$\Delta U_{21}-U_{23}$[c]	M1	57	0.47	100	0
	M1+C5	[g]	[g]	–	–
$\Delta U_{21}-C_{24}$[c]	M1	[g]	[g]	–	–
	M1+C5	[g]	[g]	–	–

In order to establish the Michaelis-Menten constants K_m and k_{cat} for M1 RNA or the holoenzyme, reactions were performed with 11 nM M1 RNA with or without 11 nM C5 protein, respectively. The concentration of the substrate ranged between 40 and 100 nM. For other reaction conditions see McClain *et al.* (13). The absence of an entry from the Table means that the value was not or could not be determined.

[a] Numbers in parentheses give the number of 3′ terminal nucleotides from the TYMV genome and the number of 5′ nucleotides derived from the polylinker region, respectively.

[b] The substrate pTyr is identical to the naturally occurring precursor to tRNA^{Tyr} and is prepared by transcription *in vitro*. Data taken from Guerrier-Takada *et al.* (15). In this reference the (82+6)mer is described as the 88-mer.

[c] The deletion mutants are derived from the wt(93+10)mer.

[d] K_m is expressed as nmol/l.

[e] k_{cat} is expressed as mol substrate/mol enzyme/min.

[f] The numbers shown represent the relative extent of cleavage at the two sites of cleavage.

[g] no reaction.

Table 2. Relative initial velocities and sites of cleavage for reactions of M1 RNA and RNase P with tRNA-like substrates of TYMV.

TYMV substrate	Enzyme	Initial velocity (relative)[b]	Sites of cleavage[a]	
			C19/U20	U20/U21
wt (93+4)mer	M1	100	53	47
	M1+C5	1945	–	–
A10→G	M1	85	24	76
	M1+C5	765	–	–
A10→C	M1	56	36	64
	M1+C5	565	–	–
U12→G	M1	20	32	68
	M1+C5	78	–	–
U12→C	M1	76	48	52
	M1+C5	545	–	–
ΔC_{11}	M1	36	40	60
	M1+C5	47	–	–
G14→C	M1	24	56	44
	M1+C5	236	–	–
G14→C/C26→G	M1	127	49	51
	M1+C5	1850	–	–

The TYMV RNA substitution mutants are derived from the wt(93+4)mer. The mutant ΔC_{11} is derived from the wt(93+10)mer. The absence of an entry from the Table means that the value was not determined.

[a] The numbers shown represent the relative extent of cleavage at the two sites of cleavage.

[b] The initial velocity of the (93+4)mer with M1 RNA is set at 100%. The other initial velocities are related to this value.

Partial digestions with RNase T1 show that G13, G15 and G17 become accessible to RNase T1 (Fig. 3), indicating that this substitution disrupts the pseudoknot. As can be seen in Table 2, the G14→C mutant is cleaved by RNase P to a small extent. This result supports the observation of Guerrier-Takada (15) that the presence of the RNA pseudoknot in the tRNA-like structure of TYMV RNA, although not an absolute requirement, markedly improves the efficiency of the reaction. Reactions of M1 RNA or the holoenzyme with the pseudorevertant (G14→C/C26→G), designed to restore the pseudoknot, have initial velocities similar to those of the *wild type* (Table 2). The restoration of the pseudoknot structure was confirmed by partial digestions with RNase T1 (Fig. 3).

The site specificity for cleavage is not significantly changed in the G14→C and the G14→C/C26→G mutants in reactions with M1 RNA.

