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Monocistronic translation of alfalfa mosaic virus RNAs

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ABSTRACT

The four alfalfa mosaic virus RNAs (respectively 24 S, 20 S, 17 S and 12 S) have been used separately as messengers in two in vitro protein synthesizing systems : wheat germ and rabbit reticulocyte lysate. In both systems a polypeptide corresponding to the translation of the entire length of the RNA can be found for RNAs 24 S, 20 S and 12 S, but not for 17 S RNA, the translation product of which is only 35,000 daltons. The number of initiation sites has been determined for each RNA by analyzing the initiation peptides synthesized in the presence of sparsomycin and show that there is only one initiation or binding site perRNA. We thus conclude that each AMV RNA behaves as a monocistronic messenger in in vitro translating systems.

INTRODUCTION

Alfalfa mosaic virus (AMV) is a multicomponent virus whose principal components are four nucleoproteins, each of which contains its own RNA, namely 24 S, 20 S, 17 S and 12 S. Its biological properties have been reported elsewhere (for review see Jaspars [1]). The entire genetic information is contained on the three heaviest RNAs, but its expression is only possible in the presence either of the fourth RNA (12 S), or of its translation product, the AMV coat protein. These observations imply a redundancy for the coat protein cistron. Genetic experiments [2] and homologies of RNA sequence [3] have clearly demonstrated that this cistron is located on both the 17 S and the 12 S RNAs.

In vitro translation experiments [4] have shown that in mammalian cell-free systems derived from Krebs-II ascites cells and from rabbit reticulocytes, it was possible to find a polypeptide product corresponding to the whole translation of the messenger when 24 S, 20 S or 12 S RNA was added separately to the system, suggesting that these RNAs are mono-

cistronic. In contrast, the only translation product of 17 S RNA is a single polypeptide of 35,000 daltons, indicating that a substantial proportion (about 45 %) of the 17 S RNA chain is not translated. The serological properties and tryptic peptide fingerprint of the 35,000 dalton protein show it to be unrelated to AMV coat protein [5]. Consequently, it appears that 17 S RNA is dicistronic but that only one of the genes is translated.
In this paper, we give additional evidence that each AMV RNA is translated in a monocistronic fashion by showing that only one ribosome binding site is present on each RNA.
Thus, although 17 S RNA must contain two potential initiation sites for protein synthesis, only one of them, that which governs synthesis of the 35,000 dalton protein is active. The site for initiation of coat protein synthesis only functions on the subgenomic 12 S RNA species.

MATERIALS AND METHODS

The virus strain, AMV-S, was multiplied and purified according to Pinck and Hirth [6]. The different RNAs were separated by electrophoresis on polyacrylamide-agarose gels [7] which was found to result in minimal contamination of the different RNAs by one another [3]. The coat protein was purified as described by Kruseman et al. [8]

Reticulocyte lysate assay. The lysate was prepared as described previously [4]. Assays were performed in 10 µl final volume containing 4 µl of the reticulocyte lysate, 75 mM KCl, 2 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 15 µM hemin, 15 mM creatine phosphate, 40 units/ml of creatine phosphokinase, 19 unlabeled aminoacids (0.1 mM each), 0.5 µCi of ^{35}S methionine (340 Ci/mmole), and various amounts of AMV RNA as messenger. Incubation was at 27° for 1 hour.

Sodium fluoride treated reticulocyte ribosomes. For initiation studies, ribosomes were prepared after incubation of reticulocytes with sodium fluoride [9]. The cell-free system, in a final volume of 100 µl, contained : 30 mM Tris-HCl pH 7.5, 96 mM KCl, 2.4 mM magnesium acetate, 1 mM dithiothreitol, 1 mM ATP, 0.1 mM GTP, 0.6 mM CTP, 5 mM creatine phosphate, 18 units/ml creatine phosphokinase, 19 unlabeled

aminoacids (0.1 mM each), 0.15 A_{260} units of reticulocyte tRNA, 0.20 A_{280} units of KCl wash fraction from rabbit reticulocyte polysomes[10], and 40 µM sparsomycin. The nature of the messenger RNA, the labeled amino acid used and the time of incubation are described in the Results for each assay performed with this system.

