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Length requirements for tRNA-specific enzymes and cleavage specificity at the 3' end of turnip yellow mosaic virus RNA

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ABSTRACT

This paper describes the minimum length of the turnip yellow mosaic virus (TYMV) RNA necessary to fulfill the tRNA-like properties of the viral RNA: 50 to 75 nucleotides and 86 nucleotides from the 3' end of TYMV RNA are sufficient for adenylation and valylation respectively by the *Escherichia coli* system. The size of the tRNA-like fragments obtained *in vitro* in the presence of an *E. coli*, a reticulocyte or a Chinese cabbage leaf extract has also been determined. Among the major fragments liberated from the 3' end of TYMV RNA by the three systems are fragments of 117 and 112 nucleotides. In addition, the *E. coli* extract liberates fragments of 139 and 61 nucleotides, and the reticulocyte lysate fragments of 109, 94, 84, 73 and 46 nucleotides. The cleavage of the viral RNA by several systems *in vitro* to yield RNA fragments encompassing the tRNA-like sequence suggests that such fragments might also be liberated *in vivo*.

INTRODUCTION

Turnip yellow mosaic virus (TYMV) contains an infectious genomic "+" RNA of Mr = 2.0 x 10^6, and a subgenomic RNA of Mr = 0.24 x 10^6 derived from the 3' region of the genomic RNA. It has been demonstrated by Pinck *et al.* (1) and Yot *et al.* (2) that TYMV RNA can be enzymatically aminoacylated with valine *in vitro*. The valyl-tRNA synthetase of various origins, bacterial, yeast, plant or animal can catalyze this reaction (2,3). The kinetic constants (V_{max} and K_m) for aminoacylation of the genomic and the subgenomic RNA using the yeast enzyme are comparable to those obtained for yeast $tRNA^{Val}$ (4). Subsequently, the capacity to undergo aminoacylation *in vitro* was generalized to several plant and animal RNA viruses; those from a given group are generally acceptors of the same amino acid (for a review see ref. 5).

A series of other tRNA-specific enzymes recognize TYMV RNA in conditions comparable to those used for a tRNA. These are *in vitro*, the tRNA nucleotidyl-transferase (2,6), an *E. coli* extract containing "RNase P" (7), a tRNA methyl-transferase (8), the elongation factors eEF-1 (9) and EF-T (10) and the

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peptidyl-tRNA hydrolase (2), and *in vivo* either after microinjection into *Xenopus laevis* oocytes (11), or in infected leaf strips (our unpublished results) the tRNA nucleotidyltransferase and the valyl-tRNA synthetase.

The 3'-terminal region of TYMV RNA has been sequenced (12-15). The tRNA-like structure is entirely encompassed within the 3' non-coding end of the viral RNA, it is devoid of modified bases (13, 14) and interestingly, CAC a valine anticodon, can be adequately positioned in a possible "anticodon" loop. However, the viral tRNA-like structure bears little resemblance to the classical secondary structure of a tRNA and contains but few sequence similarities with valine tRNAs (16-18). It has recently been reported (19) that more than 56% sequence homology exists between the 3'-terminal 80 nucleotides of TYMV RNA and the corresponding region in the RNA of the Cowpea strain of tobacco mosaic virus (TMV), a member of the tobamovirus group whose RNA accepts valine instead of histidine (20).

In this communication we report the size of the shortest TYMV RNA fragment that still retains its adenylation and aminoacylation capacity using the *E. coli* enzymes. We also indicate the specific cleavage sites at the 3' end of the viral genome occurring when Val-RNA is incubated *in vitro* in the presence of an *E. coli*, a reticulocyte or a plant extract.

MATERIALS AND METHODS

RNAs and Enzymes

TYMV-infected Chinese cabbage leaves were generously supplied by S. Astier-Manifacier and P. Cornuet, and TYMV was purified by the method of Leberman (21). The viral RNA was extracted (22) under RNase-free conditions and stored at -70°C. *E. coli* tRNA was purchased from Sigma and *E. coli* 5S rRNA was a kind gift of D. Hayes. Fragments of 208 and 112 nucleotides derived from the 3' end of TYMV RNA and labelled at their 3' terminus by α^{32}P-ATP in the presence of tRNA nucleotidyltransferase, were kindly supplied by K. Rietveld.

The *E. coli* extract (a 150,000 x *g* supernatant freed of tRNAs by chromatography on DEAE cellulose) was prepared as described by Yot *et al.* (2), and the TYMV-infected Chinese cabbage leaf extract devoid of tRNAs (a 100,000 x *g* supernatant) as described by Weavers *et al.* (23). The reticulocyte lysate prepared according to Pelham and Jackson (24) and containing low amounts of $tRNA^{Val}$ was kindly supplied by M. D. Morch and G. Drugeon. Partially purified *E. coli* tRNA nucleotidyltransferase {fractions from the DEAE cellulose chroma-

togram (25)} was a kind gift of D. Carré, and the *E. coli* valyl-tRNA synthetase was partially purified in collaboration with S. Chousterman. RNase T_1 was purchased from Sankyo and RNase A from Sigma.

