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Binding of ribosomes to 5′-terminal leader sequences of eukaryotic messenger RNAs

(initiation of protein synthesis/plant viral RNAs/wheat germ/mRNA 5′ terminus)

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ABSTRACT **Tobacco mosaic virus and turnip yellow mosaic virus genome RNAs, which have long (≥69 nucleotides) 5′-terminal leader sequences preceding the AUG initiation codon, can form disome initiation complexes when incubated with a wheat germ extract and sparsomycin. In tobacco mosaic virus RNA, whereas one ribosome in such complexes presumably occupies the AUG-containing initiation site, the other is located within the RNA leader sequence. The results favor a model of eukaryotic initiation in which ribosomes first bind to the 5′ end of the mRNA independently of AUG, then advance to the AUG initiation codon, where protein synthesis ensues.**

All cellular and viral mRNAs of eukaryotic origin examined to date are functionally monocistronic. This is also true of tobacco mosaic virus (TMV) and turnip yellow mosaic virus (TYMV) RNAs, which, although structurally polygenic, initiate translation of only that cistron closest to the 5′ end of the RNA (1, 2). Sequence studies of many eukaryotic mRNAs indicate that in virtually all instances the 5′-proximal AUG functions as the initiation codon (3–5). The mechanism limiting eukaryotic initiation to 5′-proximal cistrons and to the first AUG may not involve mRNA–rRNA interactions as observed for prokaryotic systems (6), because complementarity between the initiation region of eukaryotic mRNA and the 3′ terminus of 18S rRNA is not obvious (7). Kozak and Shatkin (8, 9) have proposed a model in which the 40S ribosomal subunit interacts initially with the 5′ end of the mRNA—in a process often facilitated by the m^7G (7-methylguanosine) cap—and moves along the RNA until it encounters the first AUG codon; at this stage joining of the 60S subunit would occur. Recent experiments performed in the presence of edeine (10) are consistent with this model.

We have studied wheat germ ribosome binding to viral RNAs differing in the length of the 5′-terminal leader sequence preceding the AUG initiation codon (Fig. 1). In the absence of elongation, RNAs with a long (≥69 nucleotides) leader sequence can accommodate, in addition to the initiating ribosome, a second ribosomal particle positioned upstream from the initiation site. Our results lend strong support to the model of Kozak and Shatkin (8, 9).

MATERIALS AND METHODS

Materials. TMV and TYMV RNAs were prepared as described (17, 18). RNase T1 and A were from Sankyo (Tokyo, Japan) and Worthington, respectively. Sparsomycin was a gift from J. Douros (National Cancer Institute, Bethesda, MD). *S*-Adenosyl[*methyl*-^{3}H]methionine (62.5 Ci/mmol) and L-[^{14}C]leucine (303 Ci/mol) were from New England Nuclear (1 Ci = 3.7 × 10^{10} becquerels). Other materials were as described (17, 19).

Preparation of [^{3}H]Methyl-Labeled RNAs. TMV and TYMV RNAs methyl-labeled on their penultimate 5′-terminal nucleoside were prepared in conditions similar to those of Moss (20) except that whole vaccinia virions were the source of nucleoside 2-methyltransferase. Reaction mixtures (250 μl) contained 50 mM Tris·HCl (pH 7.5), 2 mM $MgCl_2$, 3 mM dithiothreitol, 0.05% Nonidet P-40, 1.5 μM *S*-adenosyl[*methyl*-^{3}H]methionine, 0.5 A_{260} unit of purified vaccinia virions (a kind gift of A. Dru and G. Beaud, Paris), and 40 or 60 μg of TMV or TYMV RNA, respectively. After 60 min at 37°C, viral particles were removed by centrifugation, EDTA was added to 10 mM and the RNA was extracted with phenol/sodium dodecyl sulfate, precipitated with ethanol, and purified on a Sephadex G-50 column containing 20 mM NH_4OAc (pH 5.5). The radioactive RNA eluting in the void volume was freeze-dried. TMV and TYMV [*methyl*-^{3}H]RNAs had specific activities of 5000 and 6400 cpm/μg, respectively. Reovirus [*methyl*-^{3}H]mRNA (90,000 cpm/μg) synthesized *in vitro* by reovirus cores was a kind gift of A. Shatkin (Roche Institute of Molecular Biology, Nutley, NJ).

Binding of [*methyl*-^{3}H]RNAs to Ribosomes. Binding of [*methyl*-^{3}H]RNAs to wheat germ ribosomes was studied in conditions of translation assays (17), except that 0.3 mM sparsomycin was included, all 20 amino acids were unlabeled, and the KOAc was 100 mM. Samples (75 μl) were preincubated 15 min at 30°C with sparsomycin, [*methyl*-^{3}H]RNA was then added (12 μg/ml, unless otherwise stated), and incubation was continued for 10 min at 30°C. When protection of labeled 5′ termini of RNA by ribosomes was to be determined, samples were further incubated 10 min at 22°C with RNase A (3 μg/ml) or T1 (250 units/ml). Samples were diluted 1:2 with 20 mM Tris·HCl (pH 7.6)/75 mM KCl/2.5 mM $MgCl_2$ and loaded onto 5-ml 10–30% (vol/vol) glycerol gradients in the same buffer. After 60–75 min at 47,000 rpm (Beckman SW 50.1 rotor), fractions were collected and first monitored at A_{260} or directly assayed for radioactivity in Bray's scintillant.