Wheat germ cell-free system. The wheat germ extract was prepared according to Marcu and Dudock[11]. The cell-free system was contained in a final volume of 10 µl : 2.7 mM magnesium acetate, 20 mM HEPES, 50 mM KCl, 0.025 mM each amino acid, 2.5 mM ATP, 0.375 mM GTP, 5 mM phosphoenol-pyruvate, 0.5 mM dithiothreitol, 0.61 mM spermidine, 0.5 µCi of ^{35}S methionine and 0.4 µg of AMV RNA. Incubation was performed at 30° for 2 hours.[12]

The radioactivity incorporated into proteins was determined by the method of Mans and Novelli[13] for 3 µl aliquots. Filters were counted in 10 ml toluol-omnifluor.

Analysis of the translation products. Immunoprecipitation of coat protein was performed on 25 µl samples and cell-free products were analyzed on SDS-polyacrylamide slab-gels as described previously.[4] The gels contained a linear concentration gradient of acrylamide (8-15 %) and of urea (0-4 M).

Initiation dipeptide analysis. The cell-free system with NaF treated ribosomes was incubated 5 min at 27° in the presence of 3 µg RNA, 10 µCi ^{35}S methionine and 18 µM sparsomycin. Isolation of sparsomycin dipeptides was performed as described by Smith[14] with some modifications. After incubation, 25 A_{260} units of reticulocyte ribosomes were added as carrier. The samples were then pelleted through 1 M sucrose in 25 mM NaCl, 5 mM $MgCl_2$, 25 mM Tris-HCl pH 7.5 at 120,000 x g for 4 hours, and resuspended in 1 ml of the same buffer. Sodium acetate, pH 5.0, and EDTA were added to 50 mM each and SDS to 0.5 %. The mixture was extracted with phenol-chloroform and the aqueous phase precipitated twice with ethanol. The precipitate was collected and dissolved in 20 µl water containing 10 nmoles methionyl-alanine and 1 µg pancreatic ribonuclease. After 5 min at room temperature, 100 µl of 1 % triethylamine were added

and incubation continued for 30 min at 37°. The material was then lyophilized, washed three times with water and fingerprinted on cellulose thin layer (Macherey Nagel polygram cel 400) plates in the presence of other met-X dipeptides as markers. Electrophoresis was at 350 V for 2.5 hours with the buffer pyridine : acetic acid : water (0.5/5/95), pH 3.5. The dried plates were then subjected to ascending chromatography in pyridine : acetic acid : butanol : water (50/15/75/60) for 5 hours. The plates were sprayed with ninhydrin for marker localisation and exposed to Kodak Kodirex film for 3 weeks.

RESULTS

1) Translation products of AMV RNAs

The rabbit reticulocyte cell-free system was chosen for initial studies of AMV RNA translation because of its high efficiency and because it is devoid of RNase activity, making it particularly suitable for the translation of high molecular weight RNAs. In the absence of exogenous messenger, the lysate system synthesizes mainly globin and some other cell proteins (Fig. 1A-a). Addition of mRNA to such a lysate leads to an inhibition of the overall incorporation of amino acids, but the appearance of specific viral proteins can be detected as new radioactively labeled polypeptides by electrophoresis of the products of synthesis on polyacrylamide slab-gels (Fig. 1A b-f). We have also worked with the wheat germ cell-free system in order to compare the translation products synthesized in a mammalian cell-free system with those obtained with ribosomes derived from a plant (Fig. 1B a-f). Whichever system is used, the molecular weights of the major translation products are approximately the same for a given RNA and correspond roughly to the total translation of the messenger, in the case of 12 S, 20 S and 24 S RNAs (Table 1). It is also clear that the 17 S RNA is only partially translated to give a 35,000 dalton protein, which has been shown elsewhere to be unrelated to coat protein [5].