Adenylation and Aminoacylation of TYMV RNA

The incubation conditions were as described by Yot *et al.* (2) except that the incubation mixtures contained in 50 or 300 µl (the latter for kinetic experiments) 600 µg/ml of TYMV RNA and either 4 µM ^{14}C-ATP (512 mCi/mmole; Amersham) in the absence of unlabelled ATP, or 1.5 µM ^{3}H-valine (31 Ci/mmole; C.E.A., Saclay). The enzyme concentrations are indicated in the legends of the figures. Unless stated otherwise, after 50 min of incubation at 30°C, 5 µl aliquots were used to determine the cold trichloroacetic acid (TCA) precipitable counts, and 40 µl were brought to a final concentration of 7 M urea, 1 mM EDTA, 5 mM Tris-borate pH 7, 0.01% xylene cyanol (XC) and 0.01% bromophenol blue and analyzed by gel electrophoresis. Under these conditions the percentage of adenylation and of aminoacylation was between 50 and 80 in both cases.

Partial RNase T_1, RNase A and Alkali Digestion

For digestion with RNases, 1 mg/ml of TYMV RNA or of $^{14}C\text{-}A_{OH}$-RNA of TYMV was incubated (26) in the presence of 7 M urea, 20 mM Na citrate pH 5 and 5 mM EDTA at 50°C for 10 min after which RNase T_1 (10 or 4 units/ml) or RNase A (1 or 0.4 unit/ml) was added and incubation continued for 20 min. The samples were then boiled for 2 min. When this material was further used for adenylation or aminoacylation it was first diluted three times with water, and then phenol extracted to inactivate the RNases. For alkali digestion, 1 mg/ml of TYMV RNA or of $^{14}C\text{-}A_{OH}$-RNA of TYMV was heated in 25 mM Na carbonate buffer pH 9 for 2 or 6 min at 100°C, then chilled and ethanol precipitated.

Polyacrylamide Gel Electrophoresis

Ten or 12% polyacrylamide, 7 M urea gels (20 x 16 x 0.2 cm^3, unless stated otherwise) were used at pH 7 (11, 27, 28). The reservoir buffer was constantly recycled and electrophoresis performed at 4°C. After a pre-run at 400 Volts for at least 2 h, the samples containing 20,000, 3,000 or 100 cold TCA precipitable counts per min (cpm) of ^{3}H-, ^{14}C- or ^{32}P-labelled material respectively (unless stated otherwise) were applied onto the gel, and electrophoresis continued until the XC dye was at the desired position (see figures). The part of the gel containing the 5S rRNA or tRNA markers was stained with ethidium bromide (2 µg/ml) for 20 min and the position of the RNAs visualized

under UV light. The rest of the gel containing the radioactive material was treated with 10% TCA, dimethylsulfoxide (DMSO), DMSO-PPO and water (29), then dried (30) and exposed for about one week at -70°C using flash-activated (31) Kodak films.

RESULTS

1. "Minimum" Length Requirements of the tRNA-like Fragments for Adenylation and Aminoacylation by *E. coli* Enzymes

To determine the minimum lengths of the TYMV RNA fragments which can still be recognized by *E. coli* tRNA-specific enzymes, the following strategy was used. TYMV RNA was first partially digested (pre-digested RNA) with RNase T_1 or with alkali. The mixture of fragments obtained was then either adenylated with ^{14}C-ATP or aminoacylated with ^{3}H-valine for a short period of time with limiting enzyme concentrations to prevent further RNA degradation; in each case the same amount of RNA was analyzed by gel electrophoresis (Fig. 1). Only those fragments which are still capable of being adenylated (lanes d, e, i and i') or aminoacylated (lanes f, g and j) appear on the fluorogram. The extent of adenylation or of aminoacylation of alkali pre-digested material (lanes i, i' and j) was as efficient as that of native RNA (lane a), but somewhat lower after pre-digestion with RNase T_1 (lanes d-g) due possibly to traces of urea. A few radioactive bands of which one of 82 nucleotides visible after adenylation (lane a) or valylation (not shown) and those of 56, 54 and 50 nucleotides visible after adenylation (not shown) of the native RNA (lane a) and also present among the pre-digested labelled RNA fragments (lanes d-g, i, i' and j) were considered to result from degradation due to traces of RNases contaminating the tRNA-specific enzymes and not to pre-digested RNA fragments capable of direct adenylation or valylation. This conclusion is strengthened by the fact that adenylation or valylation of RNase T_1 pre-digested RNA (lanes d-g) does not generate other bands than those produced by RNases contaminating the enzyme preparations during adenylation of native RNA (lane a), and/or those produced by RNase T_1 digestion of the adenylated RNA (lanes b and c). This also implies that no new RNase-sensitive sites are exposed as a result of pre-digestion. The intensity of the bands obtained by partial RNase T_1 (lanes b and c) or alkali (lanes h and h') digestion of $^{14}C\text{-}A_{OH}$-RNA of TYMV corresponds to the amount of each fragment derived from the 3' end of the viral RNA available for adenylation or for aminoacylation; these same bands also served as markers.

