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Formation of ribosome-RNA initiation complexes with alfalfa mosaic virus RNA 4 and RNA 3

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ABSTRACT

RNA 4 of alfalfa mosaic virus (AMV) is a monocistronic messenger for the coat protein. We have determined the sequence of the 40±2 nucleotides in RNA 4 that were protected in the initiation complex formed with wheat germ 80 S ribosomes from digestion by T_1 or pancreatic ribonucleases. The AUG coat protein initiation codon was near the middle of this protected region. We have found two ribosome-binding sites in RNA 3. The principal one, near the 5'end, is the initiation site for the major translation product, a 35,000 dalton protein. The second site binds ribosomes only weakly, at the beginning of the "silent" coat protein cistron, and is similar but not identical to the initiation site protected in RNA 4. The possibility of a ribosome-binding at the "silent" cistron initiation site is discussed.

INTRODUCTION

Most eucaryotic cellular and viral RNAs have been found to have a blocked and methylated 5'-terminal $m^7G5'ppp5'N$ structure, which has been implicated in the early stage of translation initiation[1].

Translation studies of plant and animal viral RNAs have indicated that the cistron near the 5' end of these RNAs is efficiently translated, although the distance of the initiation codon AUG from the 5' end varies greatly, 10 nucleotides in the case brome mosaic virus (BMV) RNA 4[2] and 69 for tobacco mosaic virus (TMV) RNA[3] for instance. For alfalfa mosaic virus (AMV) RNA 4 the first AUG, initiation codon of the coat protein, has been reported in position 36[4]. In this multipartite-genomic virus[5], as in BMV, the coat protein cistron is also present in RNA 3[6,7] but in both AMV and BMV this cistron is silent : only a 35,000 protein unrelated to the coat protein may be translated in vitro[8,9]. Since the coat protein cistron is near the 3' end of AMV RNA 3[10] it may be concluded, as reported by Kozak[1], that an internal initiation site in a messenger RNA molecule cannot function in eucaryotic systems.

This observation raises a question : are ribosomes unable to bind at an

internal initiation site ? To try to answer this question, we have determined the AMV RNA 4 sequence protected from digestion with T_1 RNase by involvement in a wheat-germ ribosome initiation complex and compared it with analogous fragments from RNA 3.

MATERIALS AND METHODS

Preparation of the ^{32}P-labeled virus. AMV_S (Strasbourg strain) was grown in Nicotiana tabacum var. Xanthi nc at 26-28°C and labeled with carrier-free ^{32}P 24 hr after inoculation. As previously described[11] 15 mCi were used for each plant. Virus was extracted 5 days after inoculation[6], but without polyethylene glycol precipitation. The labeled virus had in general a specific radioactivity of about 8 x 10^7 cpm/mg. The yield of virus was about 70 mg for 100 g of leaf material. AMV RNAs were phenol extracted and fractionated on cylindrical polyacrylamide-agarose 2.4-0.5 % gels as previously described[6].

RNase T_1 oligonucleotide mapping. RNA 4 and RNA 3 uniformly labeled with ^{32}P at a specific activity of 4 x 10^8 cpm/mg, were digested with T_1 RNase for 30 min at 37°C in 10 mM Tris, 1 mM Na_2EDTA, sodium acetate to pH 7.5. One unit of enzyme was used for 20 µg of RNA. The digests were fractionated on two-dimensional polyacrylamide gels[12]. The long T_1 oligonucleotides were excised from the gel, crushed by extrusion through a syringe and eluted by agitation with 0.35 M NaCl for 4-5 hr. To remove polyacrylamide particles the eluate was filtered, mixed with tRNA as carrier and precipitated with ethanol. The oligonucleotides were hydrolysed with pancreatic RNase to establish their length and their nucleotide compositions.

Cell-free protein-synthesizing system. The wheat germ extract was prepared according to Marcu and Dudock[13], and the conditions for incubation were as described before[14]. The translation efficiency of RNA 4 and RNA 3 and the absence of coat protein translation from RNA 3 were checked.