Similar results have been obtained previously in a cell-free system derived from Krebs-II ascites cells.[4] It is remarkable that these major products are nearly the only viral specific ones found in the crude lysate, whereas

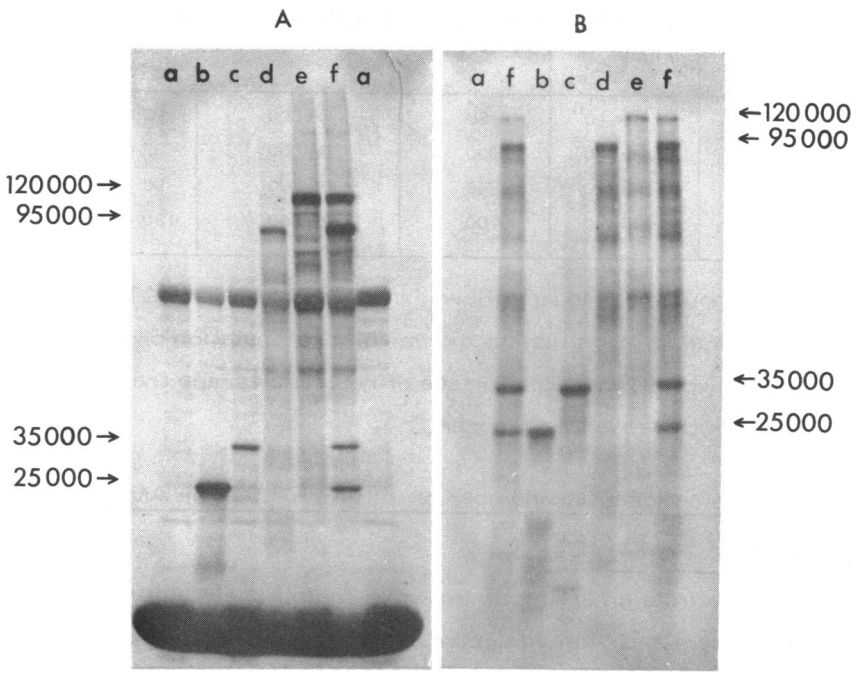

Figure 1. Comparative acrylamide slab-gel electrophoresis of AMV RNAs directed products in reticulocytes crude lysates (A) and wheat germ (B).

Each RNA was added at its optimal concentration determined for each cell-free system (results not shown). After incubation in the presence of ^{35}S labeled methionine, 10 µl reaction samples were further incubated at 37°C for 3 min. in the presence of EDTA 0.05 M and 1 µg of pancreatic RNase. Each sample was then mixed with 7 µl of a solution containing 8 % SDS, 8 % β-mercaptoethanol, 30 % glycerol and bromophenol blue. The whole mixture was heated at 100°C for 3 min. and 5 µl aliquots were layered on a linear 8-15 % polyacrylamide gradient slab-gel as described in methods (a) no added mRNA, (b) 12 S RNA, (c) 17 S RNA, (d) 20 S RNA, (e) 24 S RNA and (f) total AMV RNA.

in the other systems some lighter intermediary products can be found. The origin of the intermediary products is not understood but they might correspond to premature termination of peptide synthesis, aberrant proteolytic cleavage, or to the translation of partially degraded RNAs. This last hypothesis is favoured by the finding that the RNase-free reticulocyte system which has been preincubated with micrococcal nuclease in order to

Table 1.

AMV RNA	M.W. of the RNA	Length (nucleotides)	Coding capacity (daltons of protein)	Protein synthesized in vitro (daltons)
12 S	0.3×10^6	880	29 500	25 000
17 S	0.7×10^6	1990	66 000	35 000
20 S	1.0×10^6	2850	95 000	95 000
24 S	1.3×10^6	3700	125 000	120 000

destroy endogenous messenger, followed by treatment with EGTA to inactivate the enzyme, also gives rise to intermediate translation products (results not shown). Presumably traces of nuclease escape the inactivation and can act upon the added messenger.

2) Determination of the number of initiation sites for AMV RNAs

a. Initiation dipeptides

Sparsomycin is an antibiotic known to inhibit chain elongation [15]. A cell-free system so inhibited accumulates ribosome-bound dipeptides containing N-terminal methionine. In so far as different proteins are unlikely to have the same amino acid at the second position in their peptide chain, the number of different dipeptides found in the cell-free system inhibited with sparsomycin should be a measure of the number of separately initiated proteins. Evidently, this technique should indicate whether a given AMV RNA has more than one active initiation site and whether the N-terminal sequence of each protein is or is not the same.