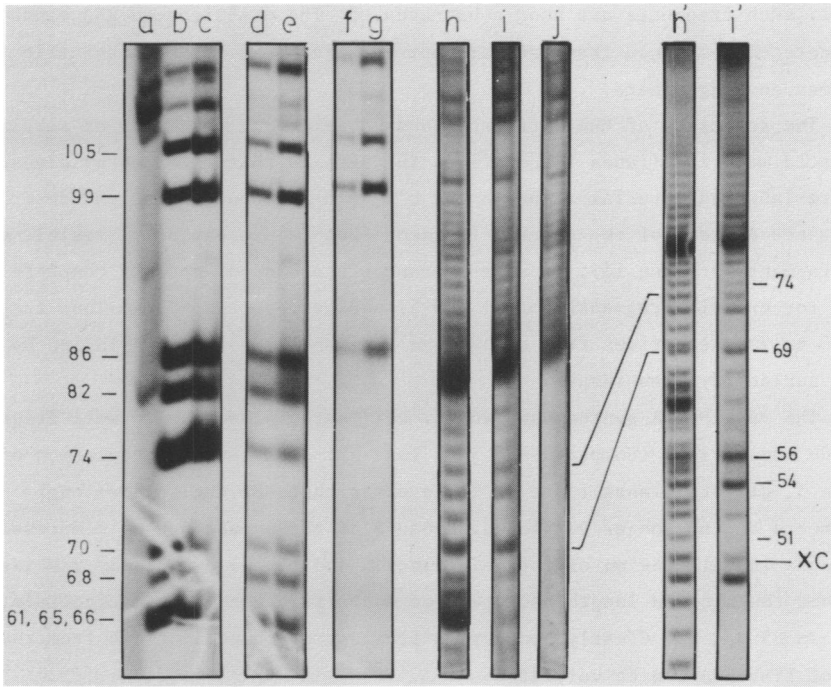

Fig. 1. - Analysis of ^{14}C-A_{OH}-RNA of TYMV and of ^{3}H-Val-RNA of TYMV on a 12% polyacrylamide, 7 M urea gel (40 x 20 x 0.2 cm^3). The RNA was adenylated or aminoacylated for 15 min using tRNA nucleotidyltransferase (0.3 mg/ml) or valyl-tRNA synthetase (0.18 mg/ml). Lane a : ^{14}C-A_{OH}-RNA (8,000 cpm) . Lanes b and c : ^{14}C-A_{OH}-RNA (8,000 cpm) partially digested using 10 and 4 units/ml of RNase T_1 respectively. Lanes d-g : TYMV RNA (90 µg) partially digested with 10 (lanes d and f), or 4 (lanes e and g) units/ml of RNase T_1 and then either adenylated (2,000 and 5,000 cpm, lanes d and e) or aminoacylated (6,500 and 20,000 cpm, lanes f and g) respectively. Lanes h (and h') : alkali digests (6 min) of ^{14}C-A_{OH}-RNA (8,000 cpm). Lanes i (i') and j : respectively ^{14}C-A_{OH}-RNA (7,000 cpm) or ^{3}H-Val-RNA (45,000 cpm) using TYMV RNA (60 µg) that was first alkali-digested for 6 min and then adenylated or aminoacylated. Lanes h' and i' : as h and i but electrophoresed for a shorter time; right-hand margin : lengths of certain RNA fragments. Left-hand margin : lengths of different RNase T_1 digests.

The intensity of the bands obtained after alkali digestion of ^{14}C-A_{OH}-RNA (lanes h and h') can be compared to that of the bands obtained when the viral RNA has first been alkali-digested and then adenylated (lanes i and i') or aminoacylated (lane j). Three situations exist. 1) The intensities of the bands obtained by these two procedures are the same: such fragments are good substrates for the tRNA nucleotidyltransferase and for the valyl-tRNA synthetase. 2) The intensities of the bands obtained with pre-digested RNA are

faint: such fragments are poor substrates. 3) The pre-digested RNA bands are not detectable: these fragments are not substrates of the tRNA-specific enzymes envisaged here.

The intensity of the different bands obtained by labelling of alkali pre-digested material (lanes i and i') is the same as that obtained by digestion of pre-labelled material (lanes h and h') down to about 75 nucleotides. It decreases in some of the shorter fragments but is usually still visible down to fragment 50 (lane i'). In some instances the band disappears completely (see for example fragments of 71 and 51 nucleotides, lane i'). Thus fragments of 50 to 75 nucleotides from the 3' end are necessary for adenylation by the tRNA nucleotidyltransferase.