Preparation of ribosome-protected AMV RNA fragments. Before introduction of RNA 4 or 3, the wheat germ cell-free system was incubated in the presence of 120 µM sparsomycin for 5 min at 30°C. The reaction mixture contained 30 µl wheat germ extract (S_{23}), 20 mM HEPES pH 7.8, 98 mM K acetate, 4.2 mM Mg acetate, 2.5 mM ATP, 0.375 mM GTP, 5 mM phosphoenol pyruvate, 1.8 mM dithiothreitol, and all 20 essential amino-acids at 0.025 mM concentration. The

^{32}P-labeled RNA was then added and the mixture was incubated for 5 min at 30°C, so that the ribosome-RNA complex could form. The RNA which did not form complexes with ribosomes was then digested with T_1 RNase (30 U/ml) or with pancreatic RNase (5 µg/ml) for 10 min at 20°C. The incubation medium was then diluted with 3 volumes of TKM buffer (50 mM Tris acetate, 100 mM KCl, 4 mM Mg acetate, pH 7.4), loaded on a 10 ml cushion of 10 % sucrose in TKM buffer, and centrifuged for 5 hr at 120,000 g. The resulting ribosome pellet was resuspended in 20 mM Tris, 2 mM Na_2 EDTA, sodium-acetate to pH 7.4. The RNA was extracted from the resuspended material with phenol/chloroform (1/1) and then precipitated with ethanol. The experimental conditions generally used to recover the labeled fragments were as follows. The RNA was redissolved in 20 mM Tris, 10 mM NaCl, sodium-acetate to pH 7.4 (buffer A) and applied to a 1 x 3 cm DEAE-cellulose column equilibrated with buffer A. The column was washed first with buffer A, then the labeled RNA fragments were eluted with 20 mM Tris acetate, 2 m NaCl, 2 M urea, pH 7.4, and precipitated with ethanol. In these conditions ribosomal RNAs are not eluted from the column. After redissolution of the eluted fragments in about 0.1 ml of Tris borate buffer pH 8.3 (90 mM Tris, 2 mM Na_2EDTA, 100 mM boric acid) containing 6 M urea, the sample was loaded on a 12 % polyacrylamide slab gel (20 x 40 x 0.4 cm) containing 8 M urea in Tris-borate buffer pH 8.3. After electrophoresis at 400 volts for 18 hr, the radioactive bands were located by autoradiography, excised from the gel, crushed through a syringe and agitated with 0.35 M NaCl. After filtration through 3 MM Whatman paper disks, the RNA fragment was ethanol-precipitated with addition of 50 µg of E. coli tRNA as carrier, before analysis of the products as described below.

RESULTS

Large oligonucleotides obtained after complete T_1 RNase digestion of RNA 3 and RNA 4. Before analysis of the ribosome-protected regions it was necessary to know the sequence of some of the T_1 RNase oligonucleotides which occur in RNA 3 and 4. A T_1 RNase oligonucleotides map on a two-dimensional polyacrylamide gel (Fig. 1) revealed large oligonucleotides which we called α in RNA 4 and α'and β in RNA 3. Their nucleotide content was determined by the classical technique of Sanger[15], with results given in Table I. We used the 5'-end-labeling technique[16] to determine their sequences. Sufficient amounts of oligonucleotides α, α' and β were prepared to be isolated and

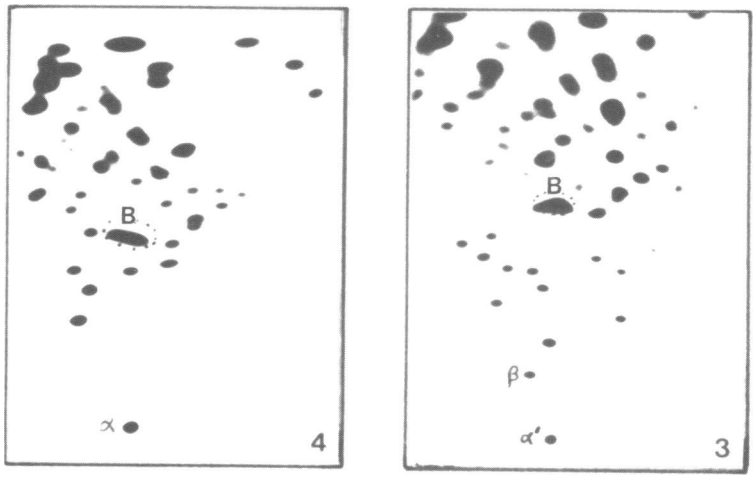

Figure 1. Two-dimensional gel electrophoresis of the T_1 RNase products of RNA 3 and RNA 4. The origin is at bottom left. B indicates the position of the bromophenol blue marker. Electrophoresis at 400 volts at pH 3.5 in the first dimension and at pH 8.3 in the second dimension, until the marker moved 15 and 23 cm respectively.

precipitated in a pure state after fractionation of a T_1 RNase digest of RNA 4 or RNA 3 on a 12 % polyacrylamide slab gel. These oligonucleotides were partially digested with pancreatic RNase (1 µg per 100,000 µg of pure oligonucleotide) for 10 min at 0°. The digestion was stopped by phenol extraction and the aqueous phase was washed with ether. The digestion products were

Table I. Pancreatic ribonuclease digestion products of T_1 oligonucleotides α from ^{32}P RNA 4, α' and β from ^{32}P RNA 3.