The cell-free system we used was derived from rabbit reticulocytes. Prior to isolation of ribosomes, the reticulocytes were treated with NaF in order to reduce the globin initiation background as low as possible. The protein synthesizing medium was then incubated for a few minutes with sparsomycin, ^{35}S methionine plus the other 19 unlabeled amino acids and a purified AMV-RNA. In the absence of added RNA, we find two major spots which have been shown to correspond to free methionine (Fig. 2 A). Their appearance in the control may reflect the binding of met-$tRNA_F^{met}$ to ribosomes even in the absence of added mRNA [16] or may represent contamination of the ribosomes by the relatively high quantities of met-$tRNA^{met}$ formed in the system. The existence of two spots on the fingerprint when

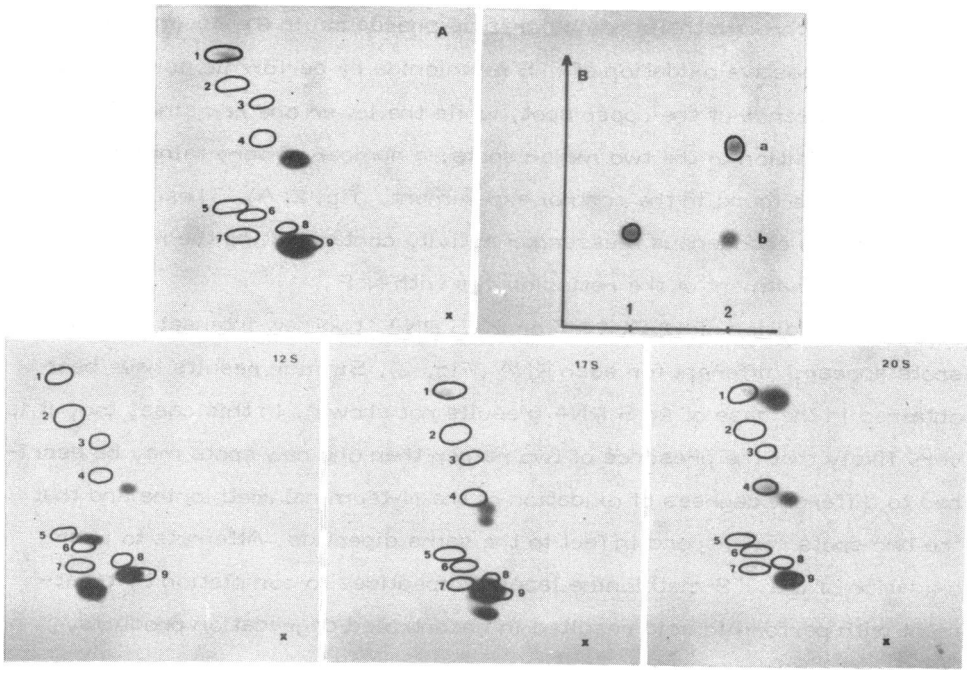

Figure 2. Autoradiogram of the fingerprint analysis of AMV-RNAs directed initiation peptides in a system primed with ribosomes derived from sodium fluoride treated reticulocytes.

The ribosomes and the reaction mixture were prepared as described in methods. 3 ug of each AMV-RNA species were added to the 100 µl reaction samples, in the presence of 10 µCi ^{35}S methionine, and 40 µM of sparsomycin. After 15 min. of incubation at 27°C, the isolation of peptides was carried out as described in methods, and unlabeled met-X dipeptides were added as markers to the mixture before fingerprint analysis. No performic oxidation was made. Circles represent ninhydrin positive dipeptides ; they have been identified as follows : 1, met-leu ; 2, met-phe ; 3, met-val ; 4, met-tyr ; 5, met-ala ; 6, met-met ; 7, met-ser ; 8, met-glu ; 9, met-asp. (A) : no mRNA added. (B) : Effect of performic oxidation on chromatographic behaviour of ^{35}S labeled and unlabeled methionine. Track 1, methionine treated with performic acid ; track 2, methionine without treatment with performic acid; spot a : methionine sulfone and methionine sulfoxide ; spot b : methionine. Circles represent the ninhydrin positive spots.

only one radioactive amino acid has been added to the system is somewhat puzzling. We have verified that the upper and lower spots correspond respectively, to the unoxidized form of methionine, and to the two comigrating oxidized forms of methionine : methionine sulfone and methionine sulfoxide ; indeed, the same two spots were obtained when free ^{35}S-labeled methionine

was carried through the chromatographic procedure in the absence of ribosomes : exhaustive oxidation of ^{35}S methionine by performic acid, leads to the disappearance of the upper spot, while the lower one remains (fig. 2, B).

