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Binding of ribosomes to the 5' leader sequence (N = 258) of RNA 3 from alfalfa mosaic virus

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ABSTRACT

RNA 3 of alfalfa mosaic virus (AlMV) contains information for two genes : near the 5' end an active gene coding for a 35 Kd protein and, near the 3' end, a silent gene coding for viral coat protein. We have determined a sequence of 318 nucleotides which contains the potential initiation codon for the 35 Kd protein at 258 nucleotides from the 5' end. This long leader sequence can form initiation complexes containing three 80 S ribosomes. A shorter species of RNA, corresponding to a molecule of RNA 3 lacking the cap and the first 154 nucleotides (RNA 3') has been isolated. The remaining leader sequence of 104 nucleotides in RNA 3' forms a single 80 S initiation complex with wheat germ ribosomes. The location of the regions of the leader sequence of RNA 3 involved in initiation complex formation with 80 S ribosomes is reported.

INTRODUCTION

The 5' non coding sequences of eucaryotic mRNAs vary greatly in length and appear to lack of any specific sequence signal for ribosome binding except the initiation codon AUG in the 5' sequence (see 1 for review). The cap structure present in many messenger and viral RNAs improves the efficiency of, but is not essential for, translation (2). The initiation of protein synthesis is a complex process in which many factors are involved. Eucaryotic ribosomes are able to recognize very efficiently the initiation codon located near the 5' end, and the subsequent translation seems to be restricted to the cistron close to this region (3). In most cases, when elongation is inhibited this ribosome binding results in the formation of monosomes (1) ; however two ribosomes were bound to the 5' end leader sequences of Brome Mosaic Virus RNA 3 (4), Tobacco Mosaic Virus genomic RNA, and Turnip Yellow Mosaic Virus RNA (5) in these conditions. We reported in an earlier paper (6) that RNA 3 of Alfalfa Mosaic Virus (AlMV), a multipartite genome plant virus, bound more than two ribosomes when elongation was inhibited, but found that the

data obtained from the analysis of the regions protected by ribosomes were too complex to deduce precise ribosome binding sites without the help of separate sequence data.

We report here the analysis of 318 nucleotides containing the 5' end leader sequence using enzymatic and chemical methods. These results helped us to locate the ribosome binding sites on this sequence. We discuss the number of ribosomes that bind on this sequence with respect to the length of the leader sequence.

MATERIALS AND METHODS

Preparation of ^{32}P labelled AlMV RNAs. AlMV infected Tobacco plants were grown on a ^{32}P carrier free solution (7). AlMV RNAs, uniformly labelled with ^{32}P, were extracted from purified virus using phenol SDS and fractionated on gels of 2.4 % polyacrylamide + 0.5 % agarose as described previously (7).

Cap removal and 5' end labelling of AlMV RNA 3. In order to label the 5' end of AlMV RNA 3 it was necessary to remove the 5' end terminal cap. The m^7Gp moiety of the cap was removed by treatment with Tobacco acid pyrophosphatase (TAP) prepared from Tobacco cell cultures as described by Efstradiatis et al. (8). 100 pmoles AlMV RNA 3 were incubated for 1 h at 37°C in a 100 µl mixture containing 1 mM Na_2EDTA, 50 mM Na acetate pH 6.0, 10 mM 2-mercaptoethanol and 10 µl TAP (1.5 U/µl according to its ATPase activity (8)). The TAP incubation was stopped by adding phenol and the RNA was extracted and precipitated with ethanol before treatment with alkaline phosphatase (calf intestine from Boehringer) purified according to Efstradiatis et al. (8). 100 pmoles of TAP treated RNA 3 were incubated for 30 min at 37°C in a 100 µl mixture containing 50 mM Tris-acetate pH 8.0 and 10 µl phosphatase at 1 U/µl and the RNA re-extracted by 2 successive phenol extractions. The treated RNA was precipitated with ethanol prior to 5' end labelling with (γ-^{32}P)ATP (1000-3000 Ci/mmole, Amersham) using T_4 polynucleotide kinase as described by Bosely et al. (9). This method was also used for 5' end labelling of RNA fragments resulting from partial RNase T_1 digest of RNA 3.