Pancreatic RNase products	Relative molar yield found in α	α'	β
AAAU	1	0.8	-
AAU	1.3	1	2.8
AAC	-	-	1.8
AU	3.6	3.1	1.3
AC	1	1	-
G	1	1	1
C	5.5	5.6	5.2
U	18	15.8	5.6

labeled at the 5'-end with [γ ^{32}P] ATP and phage T_4 polynucleotide kinase[16], and fractionated on a two-dimensional gel as used in Fig. 1. The different products thus obtained were excised, eluted with 0.35 M NaCl, and ethanol-precipitated with E. coli tRNA as carrier. After partial P_1 nuclease digestion they were analysed by homochromatography using a 3 % homomixture[17]. The results shown in Fig. 2 indicate the different subfragments and the final sequence of oligonucleotide α. This sequence is identical to that of the corresponding oligonucleotide found in RNA 4 from AMV strain 425[4]. The sequence of oligonucleotide α' from RNA 3 is identical to α. The sequence of oligonucleotide β has been obtained by the same procedure.

Binding of RNA 4 to wheat germ ribosomes. When RNA 4 is incubated for 5 min in the presence of an S_{23} wheat germ extract, polyribosomes form. Fig. 3a shows that about 30 % of the ^{32}P-RNA 4 molecules bound to 80 S ribosomes and polyribosomes, whereas 70 % of the labeled material remained unfixed. Addition of 120 μM sparsomycin, as an inhibitor of elongation, to the incubation mixture before addition of labeled RNA 4 caused the amount of polyribosomes to diminish (Fig. 3b). 23 % of the RNA 4 radioactivity sedimented with 80 S ribosomes. Although RNA 4 is monocistronic, a small amount of RNA remain associated with disomes, even if more sparsomycin was used. The presence of a small amount of disomes could be attributed to an incomplete inhibition of elongation, but this is unlikely since the amount of sparsomycin used was high, alternatively it could be attributed to the binding of a second ribosome at some internal nonspecific site. Attempts to suppress disome formation completely by increasing the sparsomycin concentration also reduced the formation of monosomes so much that insufficient labeled material was recovered for analysis. Moreover, the presence of disomes is an indication that nuclease activity in the wheat germ extracts was low and

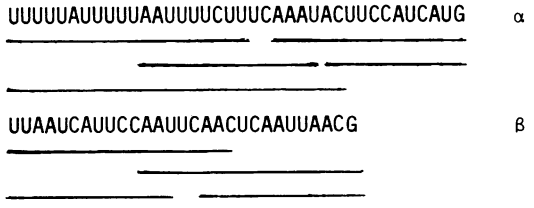

Figure 2. Sequence of oligonucleotides α and β determined from the partial pancreatic RNase digestion products and from 5'-(γ^{32}P)-ATP post-labelling. Lengths of the different overlapping subfragments are indicated by the lines underneath.

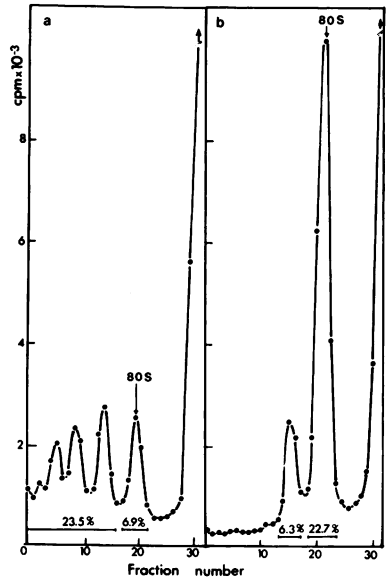

Figure 3. Sucrose density gradient radioactivity profiles of polyribosomes containing RNA 4 in the absence (a), or in the presence (b) of sparsomycin. The incubation mixture contained 2 µg of RNA 4 in 100 µl final volume. The treated sample contained 120 µM sparsomycin. Incubation conditions are described in the text. After incubation the mixture was diluted with 3 volumes of TKM buffer, layered on a 10-40 % sucrose gradient at 4° in the same buffer and centrifuged for 260 min at 25,000 rpm in a SW 27 rotor. The Cerenkov radiation of 1 ml fractions was determined in a scintillation counter. Amounts of radioactivity are indicated as percentage of the total radioactivity in the gradient.

did not significantly degrade the messenger RNA during the incubation period to yield monosomes.