The valyl-tRNA synthetase from *E. coli* esterifies equally well fragments of $\geq$86 nucleotides (compare lane j to lane h). From the aminoacylation of RNase T_1 digests (lanes f and g) it is clear that $\leq$82 nucleotide-long fragments are no longer valylated. Because of the phenomenon of compression of bands in this region of the gel (lanes h and j), we cannot say precisely whether the minimum length required for esterification is 86 nucleotides, or between 85 and 83. Clearly however, a fragment of 86 nucleotides from the 3' end of TYMV RNA can be valylated.

2. Cleavage Specificity at the 3' End of TYMV RNA

In order to envisage a possible physiological role of such a fragment, it was interesting to determine whether in the presence of various cellular extracts an 86 nucleotide-long fragment could be released from the 3' end of the viral RNA.

A. *E. coli* Extract

Total TYMV RNA was aminoacylated with ^{3}H-valine using an *E. coli* extract and the radioactive products were analyzed by gel electrophoresis (Fig. 2). With increasing concentrations of extract, the valylated RNA becomes increasingly degraded. With the highest extract concentration (lane c), four major ^{3}H-Val-RNA fragments are liberated. To determine the size of these fragments, material similar to the one applied to lane c was further analyzed (Fig. 3, lane d). Based on various RNA markers (lanes a-c and e-h), the four major fragments are 139, 117, 112 and 61 nucleotides long (± 1 nucleotide). Minor fragments of 87, 84, 60, 56, 54 and 49 nucleotides (± 1 nucleotide) are also visible upon longer exposure of the film (not shown).

The size of the material obtained after different times of valylation was also analyzed by gel electrophoresis (Fig. 4, lanes a-f): after 160 min

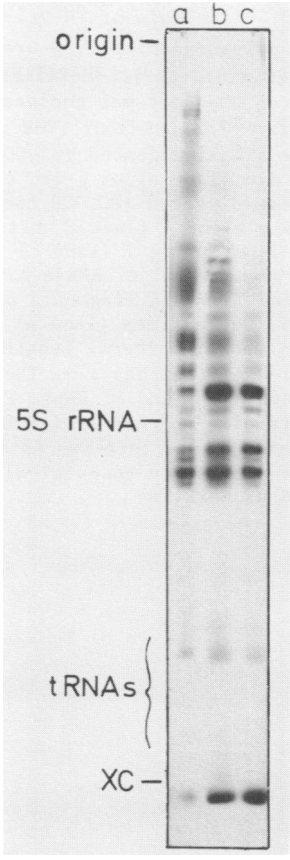

Fig. 2. - Analysis of ^{3}H-Val-RNA of TYMV on a 10% polyacrylamide, 7 M urea gel. The RNA was aminoacylated using 0.3, 1 or 1.7 mg/ml of *E. coli* extract in lanes a, b and c repectively.

only the four major fragments are visible (lane f). During aminoacylation of TYMV RNA, both acylation and fragmentation of high molecular weight (Val)-RNA occurs. To verify that the 61 nucleotide-long fragment results from the fragmentation of aminoacylated RNA as suggested by the experiments described in Section 1, the viral RNA was first pre-incubated for different times with the *E. coli* extract, then briefly allowed to be aminoacylated by addition of ^{3}H-valine (Fig. 4, lanes g-k). In these conditions, the 61 nucleotide-long fragment is barely visible. The fact that its intensity decreases with that of the high molecular weight Val-RNA, that is upon prolonged pre-incubation of the samples, suggests that it derives from the degradation of Val-RNA of higher molecular weight. Had it arisen from the direct valylation of a 61 nucleotide-long fragment, its intensity should have increased with time of pre-incubation as is the case of the other major Val-RNA fragments produced.