3' end labelling of AMV RNA 3. The 3'OH end of AlMV RNA 3 or 3'OH ends of fragments generated by RNase H digestion of RNA-DNA duplexes were labelled by adding (5'^{32}P)pCp (1000-3000 Ci/mmole, Amersham) to the 3'OH using T_4 RNA ligase as described by England and Uhlenbeck (10).

Recovery of full length 5' or 3' end labelled RNA 3. The labelled RNA was loaded on a 2.8 % polyacrylamide slab gel (20 x 40 x 0.4 cm), containing

8 M urea, 1 mM Na_2EDTA and 50 mM Tris-borate buffer pH 8.3. Electrophoresis was for 24 h at 500 V. The RNA was located by autoradiography and the major band, corresponding to full length RNA, was cut out of the gel and the RNA was eluted from the gel piece by electrophoresis.

RNA sequencing methods. The conditions for oligodeoxynucleotide-RNA hybrid formation and cleavage with RNase H (E. Coli ENZO N.Y.) of the RNA part of this duplex were as described by Donis-Keller (15). The fragments of RNA 3 resulting from these experiments were either directly labelled at their free 3'OH by addition of (^{32}P)pCp or at their 5' end with (γ-^{32}P)ATP and polynucleotide kinase after prior treatment with alkaline phosphatase.

(^{32}P)5' end labelled RNA 3 or fragments of RNA 3 were sequenced using the methods developed by Donis-Keller et al. (11). The nuclease from Neurospora crassa (12) as well as a two dimensional polyacrylamide gel analysis according to Lockard et al. (13) were used to differentiate uridine and cytidine. The chemical method described by Peattie (14) was used for sequencing the 3' end of (5'^{32}P)pCp labelled RNA fragments.

Polyacrylamide gels were made from 2 x crystallised acrylamide which had been filtered through a charcoal filter, and treated with Dowex AG 1 x 8. Gels contained 50 mM Tris-borate buffer, 1 mM EDTA pH 8.3 and 8 M urea. All glass and plastic wares were sterilized and silicone coated before use.

Preparation of ribosome protected fragments of RNA 3. RNA 3 uniformly labelled with ^{32}P was added to a protein synthesizing system derived from wheat germ (16). Sparsomycin was added to inhibit elongation. The complexes of ^{32}P-RNA and ribosomes were treated with RNase T_1, and the fragments of RNA protected from nuclease digestion were isolated and purified as described previously (6).

RESULTS

5' end sequence of AlMV RNA 3. Direct sequencing techniques have been used to sequence the first hundred nucleotides from the 5' end of AlMV RNA 3 after removal of the capped 5' end and subsequent 5' end labelling with (γ-^{32}P)ATP and polynucleotide kinase. After about nucleotide 100 the bands in the sequencing gel were compressed and did not allow unequivocal interpretation, even when the temperature was raised to increase the extent of denaturation. The 5' end of the leader sequence is reported later (see Fig. 3). The first 98 nucleotides of the sequence contain 12 T_1 oligonucleotides but no initiation codon (AUG). Such a situation is unusual for 5' ends of mRNAs.

In order to extend the 5' end sequence we tried to obtain long 5' end

fragments which could be sequenced separately, either chemically or enzymatically. Two methods were used to produce fragments of the 5' region ; in the first, specific oligodeoxynucleotide-RNA hybrids were formed and the RNA was cleaved with RNase H which yielded fragments with either a free 3'OH, which were labelled with (^{32}P)pCp using T_4 RNA ligase, or with a 3' phosphate which were labelled after dephosphorylation using kinase. The second method was the analysis of 5' end fragments of RNA 3 obtained from partial digests with RNase T_1, postlabelled at their 5' end.

RNA 3, labelled at its 5' end, was hybridized, in a presence of a large excess of cold RNA 3, with $d(pTpG)_2$ (from Collaborative Research), digested with RNase H and analysed on a 5 % polyacrylamide gel. The most abundant fragment was isolated from the gel shown in Fig. 1a which included the 5' end. This fragment was labelled with (^{32}P)pCp and chemically sequenced. The chemical sequencing method gives clear data even in the region N≈100. Fig. 1b gives the sequence from nucleotide N 89 to N 178, in which no potential initiation codon is present.