Ribosome-protected fragments in RNA 4 isolated after T_1 RNase treatment. 100 µg of RNA 4 were incubated for 5 min at 30°C in 4.8 ml of wheat germ cell-free system preincubated with 120 µM sparsomycin for 10 min at 30°C. After formation of the initiation complex, the unprotected RNA was digested with T_1 RNase (30 U/ml) for 10 min at 20°C and then diluted with 3 volumes of TKM buffer. The ribosomes were sedimented through a 10 ml sucrose cushion as described in Materials and Methods. The labeled material obtained after removal of ribosomal RNA on a DEAE-cellulose column represented 1.9 % (mean value of three experiments) of the starting labeled material. Assuming a theoretical yield of 6 %, i.e. 56 nucleotides protected among the 890 nucleotides of RNA 4, this percentage of recovery indicates that, at least,

1 in 3 molecules gave a protected fragment. The two radioactive bands obtained upon separation of this material on a 12 % polyacrylamide gel are shown in Fig.4a. The material of these bands was extracted from the gel, digested with T_1 RNase and analysed by homochromatography(Fig. 4 b-c). Band A yielded four spots, band B only three. These spots were eluted with 2 M triethylamine-bicarbonate pH 8, washed with water and digested with pancreatic RNase for determination of their nucleotide content, with results given in Table IIa. Spots from band A are numbered T_1, T_2, T_3 and T_4 ; spots from band B are numbered T'_1, T'_2 and T'_3.

Spots T_1 and T'_1 have identical nucleotide compositions suggesting that these products correspond to oligonucleotide α (see table II). Spots T_2 and T'_2 contain 1 AAAAG and 1 AC, which are characteristic of an oligonucleotide found in the complete T_1 RNase digestion products of RNA 4. The sequence of this latter oligonucleotide, established using the 5'-labeled oligonucleotide, was UUCUUACAAAAG. Spots T_3 and T'_3 correspond to AG and spot T_4 to AAAG. In order to determine the arrangement of these four oligo-

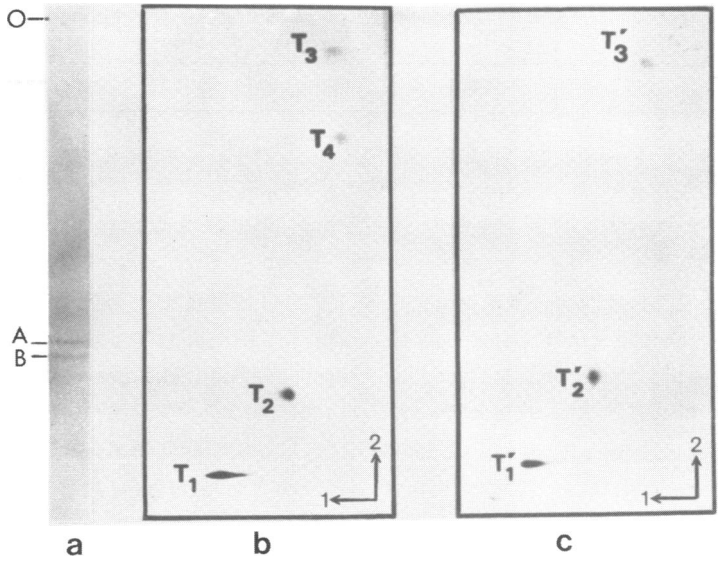

Figure 4. (a) Autoradiogram of a 12 % polyacrylamide slab gel loaded with the fragments of RNA 4 protected by ribosomes from T_1 RNase digestion. (b) and (c) Two-dimensional fractionation of bands A and B respectively, digested with T_1 RNase. The first dimension was a fractionation on cellulose acetate strips at pH 3.5 and the second dimension a homochromatography using a 3.3 % homomixture. Table II reports analysis of the umbered spots.