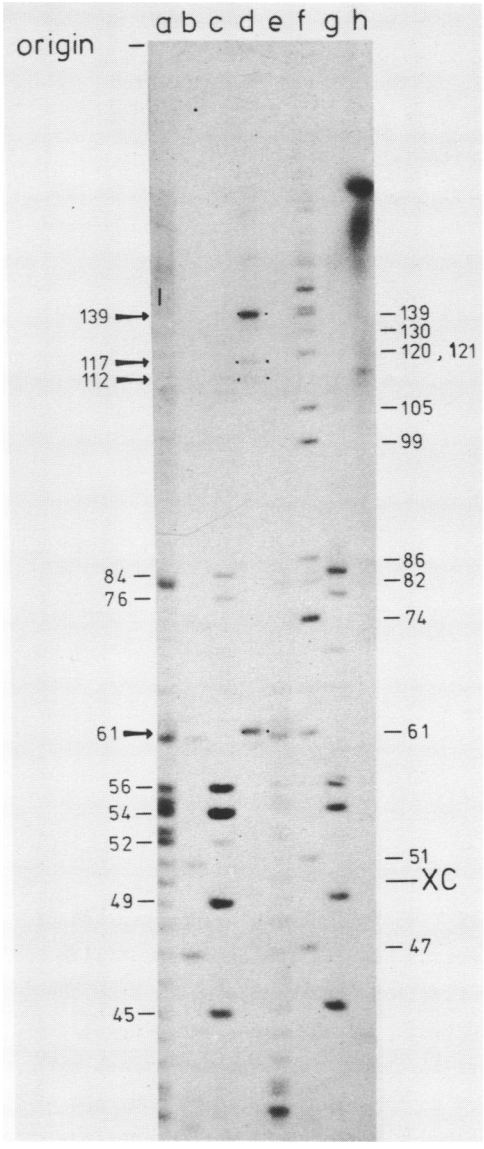

Fig. 3. - Analysis of ^{3}H-Val-RNA on a 12% polyacrylamide, 7 M urea gel (40 x 20 x 0.2 cm^3). ^{3}H-Val-RNA of TYMV (45,000 cpm) was analyzed in lane d. ^{14}C-A_{OH}-RNA of TYMV was either alkali-digested (35,000 cpm) for 2 min (lane a) or 6 min (lane e), or RNase-digested (20,000 cpm) using 10 (lane b) or 4 (lane f) units/ml of RNase T_1 or using 1 (lane c) or 0.4 (lane g) unit / ml of RNase A. The ^{32}P-labelled TYMV RNA fragments of 208 and 112 nucleotides (lane h) served as additional markers. Lengths of ^{3}H-Val-RNA fragments : →. The lengths of RNase A (—) and of RNase T_1 (—) digests of ^{14}C-A_{OH}-RNA of TYMV are indicated in the left- and in the right-hand margin respectively and served as markers.

Since fragments of 50 to 75 nucleotides can be adenylated, albeit to varying extents (Fig. 1), one can assume that the 61 nucleotide-long fragment is capable of being adenylated but not valylated.

B. Reticulocyte Lysate

The fragmentation pattern of TYMV RNA aminoacylated by the reticulocyte

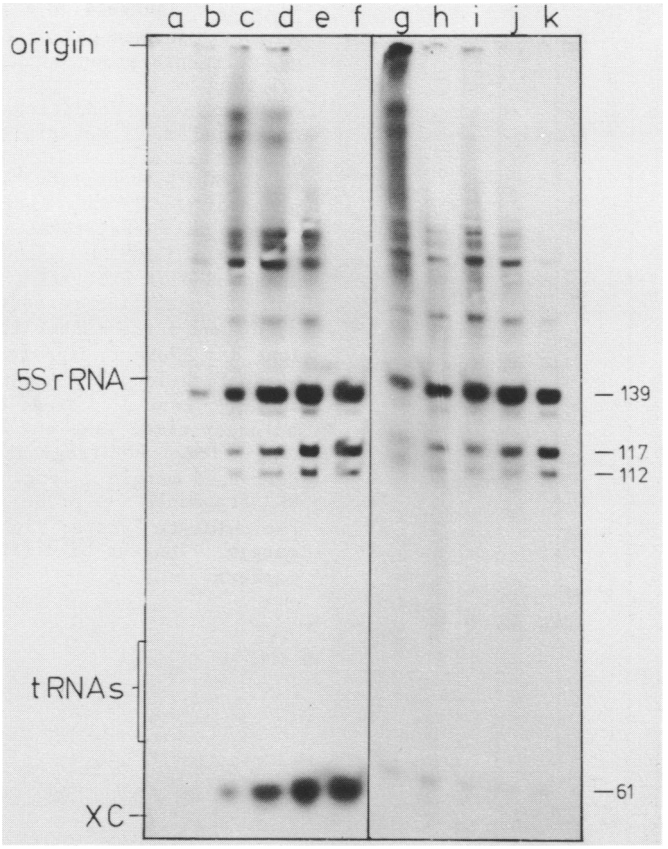

Fig. 4. - Analysis on a 10% polyacrylamide, 7 M urea gel of the size of the ^{3}H-Val-RNA of TYMV produced after different times of aminoacylation. TYMV RNA was valylated with 0.65 mg/ml of *E. coli* extract for 0, 10, 20, 40, 80 and 160 min (lanes a-f respectively). In lanes g-k, TYMV RNA was pre-incubated for 0, 10, 30, 70 and 150 min respectively with 0.65 mg/ml of *E. coli* extract in the absence of valine; 1.5 µM ^{3}H-valine and an additional 0.13 mg/ml of *E. coli* extract was then added and the incubation continued for 10 min. Aliquots (20 µl) were analyzed. The size of the major cleavage products are indicated in the right-hand margin.

lysate was likewise examined by gel electrophoresis (Fig. 5). The position of endogenous ^{3}H-Val-tRNAs (lane a) can be seen when no TYMV RNA was added during aminoacylation. Lanes b and c represent ^{3}H-Val-RNA of TYMV after 20 and 60 min of aminoacylation respectively. The length of the ^{3}H-Val-RNA fragments produced as determined based on RNA markers (lanes d, d' and e) is 117, 112, 109, 94, 84, 73 and 46 nucleotides (± 2 nucleotides).