Another interesting fragment was obtained after hybrid formation with $d(pT_8)A$ (from P.L. Biochemicals) and RNase H digestion ; this fragment of about 64 nucleotides was 5' end labelled and sequenced using the Donis-Keller method. It extends from N = 209 to N = 273 (see Fig. 3) and is overlapping with two fragments resulting from partial T_1 RNase digestion of RNA 3 (1 unit T_1 RNase for 500 µg RNA) for 15 min at 0°C at pH 5.0 in 10 mM Na acetate and 100 mM NaCl. The resulting fragments were 5' end labelled with (γ-^{32}P)ATP and fractionated on a 8 % polyacrylamide gel (Fig. 2a). Two fragments, G4 and G11, had sequences which overlapped with the RNase H fragments previously mentioned. The sequence data of fragment G4, shown in Fig. 2b were confirmed by two dimensional gel analysis (Fig. 2c) in which two AUG codons were found at position N = 258 and N = 277. A third AUG is found in position N = 294.

The codon AUG at position N = 258 is the first AUG from the 5' end and a potential initiation codon. The sequence of 318 nucleotides from the 5' end is presented in Fig. 3, in which the different fragments used to establish this sequence are indicated. Two sequences, each 29 nucleotides long, which are indicated in boxes, were identical and a third (N = 72 to N = 100) was very similar, with only 4 differences of one nucleotide.

Initiation complexes formed with RNA 3 and RNA 3'. RNA species of AlMV are found in different nucleoprotein components and RNA 3 is the major species of top component b particles. However, when RNA extracted from the nucleo-

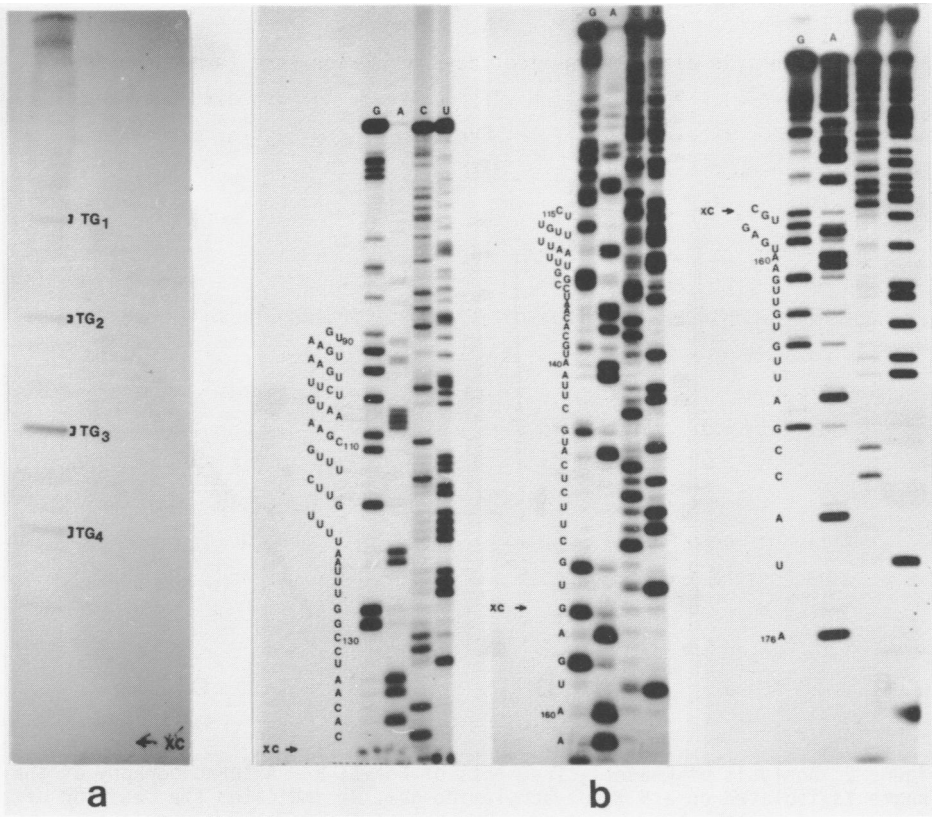

Figure 1. Analysis of fragments of RNA 3 obtained after cleavage of duplexes with $d(pTpG)_2$ by RNase H. a - Autoradiography of the RNase H fragments labelled with $(^{32}p)pCp$, analysed on 5 % polyacrylamide gel. b - Autoradiography of sequencing gels of fragment TG_3 shown in a, corresponding to the sequence from N 98 to N 178. xc indicates the position of xylene cyanol marker.