In addition to the two major spots, a number of very faint products are also to be found in the control experiment. (fig. 2, A). These are, no doubt, due to endogenous messenger activity contaminating the ribosomes even after treatment of the reticulocytes with NaF.

On addition of 12 S, 17 S or 20 S RNA, two new intensely labeled spots appear, different for each RNA (Fig. 2). Similar results have been obtained in the case of 24 S RNA (results not shown). In this case, too, it is very likely that the presence of two rather than one new spots may be ascribed to different degrees of oxidation of the N-terminal methionine and that the two spots correspond in fact to the same dipeptide. Attempts to push oxidation of the ^{35}S methionine labeled dipeptides to completion by treatment with performic acid resulted in uncontroled degradation products, and consequently were abandoned.

From the above experiments, we conclude that each AMV-RNA has only one functional initiation site. Unfortunately, it was not possible to identify the second amino acid in the newly synthesized dipeptide since none of them correspond to the dipeptides used as markers.

b. Ribosome binding of ^{32}P AMV RNA

The fact that there exists very likely only one met-X dipeptide synthesized for each AMV RNA means that there is only one functional initiation site for each RNA. It may be imagined however, that ribosomes could bind to other initiation sites, but for unknown reasons, could not participate in protein synthesis in the cell-free system. In particular, it is conceivable that the 17 S RNA could bind ribosomes at both the coat protein and the 35,000 dalton protein initiation sites but that only one, the latter, is translated in vitro. In order to rule out this possibility we determined the conditions for optimal binding of ^{32}P AMV RNA to reticulocyte ribosomes after having controled that each ^{32}P RNA was intact and able to direct in vitro synthesis of the usual viral products. The labeled RNA was incubated in the system in the presence of sparsomycin and the resulting ribosome-RNA complex was analysed by sedimentation in linear sucro-

se gradients. In preliminar experiments we found that the tRNA content of the system is a limiting factor in binding. By increasing the concentration of partially purified $tRNA_F^{met}$, the percentage of ^{32}P RNA bound to ribosomes could attain 15 % of the input. It also became evident that a better yield of fixation could be obtained by decreasing the amount of the ^{32}P RNA per assay. A plateau of binding was obtained with 2 µg of ^{32}P RNA per 100 µl assay. Changing the concentration of sparsomycin in the range from 10 to 100 µM changed neither the sedimentation profile nor the percentage of RNA migrating in the complex.