Since intensive cleavage of TYMV RNA occurred with optimum reticulocyte

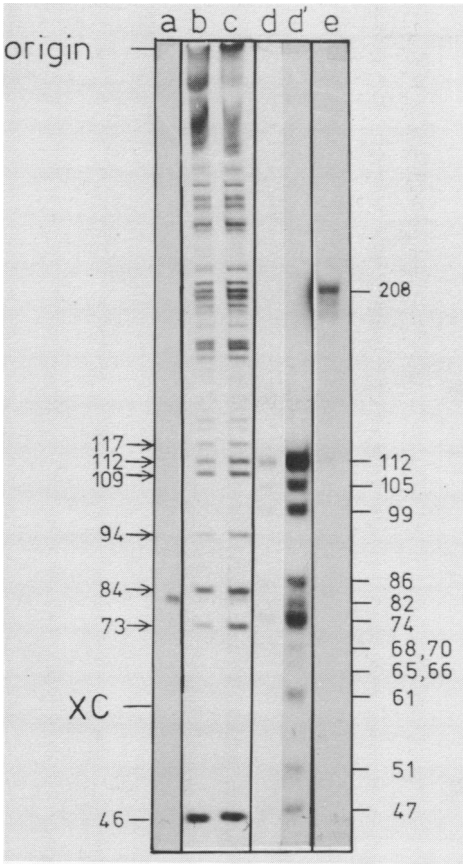

Fig. 5. - Analysis on a 10% polyacrylamide, 7 M urea gel of the ^{3}H-Val-RNA fragments produced during aminoacylation by the reticulocyte lysate. Aminoacylation conditions were as described under Materials and Methods except that a reticulocyte lysate (2 µl in 50 µl incubation) was used in place of the *E. coli* extract. Lane a : endogenous ^{3}H-Val-tRNAs (in the absence of TYMV RNA). Lanes b and c : ^{3}H-Val-RNA of TYMV after 20 and 60 min of incubation respectively. Lanes d, d' and e are marker fragments : lane d : RNase T_1 digests of the 112 nucleotide-long ^{32}P-A_{OH}-RNA fragment of TYMV; lane d' : as d, exposed for a longer time; lane e : 208 nucleotide-long ^{32}P-A_{OH}-RNA fragment of TYMV. Left-hand margin : lengths of ^{3}H-Val-RNA fragments (→) produced by the reticulocyte lysate; right-hand margin : lengths of different RNA markers.

lysate concentrations for valylation even after short incubation times (not shown), the minimum size required for adenylation and valylation using this *in vitro* system was not determined. By analogy with the *E. coli* enzymes, we can postulate that fragments between 50 and 75 nucleotides can still be adenylated and that fragments $\geq$ 86 nucleotides can be valylated. Thus the 73 and 46 nucleotide-long fragments observed after incubation with the reticulocyte lysate most probably correspond to endonucleolytic cleavage after aminoacylation.

C. Chinese Cabbage Leaf Extract

Plant extracts, *Phaseolus vulgaris* and *Triticum aestivum*, allow valylation of TYMV RNA (3). Attempts to prepare highly active Chinese cabbage leaf extracts were unsuccessful. Therefore, to visualize the fragmentation pattern produced by this homologous system, TYMV RNA was either adenylated with tRNA

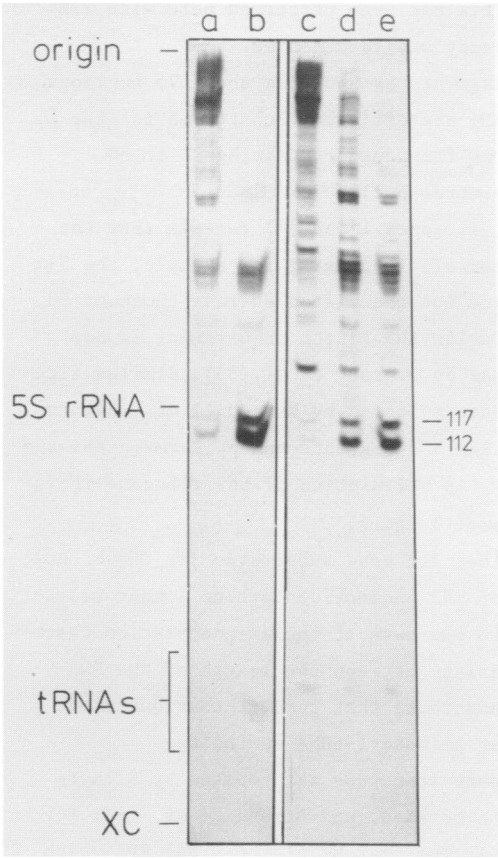

Fig. 6. - Analysis by 10% polyacrylamide, 7 M urea gel electrophoresis of ^{3}H-Val-RNA of TYMV incubated with a leaf extract. TYMV RNA was either adenylated using 0.3 mg/ml of tRNA nucleotidyltransferase in the absence (lane a) or presence (lane b) of 1.6 mg/ml of leaf extract, or aminoacylated using 0.13 mg/ml of *E. coli* extract in the absence (lane c) or in the presence of 0.8 and 1.6 mg/ml (lanes d and e, respectively) of leaf extract. The size of the major fragments are indicated to the right.