proteins which precipitated with 30 mM $MgSO_4$ (17), i.e. mostly the heavy components of AlMV, were analysed by electrophoresis in polyacrylamide-agarose gels, RNA 3 appeared as a double band which was described in an earlier paper (7) as RNA 17-S_1 and RNA 17-S_2. The minor component, now named RNA 3', can be directly labelled with $(\gamma\text{-}^{32}P)ATP$ and therefore has no capped 5' end. Sequencing data showed that RNA 3' corresponds to RNA 3 except that in RNA 3' the first 154 nucleotides and the cap are missing, as indicated in Fig. 3.

RNAs 3 and 3' provide an opportunity to study ribosome binding to RNA

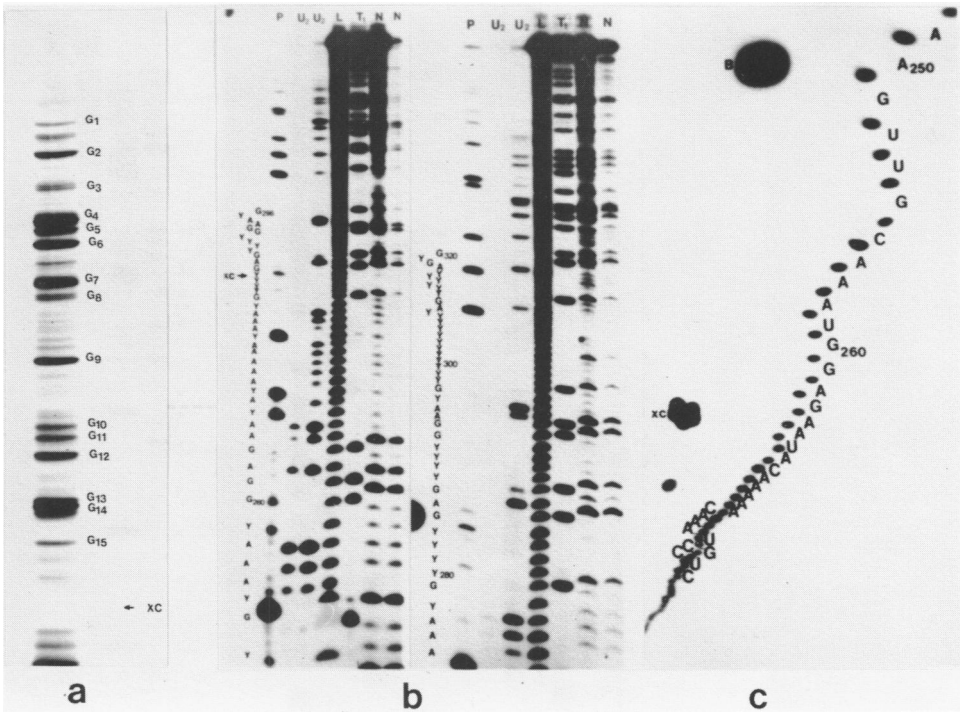

Figure 2. Analysis of RNase T_1 fragments of RNA 3. a - Autoradiography of the fragments isolated on a 8 % polyacrylamide gel. xc indicates the position of the xylene cyanol marker. b - Sequencing gel of fragment G4 shown in a. c - Two dimensional gel analysis of fragment G4 indicating the position of the two first AUG triplets (N 258 and N 277). B indicates position of bromophenol blue marker. Lanes P, U_2, T_1 correspond to digests obtained using pancreatic, U_2 and T_1 RNase ; lane N_2 is obtained with Neurospora nuclease, L lane is the ladder.

molecules having the same initiation site but with leader sequences of very different lengths, 258 and 102 nucleotides respectively. Because the cap was only present in RNA 3 and in order to avoid additional methylation by labelling with vaccinia enzymes (5) we labelled both RNAs 3 and 3' by addition of (^{32}P)pCp at the 3'OH end. The labelled RNAs were purified on 2.8 % polyacrylamide slab gels before ribosome binding experiments were performed. The experimental binding conditions were as previously used (6). The ribosome RNA complexes formed in presence or in absence of sparsomycin were sedimented in sucrose gradients (Fig. 4). Under normal conditions for protein synthesis, RNA 3' formed polysomes but when elongation was inhibited mainly monosomes