Other viral tRNA-like substrates

A number of tRNA-like structures, which have structures similar to that of TYMV RNA, have been described for members of the tymovirus group. Each structure can all be considered as a variant of that of TYMV RNA (32). In this study we examined the tRNA-like structure of the eggplant mosaic virus (EMV) for cleavage by RNase P. The RNA pseudoknot in EMV RNA is similar to that in TYMV RNA (32). The loop crossing the deep groove consists, however, of only two nucleotides in EMV. Moreover, stem I in the aminoacyl acceptor arm contains an additional A-U base pair, which is compensated for by the loss of one G-C base pair in hairpin II (compare Fig. 1A and Fig. 2D). 3′ end-labelled fragments of EMV RNA, ranging from 60 to 150 nucleotides in length, are cleaved by M1 RNA alone. On a 20% polyacrylamide gel only one 3′ cleavage product could be seen (results not shown). This result is in agreement with the single site of cleavage in the $\Delta U_{22}U_{23}$ mutant of TYMV RNA, which also has two nucleotides in the loop that crosses the deep

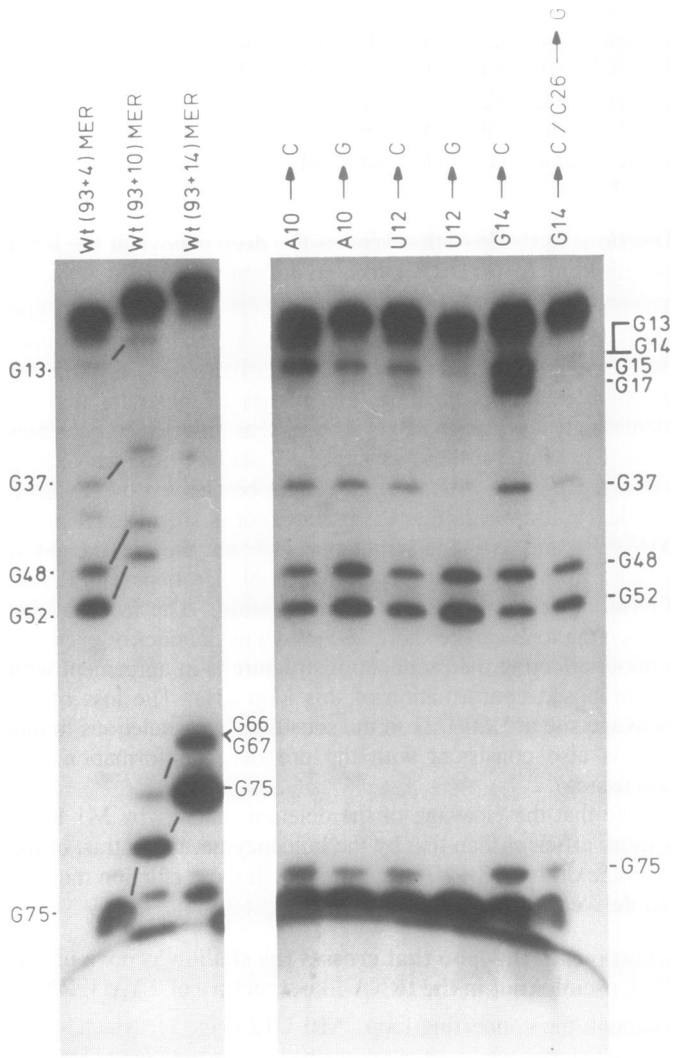

Figure 3. Partial digestions by RNase T1 of several fragments of TYMV RNA, containing the tRNA-like structure. Lanes in the left panel represent the results with wild-type fragments of TYMV RNA (see Fig. 1) that have 4, 10 or 14 nucleotides derived from the polylinker region at the 5′ side of the molecule, as indicated above the panel. Lanes in the right panel represent results with mutants derived from the wt(93+4)mer as indicated. The positions of the G residues, with numbering from the 3′ end of the RNA molecule, are given beside the autoradiogram. Reactions were performed under 'native' conditions (10 mM $MgCl_2$), as described by Van Belkum *et al.* (30).

groove. As a control, a 3′ end-labelled fragment of TYMV RNA of about 60 nucleotides was found to yield the expected pair of 3′ cleavage products after cleavage by M1 RNA when analyzed on the same 20% polyacrylamide gel.