Table II. Pancreatic RNase digestion products of bands A and B from the ribosome-protected fragments of RNA 4 shown in Fig. 4.

Spots	Digestion products	Relative molar yields found	expected
T_1 and T'_1	AAAU	0.96	1
	AAU	1.0	1
	AU	2.7	3
	AC	0.9	1
	G	1	1
	C	4.6	5
	U	15.4	17
T_2 and T'_2	AAAAG	1	estimated
	AC	1	-
	C	1-2	-
	U	2-4	-
T_3 and T'_3	AG		
T_4	AAAG		

The digestion products from spots T_2, T_3 and T_4 were determined from their position on the electrophotogram on DE 81 paper electrophorised at pH 3.5 for 100 min at 2,000 volts.
The relative content of A and G in AAAAG and AAAG were controlled by base composition.

nucleotides in the final sequence, the material of bands A and B was digested with pancreatic RNase and analysed by fingerprinting. Two characteristic spots were found in the digestion products of band A : P_1 (G,AG)U and P_2 (AAAAG,AAAG). In the products of band B, P_1 (G,AG)U and AAAG were found.

From these data it is possible to arrange the different oligonucleotides in their proper order. Pancreatic RNase product P_2 contains no pyrimidine and is wholly contained within band A : therefore P_2 must be the 3'-terminus of band A. Similarly, AAAAG is the 3'-terminus of band B. In addition, the position of the product T_3 (AG) can be deduced from the existence of the pancreatic RNase product P_1 (G,AG)U, which is possible only if T_3 lies between T_1 and T_2. The sequence corresponding to the ribosome-protected region of RNA 4 obtained in band A is thus T_1-T_3-T_2-T_4, band B contained the

same subfragments except T_4. The corresponding sequences are shown in Fig. 5. Translation of this nucleotide sequence would yield the N-terminus of the viral coat protein. The 5'-end of fragment T_1 is the cap structure identified as $m^7G^{5'}ppp^{5'}Gp$ for RNA 4[18].

Ribosome-protected fragments in RNA 4 isolated after pancreatic RNase treatment. Next, 50 µg of ^{32}P-RNA 4 were incubated in 2.4 ml of wheat germ extract, in the same conditions as above except that pancreatic RNase, at 5 µg/ml for 10 min at 20°C, was used instead of T_1 RNase. After removal of the ribosomal RNA and electrophoresis on a 12 % polyacrylamide slab gel, several bands were revealed (Fig. 6a). Each of these bands was eluted from the gel, digested with T_1 RNase, and analysed by homochromatography. Products of bands B and D contained enough radioactivity for further pancreatic RNase analysis of the spots from the homochromatograms (Fig. 6 b-c) and for characterization of the oligonucleotides by separation on Whatman DE 81 paper (see table III). For the other bands the relative molar yields of the pancreatic RNase digestion products were estimated visually on the autoradiograms. Visual examination of the T_1 RNase digestion products from band A indicated that band A is the same as band B with the addition of one C and two or three Us. Band C did not differ noticeably from band D. In bands E and F, the products corresponding to spots 6, 7 and 8 (Fig. 6) were still present, but spot 5 was absent and only AUG remained from oligonucleotide α, indicating a lower ribosome protection on the 5'-side of these fragments. The various fragments obtained after digestion of the ribosome-protected regions with pancreatic RNase are shown in Fig. 7. The cap structure and the 5'-part of oligonucleotide α were not protected from pancreatic RNase by the

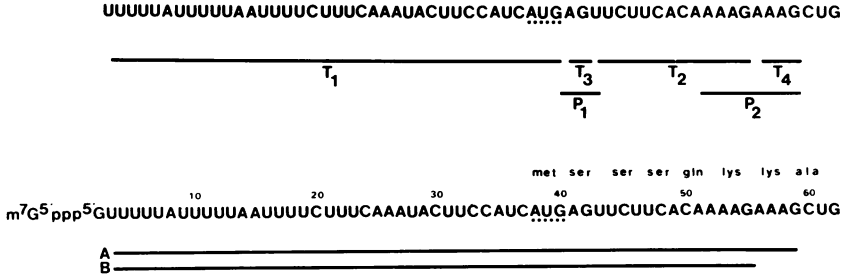

Figure 5. a - Oligonucleotide arrangement in band A as deduced from the information in Fig. 4 and from the pancreatic RNase digestion products (see text).
b - Sequences corresponding to bands A and B in RNA 4.