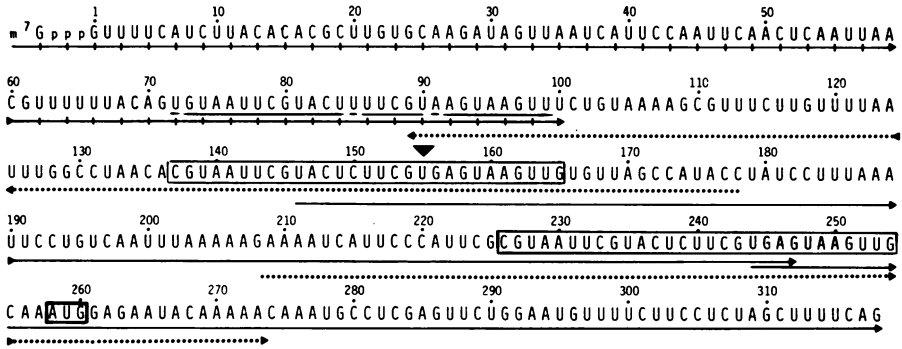

Figure 3. Sequence of 318 nucleotides from the 5' end of AlMV RNA 3 determined by direct sequencing (⊣——⊢) from N1→N100 or by analysis of fragments obtained from RNase H digests (.....) N89→N178 and N209→N273, or fragments obtained from partial T_1 RNase digests (——) N146→N247 and N244→N318. The arrow indicates the origin of RNA 3' at N = 155. Underlined sequence N72 to N99 indicate partial homology with the boxed sequences.

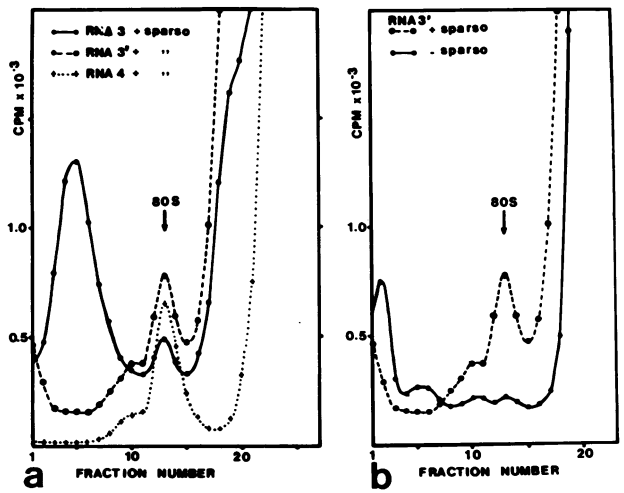

Figure 4. Sucrose density gradient radioactivity profiles of ribosome-RNA complexes formed in the absence or in the presence of sparsomycin (120 µM). Samples of 60,000 cpm of each 3'OH (^{32}P)pCp labelled RNA 3, RNA 3' and RNA 4 were used in a 100 µl incubation mixture. The ribosome-RNA complexes are analysed on 10-40 % sucrose gradients in 50 mM Tris-acetate, 100 mM KCl, 4 mM Mg acetate pH 7.4, centrifuged for 150 min at 40,000 rpm in a SW 41 rotor.

were formed (Fig. 4b). With RNA 3 however, polysomes were present even when sparsomycin was added (Fig. 4a). From the position of the heavy polysome peak in the gradient compared to that of normal polysomes, run in parallel, the complex formed with RNA 3 probably contained 3 ribosomes (Fig. 4a and b).The position of 80 S monosomes was indicated by the initiation complexes formed with AMV RNA 4 (Fig. 4a), which have been studied earlier (6).