nucleotidyltransferase (Fig. 6, lanes a and b) or aminoacylated with limiting amounts of an *E. coli* extract (lanes c-e), in the absence (lanes a and c) or in the presence (lanes b, d and e) of varying concentrations of leaf extract. Both by adenylation and aminoacylation, the 117 and 112 nucleotide-long fragments appear as the major fragmentation products.

DISCUSSION

Many plant RNA viruses contain a tRNA-like structure at the 3' end of their genome. The minimum size of a viral RNA fragment that still possesses tRNA-like properties has usually been considered to be in the range of 100 to 200 nucleotides, whereas that of the longest tRNA sequenced to date is 92 nucleotides (32). Consequently, it seemed of interest to determine more precisely the length of the viral RNA fragments that could still be used as

substrates by tRNA-specific enzymes. This has been performed here with TYMV RNA whose tRNA-like properties are already well documented.

The results reported show that fragments as short as about 75 nucleotides derived from the 3' end of the viral RNA are efficient substrates for the *E. coli* tRNA nucleotidyltransferase, whilst fragments containing 74 to 50 nucleotides are less efficient. Furthermore, a close examination after adenylation of pre-digested TYMV RNA (Fig. 1, lanes i and i') reveals that the level of adenylation is not the same for all fragments; for exemple, the 51 and the 71 nucleotide-long fragments are not adenylated whereas those of 50 and 69 are adenylated. Thus for adenylation the length requirement is not stringent as already observed with tRNAs by Overath *et al.* (33). Similar situations of multisite recognition have been reported by Renaud *et al.* (34) for the aminoacylation of purified yeast $tRNA^{Phe}$ fragments and by Parmeggiani and co-workers (personal communication) for the stimulation of the ribosome-EF-Tu GTPase by Val-tRNA fragments of different lengths.

Fragments containing $\geq$86 nucleotides are good substrates for the *E. coli* valyl-tRNA synthetase, whereas those of $\leq$82 nucleotides are no longer valylated. It is interesting to note that in the case of the aminoacylation reaction examined here, a sharp transition exists between the length of the RNA fragment that can serve as substrate and those that cannot; this would be expected of an enzyme as specific as an aminoacyl-tRNA synthetase.

Fig. 7 presents a possible secondary structure as proposed by Silberklang *et al.* (14) for the first 112 nucleotides, and Briand *et al.* (13) for nucleotides 113 to 160 of the viral RNA. The regions of the viral RNA which serve as substrates for the *E. coli* tRNA nucleotidyltransferase and valyl-tRNA synthetase are indicated. Since RNA fragments of 50 to 75 nucleotides and of 86 nucleotides from the 3' end are sufficient for adenylation and aminoacylation respectively, base-pairing between nucleotides 105 to 98 and nucleotides 4 to 10 to form the "aminoacyl" stem is excluded. If an "aminoacyl" stem is required for adenylation or valylation, then another folding of the first 75 or 86 nucleotides of TYMV RNA must be envisaged. On the basis of chemical modification studies and digestion with several RNases, in the accompanying paper, Rietveld *et al.* (35; Fig. 6) have proposed a secondary structure for the 3' end of TYMV RNA. The model proposed predicts that nucleotides beyond 42 are not required to complete the "aminoacyl" arm; this is in line with our results which show that nucleotides 50 to 75 or 86 are sufficient for proper folding of the RNA molecule to allow adenylation or aminoacylation respectively.