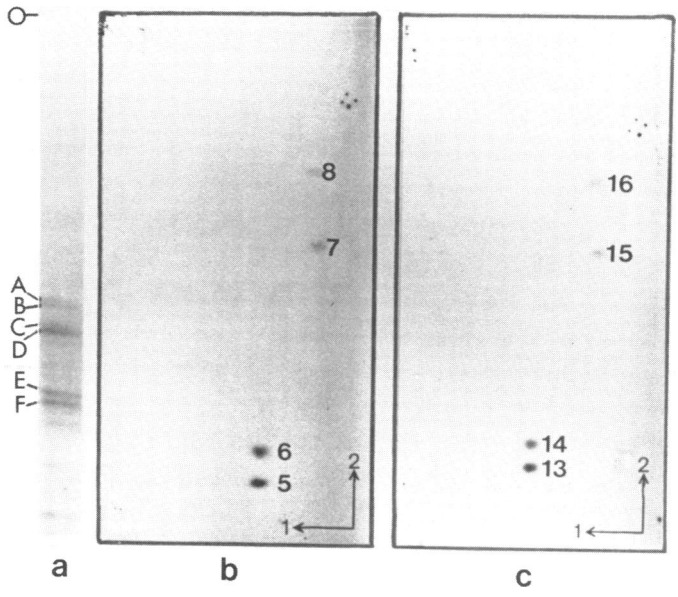

Figure 6. Autoradiogram of the fractionation on a 12 % polyacrylamide gel of RNA 4 ribosome-protected fragments after pancreatic RNase digestion (a). (b) and (c) T_1 RNase digestion products of bands B and D obtained as in Fig. 4 b-c. The numbers refer to the spots analysed in table III.

80 S ribosomes. Furthermore it appears that the protection at the 3'-terminus stopped at nucleotide 59 when T_1 RNase acted and at nucleotide 60 when pancreatic RNase acted.

When the experiments were performed in the conditions needed for 40 S ribosome binding[19], and followed by T_1 RNase treatment, the cap was protected. The presence of the cap in the 40 S ribosome-protected fragments was established after digestion with T_1, T_2 and pancreatic RNases and electrophoretic analysis on Whatman DE81 paper as indicated in reference 18.

Binding of RNA 3 to wheat germ ribosomes. The formation of the ribosome-RNA 3 complex was performed under the same conditions as described for RNA 4. The analysis of the reaction products by sedimentation through a sucrose gradient is shown in Fig. 8, together with the sparsomycin-untreated control. In contrast with the results obtained with RNA 4 the radioactivity profile indicates that RNA 3 is able to bind with two ribosomes, as shown by the presence of a disome peak as large as the 80 S ribosome peak. The radioactivity bound with disomes and monosomes represents 20 % of the input

Table III. Molarity of the pancreatic RNase digestion products in bands B and D from RNA 4 ribosome-protected fragments.

Band	Spot	Digestion products	Relative molar yields	Comments
B	5	AAAU AU AC C U G	1 2 1 4 3 1	Fragment of oligonucleotide T_1 (α) UCAAAUACUUCCAUCAUG
	6	AAAAG AC C U	1 1 2 4	Corresponds to oligonucleotide T_2 or T'_2
	7	AAAG		Corresponds to T_4 or T'_4
	8	AG		Corresponds to T_3
D	13	AAAAG AC C U	1 1 2 4	Corresponds to T_2 or T'_2
	14	AU AC C U G	2 1 2 2 1	Corresponds to a part of oligonucleotide T_1 (α) ACUUCCAUCAUG
	15	AAAG		Corresponds to T_4
	16	AG		Corresponds to T_3

material. Aggregation products, heavier than the polysomes in the untreated sample, were also observed ; they were always obtained in various sparsomycin concentration conditions, but their significance is unknown.

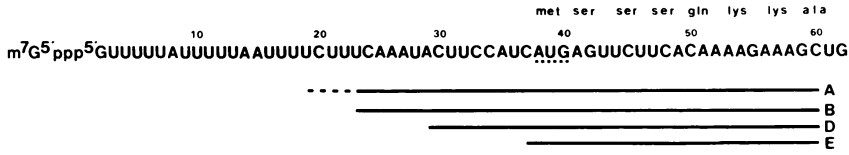

Figure 7. Sequences of the various fragments protected by ribosomes from pancreatic RNase digestion in RNA 4.