Location of the ribosome binding sites. We reported in a previous paper (6) that in addition to monosomes and disomes heavy aggregates formed in ribosome binding experiments performed with (^{32}P) RNA 3. In these experiments the specific radioactivity of the fully labelled (^{32}P) RNA 3 was however too low to permit the different analyses necessary for the sequence determination of the ribosome protected fragments found in the initiation complexes, monosomes or polysomes, separated on sucrose gradients. Therefore, in order to increase the amount of radioactive fragments available for analysis, we incubated the initiation complexes with T_1 RNase immediately after their formation. The resulting initiation complexes, 80 S ribosome-(^{32}P) RNA 3 fragments, were collected by sedimentation through a sucrose layer to remove all unbound RNA fragments (not shown). The labelled fragments were recovered by phenol extraction of the pelleted material and analysed as previously described (6). Fig. 5a shows RNA fragments recovered in this way analysed on 8 % polyacrylamide gels. The size of the fragments, estimated by comparison with fragments of known length obtained from AlMV RNA 4 (6) was 36, 30 and 22 nucleotides, respectively, for bands A, B, and C. The analysis of bands A and B by complete T_1 RNase digestion and separation by electrophoresis and homochromatography gave complex patterns (Fig. 5 b - c). The nucleotide content of the spots is shown in Table I. The total number of oligonucleotides shown by this analysis indicated that the bands isolated from the gels contained two or three different fragments of the same length which had comigrated. Table I indicates also which of the spots on Fig. 5 b - c correspond to which T_1 RNase oligonucleotide as numbered in the sequence (Fig. 6). Some spots, such as T_6 or T_8, have at least 3 different possible positions.

It was not possible to get unambiguous sequence information from the protected fragments because of the limited amount of radioactivity available. It was possible, however, from the 5' end sequence and the lengths of the ribosome-protected fragments, to locate three different possible binding sites by analysis of the data reported in Table I, as shown in Fig. 6. The binding site containing the two first AUG triplets (N 258 and N 277 : site 3)

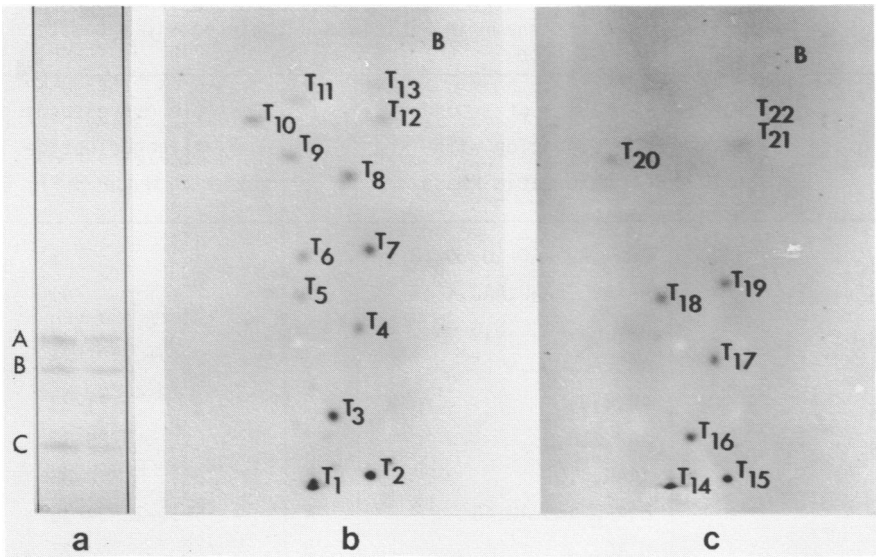

Figure 5. Autoradiogram of an 8 % polyacrylamide slab loaded with ribosome protected fragments obtained after T_1 RNase digestion (a). Homochromatographic fractionation of band A (b) and band B (c) digested with T_1 RNase. The analyses of the different spots are given in Table I.

was found in bands A and B (Fig. 5). Since the initiation triplet AUG is usually found close to the middle of the ribosome-protected sequence (1,6) it is likely that the AUG codon (N 258-260) constitutes the initiation codon of the 35 Kd protein. This site includes oligonucleotides 37 to 41 and could extend to oligonucleotides 34 - 35 - 36 but a greater extension is not in agreement with the size of the ribosome-protected fragments. The putative amino acid sequence corresponding to the reading frame starting with the first AUG is indicated in Fig. 6.

The two other ribosome binding sites contain the following oligonucleotides : for site 1, oligonucleotides 5 and 6 but not 4 or 7 which are not among the products listed in Table I ; for site 2, oligonucleotides 19 to 23 with a possible extension to 24 and 25. Oligonucleotide 17 is not found and oligonucleotide 33 is beyond the size limit of the fragment. Some variability may, however, exist in the extent of ribosome protection so that it would be better to consider the ribosome-protected sequences as corresponding to a ribosome binding domain.