Pseudoknotted structures have also been proposed for the 3′ termini of other plant viral RNAs, for example those of the tobamoviruses and the tobraviruses (33,34). It is noteworthy that the loop at the 5′ side of the aminoacyl acceptor helix in tobamoviral RNAs resembles the T loop of tRNAs more closely than does the equivalent loop in tymoviral RNAs (35). However, RNA fragments of TMV, obtained after transcription with T7 RNA polymerase, can be cleaved neither by M1 RNA nor by the holoenzyme. Deletions at the 5′ side of the cDNA of the TMV RNA clone were made to remove potentially inhibitory 5′ proximal sequences (see Materials and Methods). Transcripts of 108 and 44 nucleotides were obtained, respectively. The

(35+9)mer of TMV has a sequence at the 5′ end that differs slightly from the wild-type sequence (Fig. 2F,G). Neither M1 RNA nor the holoenzyme can cleave either of the shortened fragments of TMV RNA. Higher concentrations of M1 RNA or higher temperatures of incubation did not alter these results.

A fragment of tobacco rattle viral (TRV) RNA, containing 111 nucleotides from the 3′ terminus of the genome and 10 nucleotides of the polylinker region at the 5′ end, was also not cleaved either under the conditions used for cleavage of the RNA fragments of TMV. A proposal for the secondary structure of the 42 nucleotides at the 3′ end of the genome of TRV is shown in Figure 2E (see also Ref. 34).

The cucumoviruses and the bromoviruses also have a pseudoknot-containing tRNA-like structure at the 3′ end of their three genomic RNAs (36,37). In these cases the aminoacyl acceptor domain is, however, constructed in a different way.

Consequently, if cleavage by RNase P in the loop that crosses the deep groove were to occur as it does in TYMV RNA, a 3′ terminal RNA product of about 100 nucleotides would be anticipated. Cleavage of genomic RNAs from cucumber mosaic virus (CMV) produces a fragment of the expected size in reactions both with M1 RNA and with the holoenzyme. The C5 protein appears to have an inhibitory effect on the reaction (results not shown).

In order to determine why TYMV, EMV and CMV RNA fragments are cleaved by RNase P and those from TMV and TRV are not, it is appropriate to examine the 3′ terminal single-stranded region of these RNAs. An ACC(A) sequence is present in TYMV, EMV and CMV RNA, whereas CCC(A) is found in TMV and TRV RNA. The nature of the nucleotide at position 4, which is equivalent to position 73 in tRNAs may, thus, be crucial for cleavage. To test this hypothesis, two mutants of TYMV RNA, A4→G and A4→C (a gift from Dr. C. Florentz, University Louis Pasteur, Strasbourg) were assayed in a reaction with M1 RNA. Both mutants were cleaved by M1 RNA. The nature of the nucleotide at this position appears, therefore, not to be important for cleavage.

Further examination of the tRNA-like sequences from these viruses revealed that the 3′ terminal nucleotide in the loop that crosses the deep groove, which is equivalent to the −1 position in tRNA precursors, is a pyrimidine in TYMV, EMV and CMV RNA and a purine in TMV and TRV RNA. The nature of the base at this position could, therefore, determine the efficiency of cleavage. A fragment of brome mosaic viral (BMV) RNA, transcribed from the clone mpSPB3HT, was tested for susceptibility to cleavage. This fragment of RNA contains the tRNA-like structure of BMV, which closely resembles the tRNA-like structure of CMV. This viral RNA, which has a purine at the −1 position instead of the pyrimidine as in CMV, is cleaved by M1 RNA only at the lower limits of detection, whereas the holoenzyme does not appear to cleave it at all. This result supports the finding that the nucleotide at the −1 position must be a pyrimidine if efficient cleavage is to occur. The role of the nucleotide at the −1 position was further investigated by the introduction of substitutions at that position in tRNA-like structures that cannot be cleaved by RNase P (TMV and TRV RNA), and in a tRNA-like structure that can be cleaved (TYMV RNA). In the case of TMV and TRV RNA the purine at the −1 position, an A residue, was replaced by a pyrimidine. These mutants, which were expected to be substrates for M1 RNA, were not cleaved by M1 RNA. In TYMV RNA the pyrimidine at the −1 position, a U residue, was replaced by an A, and this mutant was cleaved by M1 RNA (see Discussion).