Fig. 3 A shows that the incubation of total AMV-RNA for 4 min. in

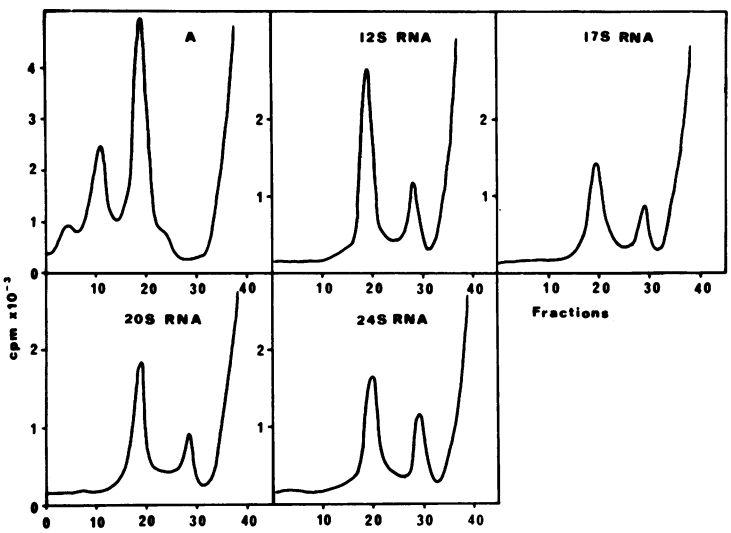

Figure 3. Binding of separated ^{32}P labeled AMV RNAs to ribosomes from NaF treated reticulocytes.
The incubation medium (100 µl) was realized as described in methods.
Each sample, except A, contained 40 µM of sparsomycin. 2 µg of total or separated ^{32}P labeled RNA (about 2.5×10^5 counts/min) were added to the reaction mixture ; after an incubation of 4 min. at 27°C, samples were diluted to 1 ml with 25 mM Tris-HCl, pH 7.5 buffer, containing 25 mM NaCl, 5 mM $MgCl_2$, and layered on 10-30 % sucrose linear gradients. Centrifugation was in SW-27 rotor for 4 1/2 hr at 25,000 rpm. Sedimentation was from right to left and 44 fractions were collected for each gradient. Radioactivity in each fraction was estimated by Cerenkov counting. Position of the monosomes peak was determined by optical density, usually at the fraction 19. (A) contained total AMV-RNA and incubation was without sparsomycin.

the absence of sparsomycin leads to the formation of polysomes and gives the exact position of sedimentation of the monosomes, disomes and trisomes in the gradient profile. The other profiles show the complexes obtained with each purified ^{32}P AMV RNA in the presence of 40 µM sparsomycin. It can be clearly seen that only monosomes were formed with each RNA species.

c. Further studies on the translation of 17 S RNA

The above experiments clearly show that under optimum conditions of translation there is only one ribosome fixation site on 17 S RNA with a cell-free protein synthesizing system of mammalian origin. It may be argued, however, that treatments designed to modify the secondary structure of the RNA or use of a synthesizing system obtained from a nonmammalian source (wheat germ) might permit the masked second binding site to be recognized. In the experiments described below we tested for coat protein synthesis by immunoprecipitation with antibodies against coat protein. The results are summarized in Table 2.
When the wheat germ extract is incubated with increasing amounts of 17 S RNA, the radioactivity found in the immunoprecipitate does not significantly increase above the control background which was a system incubated in the presence of TMV RNA. In comparison, we have found, that up to 17 % and 30 % respectively, of the incorporated counts can be immunoprecipitated by coat protein antibodies when total or 12 S RNA are translated in the system.

In view of the particular biological activity of AMV coat protein on the expression of AMV genome it is conceivable that it could have some influence on 17 S RNA translation. Hence, we have translated 17 S RNA previously incubated with coat protein under conditions where AMV coat protein is biologicaly active and can form an RNA-coat protein complex.

Preincubation of a constant amount of 17 S RNA with increasing quantities of coat protein leads to a noticeable decrease of amino acid incorporation, suggesting that the interaction between RNA and coat protein prevents further translation. Nevertheless, gel analysis of the translation products obtained in this case does not differ from the control, nor does the serological assay for coat protein synthesis.

RNA	AMV coat protein added	total cpm incorporated	cpm in immunoprecipitate	% immuno-precipitate
-	-	2115	409	-
AMV	-	61000	8835	14.4
12 S	-	53320	15820	30.0
2 µg	/	23835	1108	3.2
4 µg	/	28930	1592	4.4
8 µg	/	32040	1801	4.6
12 µg	/	30190	1158	2.6
17 S	-	21203	1205	4.1
	0.1	22195	1038	3.1
	0.5	19822	809	2.2
	1	18331	975	3.4
	5	16978	850	2.9
17 S F_8a	-	19520	980	3.2
17 S $a_2$fi	-	15260	732	2.4
17 AMV_{246}	-	21036	1092	3.6
VMT 1 µg	-	58800	2309	3.7
VMT 2 µg	-	100045	3253	2.9

Table 2. Immunoprecipitation test for translation products of 17 S or total AMV RNA synthesized in wheat germ cell-free system.

Immunoprecipitation tests were performed on 25 µl samples. To these samples were added : 5 µl of 0.3 M cold methionine, 4 µl of AMV coat protein (1 µg/µl), 15 µl of a mixture of DOC-Triton X100 (10 % each), 1 µl NaCl 2.5 M, and 25 µl of serum anti-AMV ; the medium was left at room temperature for two hours. After incubation, the reaction mixture was layered over 150 µl of 1.0 M sucrose in 10 mM sodium phosphate pH 7.2, 1.5 M NaCl, 1 % DOC and Triton, 0.1 M cold methionine in a 400 µl propylene tube, and centrifuged for 10 min at 10 000 g. The tip , frozen, containing the immunoprecipitate was cut off and evaluation of incorporated radioactivity was performed as described.[4] The percentage were calculated after substracting the blanks. Unless indicated, the quantity of added RNA to the cell-free system was 2 µg/25 µl.