Among the ribosome protected fragments, one T_1 RNase oligonucleotide

Table I. Analysis of secondary digestion products obtained from ribosome protected fragments shown in Fig. 5a and their possible position in the leader sequence.

T_1 oligonucleotide	Products of secondary digestion with pancreatic RNase	Size	Possible corresponding T_1 oligonucleotide number in sequence Fig. 6
T_1 , T_{14}	AAU_3,AU,AAC_2,U_6,C_6,G(*)	29	6
T_2 , T_{15}	AAAAAC,AAAU,AAU,AC,G	16	41
T_3 , T_{16}	AAU,AU,AC,C,U,G		n.d.
T_4 , T_{17}	AAC,AC,C,U,G	9	19
T_5	AC,C,U,G	9	21-33
T_6 , T_{18}	AAU,C,U,G	7	9-20-32
T_7 , T_{19}	AAAU,C,G	6	38
T_8	U,AAG and AU,AG	4	5-11-12-24-36
T_9	U,AG	3	27
T_{10} , T_{20}	U,G (UUG)	3	25-37
T_{11}	UG	2	3-8-22-26-34
T_{12} , T_{21}	AG	2	23-35-40-43
T_{13} , T_{22}	G	1	18-39-45

(*) The relative molar yield of pancreatic digestion products was calculated for oligonucleotides T_1 and T_2 ; for the others it was estimated, underlining denotes spots present in more than one copy.

(T_3 or T_{16} in Fig. 5) could not be located within the leader sequence of RNA 3 or 3'. This oligonucleotide may result from spurious ribosome binding to RNA fragments arising from breakage in a region highly sensitive to fragmentation within the coding sequence. Nevertheless, the fact that only monosomes formed initiation complexes with RNA 3' indicates that only one ribosome binding site exists on each RNA molecule or RNA fragment. The existence of a mutually exclusive binding site cannot, however, be ruled out.

DISCUSSION

The distance between the 5' end and the beginning of the first initiation codon varies greatly in the different viral RNAs and mRNAs analysed until now (1). The two most common situations are illustrated by Reovirus

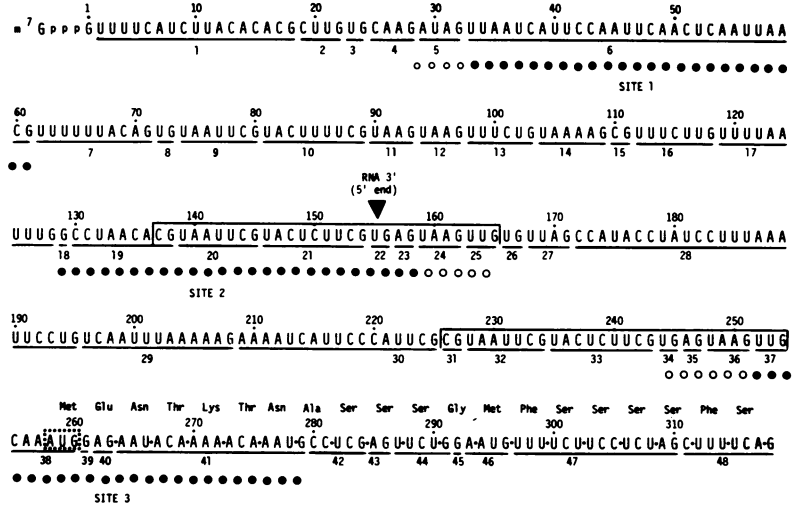

Figure 6. Ribosome binding sites on the leader sequence of AlMV RNA 3. The T_1 RNase oligonucleotides are numbered from the 5' end, they correspond to the numbers listed in Table I. The ribosome protected regions are indicated by •••, the circles ooo indicate a possible extension of the ribosome protection.

mRNAs in which the initiation triplet AUG is between 20 and 40 nucleotides from the 5' end (18) or by TMV RNA (19) or BMV RNA 3 (4) for which this distance is close to 70 or 90 nucleotides.