Positions in tRNA precursors				tRNA-like structures	RNase P cleavage
-1		73			
49	U	31	A	TYMV	+
		14	G	TYMV (A4-G)	+
		3	U		
		1	C	TMV (A18-U)	-
				TYMV (A4-C), T4 RNA substrate	+
13	C	8	A	CMV, EMV	+
		4	G		
		1	U		
		0	C	TMV (A18-C), TRV (A20-C)	-
8	A	3	A	BMV	+/-
		2	G	TYMV (U21-A)	+
		3	U		
		0	C	TMV, TRV	-
3	G	3	A		
		0	G/U/C		

Figure 4. Nucleotides at positions −1 and 73 in tRNA precursors from *E. coli* strain K12 compared to those at the equivalent position in tRNA-like structures of viral origin. In the left panel, the sequences, of 73 out of 79 mapped genes for tRNA in *E. coli* strain K12 have been used in this analysis ((42) and references therein). The right panel summarizes the results for the RNA substrates described in this paper (see text for details). Cleavage by RNase P of these substrates, indicated by + or − is shown in a separate column. Note that the −1 and 73 position in tRNAs are equivalent to the 3′ terminal nucleotide of the loop that crosses the deep groove and position 4, respectively, in tRNA-like structures.

DISCUSSION

The observation that the tRNA-like structure of TYMV RNA can be cleaved by RNase P (15) gave us the opportunity to study recognition by RNase P of its substrates as well as the structural requirements of the RNA pseudoknot that allow cleavage catalyzed by RNase P. The reactions of the various substrates used in this study with RNase P support and extend both the results on substrate recognition by RNase P of previous studies ((2) and references therein) and our current model of the RNA pseudoknot (31). The size of the loops and the nature of the nucleotides in the loop that crosses the shallow groove are significant structural features of the RNA pseudoknot that are required for efficient cleavage by RNase P. Furthermore, new data on the importance of the nature of the nucleotides at positions −1 and 73 were obtained from our study of other tRNA-like structures.

Rates of cleavage of RNA substrates derived from the 3′ end of TYMV RNA

The wild-type tRNA-like structure of TYMV RNA is a much better substrate for RNase P than the natural precursor to $tRNA^{Tyr}$. This property, reflected by a higher k_{cat} value, is probably due to the fact that the bond to be cleaved in the tRNA-like structure is more constrained. Part of the single-stranded structure, containing the cleavage sites, makes a sharp turn and crosses the deep groove of the RNA double helix, according to the present model of the structure of the pseudoknot (31,38). The cleavage site in the $tRNA^{Tyr}$ precursor is located, by contrast, in a 'dangling' strand of RNA (11).

The differences now found among the kinetic behaviour of the various wild-type tRNA-like substrates from TYMV RNA are rather unexpected. They are correlated with a difference in the length of the 5′ proximal region of these tRNA-like structures. Apparently, the 5′ proximal region either causes steric hindrance

near the 3′ end or somehow changes the conformation of the tRNA-like structures. Recently from probing of these structures with nucleases, we obtained evidence to suggest that the various wild-type substrates can have different conformations (Mans *et al.*, manuscript in preparation). It is noteworthy that the effects of these changes are also apparent in the process of aminoacylation of TYMV RNA substrates ((39), Mans *et al.*, manuscript in preparation).