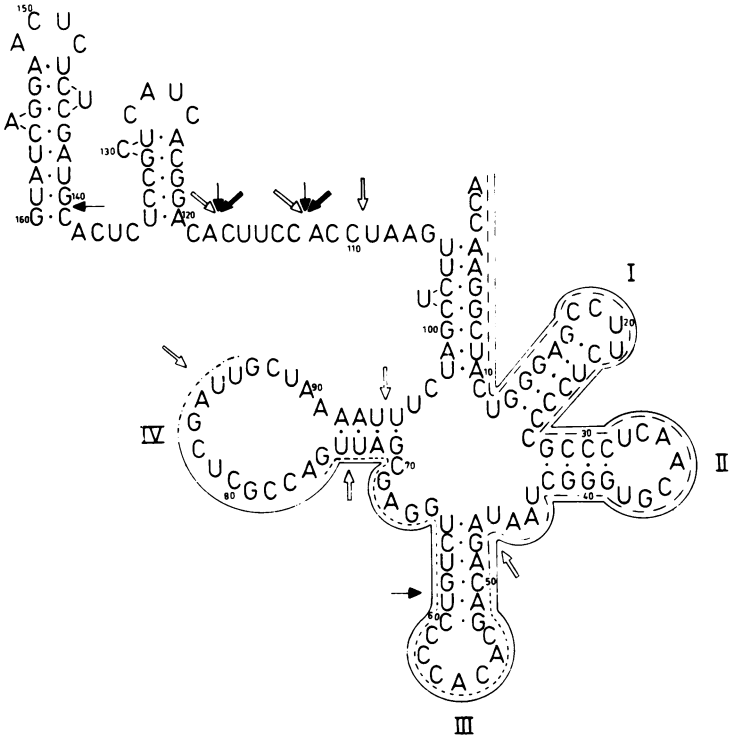

Fig. 7. - A possible secondary structure for the 3'-terminal 160 nucleotides of TYMV RNA (13, 14). TYMV RNA lacks the 3'-terminal A residue; the sequence is presented here with this residue added by the tRNA nucleotidyltransferase. Numbering of the nucleotides is from the 3' end. The lengths of the RNA fragments that are indispensable for (– –) or enhance (----) adenylation, and of those required for valylation using the *E. coli* enzymes (——, or ····· depending on the exact length required) are indicated. The major cuts produced by the *E. coli* extracts (⟶), the reticulocyte lysate (⟹) and the leaf extract (➡) are presented. I to IV refer to the four loops and stems within the tRNA-like fragment.

The minimum length of the viral RNA that can serve as substrate of the tRNA nucleotidyltransferase extends beyond stems and loops II and III (Fig.7). It is possible that the region of the RNA between nucleotides 66 and 74 participates in bridging the distance between the "anticodon" stem and loop III, and the "aminoacyl" arm, thereby favoring adenylation. This would however be only partly successful since 75 nucleotide-long fragments cannot be aminoacylated by the *E. coli* valyl-tRNA synthetase. As fragments of $\geq$86 nucleotides are necessary for esterification, stem and loop IV might have to be folded in a different manner from the one presented in Fig. 7 to facilitate

the orientation of the "aminoacyl" versus the "anticodon" arms.

The tRNA-like region of the genome of the Cowpea strain of TMV whose RNA accepts valine, contains significant sequence similarity (56%) with TYMV RNA within the first 80 nucleotides from the 3' end (19). This observation and our finding that 86 nucleotides of the TYMV genome are sufficient for valylation and thus for the formation of the tRNA-like structure, together with the proposed model of Rietveld *et al.* (35) for this region of the viral RNA, suggest strongly that about 80 nucleotides should also be required by the Cowpea strain of TMV RNA to serve as substrates for tRNA-specific enzymes.

In the case of brome mosaic virus RNA, fragments of 65 nucleotides lose their tyrosylation capacity (36), whereas those of 161 nucleotides can be tyrosylated (37), indicating that regions between nucleotides 65 and 161 are also necessary to allow recognition by tRNA-specific enzymes. It is not unlikely that the active portion of the molecule is contained within the first ∿80 nucleotides.

The major cleavage products produced at the 3' end of TYMV RNA by an *E. coli*, a reticulocyte or a plant extract are presented in Fig. 7. Of the four major cuts occurring with the *E. coli* extracts, three (yielding fragments of 139, 117 and 112 nucleotides) are aminoacylatable; the fourth is produced by cleavage within the tRNA-like structure, in the "anticodon" stem, liberating a non-aminoacylatable fragment of 61 nucleotides. Reticulocyte lysates provide the following major fragments of 117, 112, 109, 94, 84, 73 and 46 nucleotides. When using extracts from Chinese cabbage leaves, the host plant, only two major cuts yielding fragments of 117 and 112 nucleotides are produced. The major cuts obtained with the different systems used here do not derive solely from cleavages in exposed regions in the viral RNA but appear to be specific of the system since different fragmentation patterns and different relative intensities of cleavage are obtained.

The shortest aminoacylatable fragment liberated using an *E. coli* or a leaf extract, or the *X. laevis* system (mentioned as a 4.5S RNA fragment in ref. 11) is 112 nucleotides long. This is somewhat longer than required for recognition by the valyl-tRNA synthetase, *i.e.* 86 nucleotides. Only with the reticulocyte lysate is a major fragment of 84 (±2 nucleotides) released that can be esterified with valine. Since with the three former systems no major cut occurs between nucleotides 112 and 86, one can assume that this part of the structure is compact and/or interacts strongly with the rest of the molecule, preventing release of an 86 nucleotide-long fragment. Our preliminary data suggest that the viral RNA is aminoacylated *in vivo* in infected

Chinese cabbage leaf strips (manuscript in preparation). Experiments are under way to define the size of the charged viral RNA. It is tempting to speculate that a fragment of 112 or of 86 nucleotides is also formed *in vivo* that might play an as yet undefined function during the life-cycle of the virus.

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