The leader sequence of AlMV RNA 3 constitutes with polyoma virus mRNAs (20) and foot-and-mouth disease virus (21) an example of a leader longer than 250 nucleotides. Repetitive sequences reported here for AlMV RNA 3 have been postulated in polyoma mRNA to explain multiple ribosome binding (20). In the case of AlMV RNA 3 an analogous situation is possible since the second ribosome binding site contains a part of the repeated sequence involved in the third binding site, at the initiation codon. It is however interesting to note that the region from N 72 to N 99, which is very similar to the repeated sequences, is not protected by ribosomes. The position of the first binding site is, however, close to this region. The absence of any additional ribosome binding may be due to the hindrance of the previously bound ribosomes since the elongation is inhibited with sparsomycin. Both 80 S and 40 S ribosomes could be involved in heavy aggregates but treatment of the initiation complexes formed with ^{32}P labelled RNA 3 in the presence of sparsomycin

with T_1 RNase before analysis on sucrose gradients eliminated these heavy aggregates and disomes ; the radioactivity shifted from the heavy region to the 80 S monosomes (results not shown) indicating that the bound ribosomes are principally 80 S ribosomes. This situation, similar to that found with BMV RNA 3 (4) and TMV RNA (5), is not in agreement with the scanning model of Kozak (1), which predicts that 40 S subunits slide along the leader sequence and join the 60 S subunit at the initiator AUG, unless sparsomycin is responsible for a premature joining with 60 S subunits.

The single ribosome binding observed with RNA 3' is surprising since this RNA has a long leader sequence (104 N). The minor disome peak (Fig. 4) is comparable in size to the disome peak found with RNA 4, which is not considered to be significant. The length of the RNA 3' leader sequence could allow the binding of at least a second ribosome by analogy to TMV RNA or to BMV RNA 3 but the absence of an additional ribosome binding may be related to the absence of a cap structure.

We compared the amino acid incorporation activities of RNA 3 and RNA 3' at concentrations ranging from 10 to 100 µg/ml of wheat germ reaction mixture and found that the activity of RNA 3' was only 10 to 15 % lower than that of RNA 3. This indicates that overall rate of translation is not affected in an important way by the absence of the cap and of a part of the leader sequence. This absence, however, has a slight effect at the ribosome binding level since 16 % of RNA 3 binds to ribosomes versus 10 % for RNA 3' as calculated from the data reported in Fig. 4. This is in agreement with the facilitating effect of the cap structure at the initiation level proposed by Shatkin (2). The length of the leader remaining in RNA 3' is very long compared to that of RNA 4 which is the most efficient messenger among AlMV RNAs (22). An analogous situation exists when RNA 3 and RNA 4 of BMV are compared (23), suggesting that subgenomic RNAs characterised by short leader sequences are more efficient than messengers with long leaders.

A possible local secondary structure of the 318 nucleotide fragments

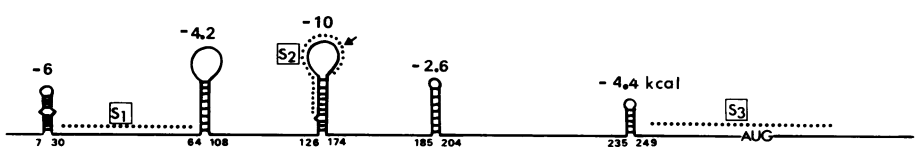

Figure 7. Possible secondary structure of the 5' end sequence of RNA 3. The free energy calculated according to (24) is indicated for each structure.

can be constructed, according to Tinocco (24), taking into account the accessibility to RNase T_1 and the facility of hybrid formation to deoxyoligonucleotides. Figure 7 represents a schematic drawing of this structure characterised by 5 small stable hairpin stems. The location of the 3 ribosome binding sites S1, S2 and S3 shows that only the site 2 corresponds to a structured region. The major part of the sequence that interacts with site 2 is on the 5' side of the cleavage point corresponding to the origin of RNA 3' (indicated by an arrow in Fig. 7). Sites 1 and 2 both contain a nearly central triplet AUU (N 44 - 46 and N 140 - 142) which could interact with the Met-tRNA anti-codon as suggested for the TMV RNA secondary binding site (5). In the leader sequence of RNA 3', however, at least 3 AUU triplets are available to allow a second ribosome to bind, but no binding is observed suggesting that other or additional structural signals are involved in extra ribosome binding.

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