Mutants in the RNA pseudoknot of TYMV RNA

Shallow groove

The observed reduction in the rate of cleavage by RNase P of fragments of RNA with a substitution or deletion in the connecting loop that lies over the shallow groove may be due to the loss of any interaction between the nucleotides in this loop and M1 RNA. Such interactions are plausible, since the base residues are exposed to the solvent (31). However, the K_m values of the wild-type tRNA-like substrates do not differ significantly from the K_m value of the $tRNA^{Tyr}$ precursor, suggesting that the nucleotides in this loop (but also the nucleotides in the loop that lies over the deep groove) have no influence on the efficiency of binding of M1 RNA. The reduction in the rate of reaction is, therefore, most likely to be due to conformational changes in the TYMV substrates, which can be inferred from the results of partial digestions with RNase T1, even though their exact nature is not yet known. The mutations in the loop that crosses the shallow groove also decrease the rate of aminoacylation with valyl-tRNA synthetase (Mans *et al.*, manuscript in preparation). The parallel results obtained in analyses of cleavage by RNase P and aminoacylation indicate that the loop over the shallow groove may have a constrained conformation. These observations are also in line with the high degree of conservation of this loop among related tymoviral RNAs (32).

Deep groove

Deletions in the loop that crosses the deep groove also reduce the rate of reaction of the cleavage by RNase P. The K_m values of these mutants, which do not differ significantly from the K_m value of the wild-type substrate, demonstrate that the decrease in the rate of the reaction is not due to a less efficient binding of the substrate. This result supports our conclusion, mentioned in the previous paragraph, as to the influence of the nucleotides in the loops on the binding to M1 RNA. The more nucleotides that are deleted in the loop that spans the deep groove, the more difficult does it seem to be for RNase P to cleave the substrate (a decrease in k_{cat}, loss of cleavage at the U20/U21 site). This conclusion is in agreement with the proposed conformation of this loop. The nucleotides of the loop are probably hidden in the groove (31). The smaller the number of nucleotides available to span the deep groove, the deeper must these nucleotides be buried within the groove. If all nucleotides are deleted, as in the ΔU_{21}-C_{24} mutant, cleavage by RNase P does not occur. However, this deletion mutant, in which the deep groove has to be spanned by a single phosphodiester bond, the formation of an RNA pseudoknot is very unlikely. Thus, an alternative structure may be present, which is not a substrate for RNase P.

The sequence of three G-C base pairs ($G_{13-15}C_{25-27}$)

The disruption and restoration of the RNA pseudoknot in TYMV RNA, achieved by the introduction of the appropiate substitutions in its shortest stem structure, are coupled with a decrease and an increase in the rate of cleavage by RNase P, respectively. These substitutions confirm, therefore, the earlier results of Guerrier-Takada *et al.* (15) that the presence of the RNA pseudoknot in the aminoacyl acceptor arm of the tRNA-like structure of TYMV markedly improves the efficiency of the reaction catalyzed by RNase P, but is not absolutely required. The same rates of cleavage found for the revertant and the wild-type substrate indicate that this base pair of the RNA pseudoknot does not contribute to the site specificity of cleavage by RNase P.

Site specificity of cleavage

RNase P cleaves the tRNA-like structure of TYMV at two sites (15). The results presented in this paper show that the extent to which each cleavage site is susceptible to M1 RNA or to the holoenzyme depends on several structural features, such as the number of base pairs in the aminoacyl acceptor arm and the number of nucleotides in the loop that crosses the deep groove. The way in which these structural features determine the site of cleavage depends on the positioning of the substrates in the catalytic center of M1 RNA. This positioning will depend, in turn, on the conformation and the sequence in those regions of the substrate that interact with certain nucleotides in the M1 RNA during the cleavage reaction. An illustrative example of the way in which subtle conformational differences influence the site specificity of cleavage is provided by the difference between reactions of M1 RNA with the $44mer_{synthetic}$ and with the (42+G)mer. Both substrates have an aminoacyl acceptor arm of 13 base pairs and differ in sequence in hairpin II only (see Fig. 2B,C). This difference must be responsible for the observed difference in the site of cleavage in reactions by M1 RNA. It should be noted, however, that the proposed structure of the (42+G)mer is not proven experimentally in contrast to the $44mer_{synthetic}$ (23).