Ribosome-protected fragments in RNA 3 isolated after T_1 RNase treatment. The conditions used for the ribosome-RNA 3 complex formation and for the isolation of the ribosome-protected fragments of RNA 3 after treatment with T_1 RNase were the same as reported above for RNA 4. The recovered ribosome-protected material represents 0.8 % of the input material. If we assume that two binding site exist, each of about 50 nucleotides, the expected protection in the 2000 nucleotides-long RNA 3 should be 5 %.

The ribosome-protected fragments were analysed on polyacrylamide slab gels. Several bands were isolated and digested to completion with T_1 RNase before fractionation by homochromatography. Fig. 9a indicates the positions

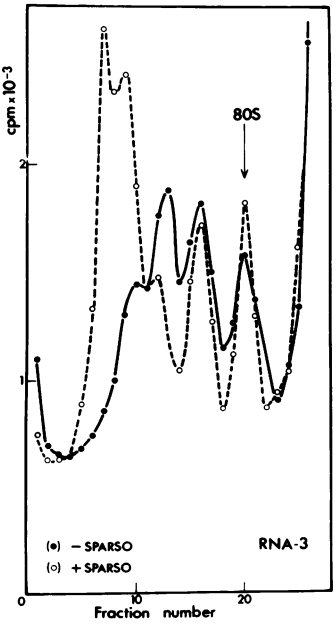

Figure 8. Sucrose density gradient radioactivity profiles of ribosome-RNA 3 complex in the presence (o) and in the absence of sparsomycin (•). Experimental conditions were as described in Fig. 3.

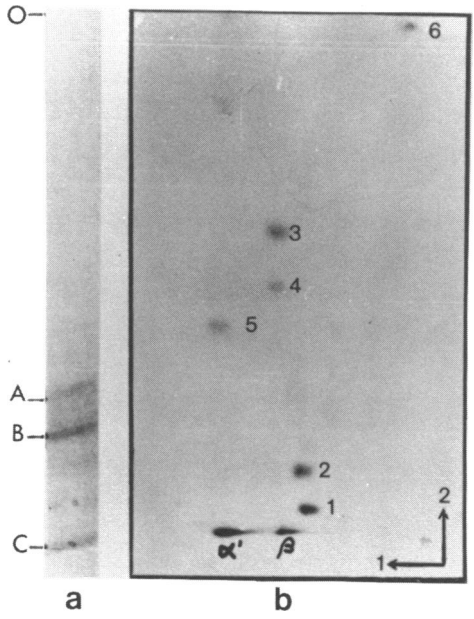

Figure 9.
a - Autoradiogram of a 12 % polyacrylamide slab gel loaded with the ribosome-protected fragments obtained after T_1 RNase digestion.
b - Homochromatographic fractionation of band A digested with T_1 RNase.

of three bands from the T_1 RNase fragments. The major band, band B, was analysed by homochromatography after complementary T_1 RNase digestion, and compared with the T_1 digestion products of band A. The oligonucleotides named β and 2, 3, 4, 5 were also found in band B, Fig. 9b. All these oligonucleotides were assigned to the 5'-end of RNA 3 since we have sequenced the first 100 nucleotides of this region by the method of Donis-Keller et al[20]. after enzymatic removal of the cap[21]. The first AUG is at position 98 from the cap, with 12 T_1 RNase oligonucleotides between the cap and this first AUG. The complete ribosome-protected region at the 5'-end will be reported in a subsequent paper.

We were interested in the analysis of band A because the homochromatogram of the T_1 RNase digestion products of this band, shown in Fig. 9b, indicates the presence of oligonucleotide α' among these products. The oligonucleotide α' has been identified after analysis of the pancreatic RNase digestion products as reported above for α in RNA 4. Oligonucleotide 6 is AG. Oligonucleotide 1, of sequence AAUACAAAAACAAAUG, has not yet been identified in the 5' end sequence of RNA 3. The presence of this great number of T_1 RNase subfragments indicate that band A was not pure and probably contained two fragments of the same length. However, none of the spots isolated from the different bands obtained from the ribosome-protected fragments

contained an oligonucleotide like oligonucleotide T_2 (Fig. 5a) characteristic of the ribosome-protected sequence in RNA 4. It appears that in the case of RNA 3 only oligonucleotides α' and AG are among the ribosome-protected fragments corresponding to a part of the ribosome binding site identified in RNA 4. This suggests that the 80 S ribosome binds differently in the 5'-end region of the coat protein cistron than in RNA 4.