We also failed to obtain coat protein translation from 17 S RNA after heating or denaturing treatments of that RNA.

Similar results have been obtained with the 17 S RNA originating from chemical mutants derived from AMV-S strain [18], for which we have demonstrated that both the 35,000 dalton protein and the coat protein have

undergone mutations, but without giving any antigenic differences from wild type coat protein in immunoprecipitation. From these results we conclude that the 17 S RNA is translated in a monocistronic fashion whichever system is used. We can add in proof that ribosome binding experiments of ^{32}P 17 S RNA in the presence of sparsomycin realized in the wheat germ cell-free system lead to the formation of monosomes, indicating that in this system too, one single binding site is recognized by the ribosomes.

DISCUSSION

The above experiments show unambiguously that each AMV RNA leads to the synthesis of a single protein. This result is obtained whichever translating system is used : mammalian or wheat germ. Consequently we believe that this behaviour may be imputed to the nature of the RNA rather than to the translating system. Contrary to the procaryotic messengers, including bacteriophage RNA, in which the genome is polycistronic and is known to be translated in a polycistronic fashion, most eucaryotic messenger RNAs have been shown to be translated in a monocistronic way. The animal picornavirus and most plant virus obey this law : the picornavirus give rise to a large polypeptide corresponding to the translation of the whole message which is soon cleaved to provide the functional products observed *in vivo*. Some plant viruses have their genetic information distributed on several RNAs, each of them being monocistronic.AMV represents still another situation : three of its genes are carried by three monocistronic messenger RNAs, and the other one (the 35,000 dalton protein) by a dicistronic messenger RNA, the other gene being the coat protein gene.

The reported results showing that the 35,000 daltons protein is the only translation product of the 17 S RNA may be related to those obtained with TMV [19] and TYMV [20] RNAs, for which it has been shown that the coat protein gene is located near the 3'end of the genome and is not translated *in vitro*. By analogy, one might reasonably suggest that the 35,000 daltons protein gene is close to the 5'end of the 17 S RNA whereas the coat protein gene lies near the 3'end. If we grant this assumption the impossi-

bility of translating coat protein from 17 S RNA can be explained by a secondary or tertiary configuration of 17 S RNA making the internal initiation site inaccessible to ribosomes. Alternatively, the coat protein gene may remain untranslated because it lacks the correct "cap" close to its initiation sequence, thus explaining why the coat protein cistron has to be converted into a subgenomic fraction (the 12 S RNA) which would be translatable only after it has been capped.

In this regard, it must be kept in mind that no replicative form corresponding to the 12 S RNA has been identified, implying that it is synthesized from one of the other RNAs, very likely from the 17 S RNA. The conversion of the 17 S RNA to the 12 S monogenic form must include the capping of the new 5'end since it has been shown that each AMV RNA possesses the m^7G structure at its 5'end [21].

The situation for AMV 17 S RNA translation is very similar to the one found by Shih and Kaesberg [22] for BMV RNA 3 : the translation product consists only of protein 3a ; even though the coat protein gene is also present on this RNA, it is not translated.

AMV coat protein is the only known viral protein. Molecular weight estimation, serological properties and tryptic peptide analysis have shown that the 12 S RNA translational product can be unambiguously identified as coat protein. Nevertheless, in this paper, we show by analysis of initiation dipeptides, that the N-terminal dipeptide arising from 12 S RNA translation was different from met-ser, which is expected from knowledge of the N-terminal coat protein sequence [17]. Similar results have been found for encephalomyocarditis virus RNA translated in a Krebs-II cell-free system [14] and for Semliki Forest virus RNA [23], where it was found that initiating dipeptide formed in a cell-free extract from mouse L. cells differed from that expected on the basis of the coat protein sequence [24]. Since there is no reason to think that the in vitro initiation site is not the physiological one, it seems likely that there exists, at least for the 12 S RNA, a lead-in sequence of amino acids which is rapidly processed in vivo, so as to give the AMV coat protein. This lead-in sequence is probably short since the electrophoretic mobility of in vitro synthesized product is not different from that of AMV coat protein.

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