Other substrates with RNA pseudoknots and the importance of the nature of the nucleotides at position −1 and 73 for cleavage RNase P

The $tRNA^{His}$ in all organisms examined has an aminoacyl acceptor stem of 8 base pairs instead of 7 as in other tRNAs. A previous study of mutants of the precursor to $tRNA^{His}$ in *E. coli* (40) showed, that in *E. coli* this difference in length was due to an aberrant cleavage by RNase P, which was almost exclusively determined by the nature of the base at position −1 in reactions with M1 RNA. The same phenomenon was found for the precursor to $tRNA^{SeCys}$ in *E. coli* (41). The importance of the nature of the base at the −1 position for cleavage by RNase P was also recognized in our comparison of tRNA-like structures in various plant viral RNAs. Analysis of the nucleotides that might be important for cleavage led to the demonstration of an important role for the 3′ terminal nucleotide in the loop that spans the deep groove, which occupies a position analogous to that of nucleotide −1 in tRNA precursors. This nucleotide is a pyrimidine in cleavable tRNA-like structures (TYMV and CMV RNA) and a purine in structures that cannot be cleaved or are cleaved very inefficiently by RNase P (TMV, TRV and BMV RNA).

In order to clarify the role of this nucleotide in a cleavage by RNase P we replaced the pyrimidine at the −1 position in TYMV RNA with a purine and the purine at the −1 position in TMV and TRV RNA with a pyrimidine. These mutants are cleaved in the same fashion as the wild-type substrates, indicating that the nucleotide at position −1 is not the sole nucleotide that determines the efficiency of cleavage. Therefore, we have had to refine our hypothesis on the recognition of its substrate by

RNase P and we now propose that both nucleotides at positions −1 and 73 are important for efficient cleavage by RNase P, as inferred from a comparison between the sequences of the tRNA-like substrates, the mutants of these substrates used in this study, and tRNA precursors from *E. coli* strain K12. Figure 4 shows a statistical analysis of the nucleotides at these positions in 73 tRNA precursors from *E. coli* strain K12 ((42) and references therein). It is striking that the combinations of those nucleotides in tRNA-like structures, which are not cleaved by RNase P, never occur in the tRNA precursors from *E.coli* strain K12, whereas the combinations of the nucleotides in the tRNA-like structures that can be cleaved are also present in these canonical tRNA precursors. An RNA substrate derived from the 5′ end of the mRNA of gene 32 of bacteriophage T4 (43) can also be cleaved by M1 RNA or the holoenzyme (Guerrier-Takada, unpublished results). The characteristics of the cleavage site in this substrate conform to our conclusions (see Fig. 4). The BMV RNA fragment is an exception to this rule. However, a closer look at the three tRNA precursors that also have an A at both positions −1 and 73 (Leu_4 and the two Met_{f1}) show that the first base pair at the 3′ end of the aminoacyl acceptor arm is a G-U or a C-A. The U21→A mutant of TYMV RNA, which is cleaved by RNase P, has a weak base pair (A-U) at that position, while the BMV RNA fragment has a G-C base pair at the analogous position. Apparently, a weak base pair or a mismatch may be needed in this part of the molecule when an A is present at both positions −1 and 73. The TMV(35+9)mer (A18→U) is the second exception to the rule. The explanation for the resistance of this fragment of RNA to cleavage, may be found in the differences between the RNA pseudoknots in TMV RNA and TYMV RNA. The aminoacyl acceptor arm in TMV RNA differs with respect to three structural features from the one in TYMV RNA: the aminoacyl acceptor arm of TMV RNA has only 11 base pairs; all TMV RNA fragments used in this study have an extra 3′ terminal CGCG sequence downstream from the CCA end; and the loop that crosses the shallow groove consists of only two U residues in the L-strain of TMV RNA. As shown here for TYMV RNA, mutations in this loop have a dramatic effect on the susceptibility to cleavage by RNase P.

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