The intensity of band A in Fig. 9a indicates a lesser amount of material than in band B. If we assume that this ratio is about 1 to 3, a rough estimation, and take into account the 0.8 % of protection obtained with RNA 3, we arrive at a figure of about 1 in 10 RNA molecules involved in ribosome binding at the major 5'-end site and 1 in 30 molecules at the internal site.

DISCUSSION

The various 80 S ribosome-protected sequences found after digestion with either T_1 or pancreatic RNase lead to the conclusion that the region of AMV RNA 4 involved in the initiation complex with an 80 S ribosome extends from nucleotide 21±2 on the 5'-side of the initiation codon AUG to nucleotide 59 or 60 on the 3'-side. Thus, 40±2 nucleotides are involved in this 80 S ribosome complex. The extent of this protection resembles that found in the initiation complex of 80 S wheat germ ribosomes with reovirus mRNAs[22], or with vesicular stomatitis virus mRNAs in reticulocyte systems[23]. The cap protection in the 80 S ribosome-mRNA complexes occurs only if the distance from the initiator AUG is short, as in BMV RNA 4[8] and some vesicular stomatitis virus mRNAs[23]. For AMV RNA 4, the distance between the cap and the first AUG is long, and, as for reovirus mRNAs[22], the cap structure is protected in AMV RNA 4 if 40 S ribosomes are used for ribosome-mRNA complex formation. The lack of cap protection by the 80 S ribosomes might reflect a loss of initiation factors and changes in ribosome configuration after conversion of 40 S to 80 S ribosomes. The 80 S ribosome-protected region of RNA 4 contains a short sequence CUCC (30-34) that can base-pair with the 3'-end sequence conserved in the 18 S ribosomal RNAs of eucaryotic cells ; as suggested by Hagenbüchle et al[24], such an interaction occurs for several eucaryotic mRNAs with a long leader sequence. In translation experiments with uncapped RNA 4 we found no modifications in the pattern of translation products (unpublished results) : this suggests that the cap is not determinant for a correct translation of RNA 4 but only enhances the translation

efficiency by a factor of two.

Our previous studies of AMV RNA homologies[6] have indicated that the whole sequence of RNA 4 is present in RNA 3. In addition, Fig.1 indicates that the leader sequence for the coat protein is identical in both RNAs. The 3'-end sequences of both RNAs are also identical (to be published). Gould and Symons[10] have demonstrated that the coat protein cistron is in the 3'-part of RNA 3. The ribosome-binding profile obtained with RNA 3 after sparsomycin treatment (Fig. 8) suggests the presence of at least two ribosome-binding sites. As mentioned previously, the 5'-end sequence of RNA 3, before the first AUG, is unusually long (95 nucleotides) and contains several G residues, characteristics that could perhaps explain the aggregates observed in Fig. 8, if there were additional ribosome binding capacity in this region.

We believe that the presence of the prominent disome peak results from true binding of ribosomes at an internal site. Our RNA 3 was not detectably contaminated (less than 1 %) with RNA 4, nor with cleavage products in which a cryptic ribosome-binding site might have been activated in the way that Pelham[25] has suggested for some mRNAs. Disomes were always present during short incubations, but incubation periods longer than 30 min (which presumably allowed some RNA degradation) or the use of partially digested RNA 3 yielded no disomes.

The possibility that oligonucleotide α', formed during the experimental procedure, could have bound specifically to the ribosomes can be ruled out by the following observations. In wheat germ extracts, to which oligonucleotide α' was added in the absence of RNA 3, 30% of the α' bound, as calculated from the distribution of radioactive label after a sucrose gradient analysis. But if α' was added 1 min after RNA 3, the binding fell to 9%. And if α' was added during the T_1 RNase digestion step, only 4% bound, which is approximately equal to the background level obtained with control oligonucleotides that were not recognized by the ribosomes and yielded no well defined bands in gels. From these various controls we conclude that the weak ribosome binding site at the internal coat protein initiation site is not artefactual. However, the absence of a part of the sequence on the 3'-side of AUG (especially oligonucleotide T_2 was not found in band A,Fig.9b), from the region that was protected in RNA 4 indicates that the ribosome binds differently in this region of RNA 3. The local configuration of this region of RNA 3 may prevent a correct ribosome binding and subsequent translation of the coat protein. We are presently trying to determine the

sequence of the intercistronic region of RNA 3 in order to find support for this hypothesis.

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