Polyacrylamide Gel Electrophoresis. Electrophoresis of RNase T1-resistant and ribosome-protected fragments (dissolved in 8 M urea and heated 3 min at 60°C) was performed in cylindrical 15% acrylamide/0.5% bisacrylamide gels in 50 mM Tris/50 mM boric acid/2.5 mM EDTA/8 M urea. After 15 hr at 23°C and 80 V in the same buffer without urea, gel slices (2 mm thick) were treated with NCS solubilizer (Amersham) and assayed for radioactivity in toluene-based scintillant.

Thin-Layer Chromatography of [^{3}H]Methyl-Labeled Nucleosides. Samples of [*methyl*-^{3}H]RNA were digested with *Penicillium* nuclease P1 followed by snake venom nucleotide pyrophosphatase and alkaline phosphatase as described (19).

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Abbreviations: TMV, tobacco mosaic virus; TYMV, turnip yellow mosaic virus; 40S, 40S ribosomal subunit.

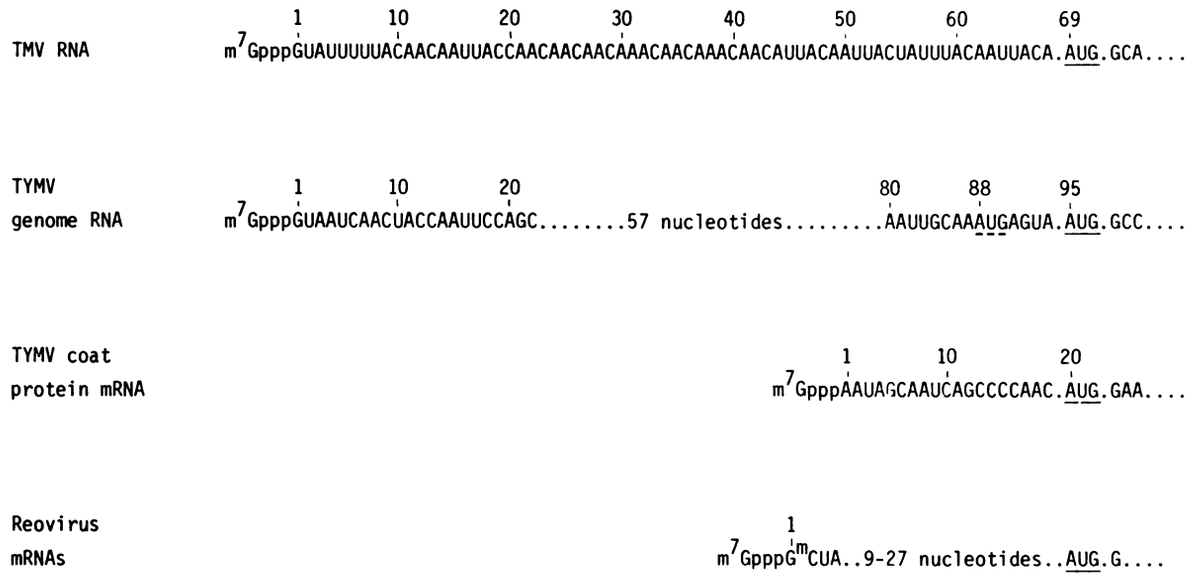

FIG. 1. 5′-Terminal sequence of TMV RNA (11, 12), TYMV genome RNA (13), TYMV coat protein mRNA (14), and reovirus mRNAs (15). Underlined are the initiation codons; the AUG selected as initiator in TYMV genome RNA is the more likely, and AUG 88–90 (---) is the less likely candidate (13). Data on reovirus mRNA are based on sequence studies of 6 out of 10 mRNA species (15); the remaining mRNAs probably also have short leader sequences (16). Numbering begins at the penultimate 5′-terminal nucleoside.

The resulting labeled 2′-*O*-methylnucleosides were separated on thin-layer cellulose plates in 1-butanol/NH_4OH/H_2O (86:5:14, vol/vol) and the distribution of radioactivity was determined (19).

RESULTS

5′-Terminally labeled [*methyl*-^{3}H]RNAs

Plant viral RNAs with the 5′-terminal structure m^7GpppN can be labeled with methyl at the 2′-O position of the penultimate nucleoside (N) by the mRNA (nucleoside-2′-)-methyltransferase associated with vaccinia virions (20, 21). We used purified vaccinia virions to methylate TMV and TYMV RNAs. To check for reaction specificity, we verified that the radioactivity in the resulting [^{3}H]methyl-labeled RNAs was associated exclusively with 5′-terminal m^7GpppNm, and that TMV RNA enzymatically decapped by treatment with potato nucleotide pyrophosphatase (17, 19) was not a substrate of the methylation reaction. Glycerol gradient analyses of the methylated RNAs indicated that incubation with vaccinia virions resulted in limited nicking of the RNAs. However, this was not considered disadvantageous because *5′-terminally* labeled TMV and TYMV RNAs were exclusively used to assay ribosome binding, a reaction occurring in the 5′ regions of these mRNAs (12–14).

Ribosome binding to TMV RNA

Incubation of TMV [*methyl*-^{3}H]RNA with a wheat germ extract and sparsomycin, an inhibitor of elongation, results in the binding of about 70% of the input RNA to a region corresponding either to an 80S complex or to a complex cosedimenting with a disome marker (Fig. 2 *A*, *B*, and *D*); the amount of disome complex was particularly pronounced with low concentrations (6 rather than 12 μg/ml) of TMV RNA.

Because TMV RNA is considered functionally monocistronic (12, 17, 22, 23), a high yield of disomes in the presence of 0.3 mM sparsomycin was unexpected. Disomes probably did not result from incomplete inhibition of elongation, because this antibiotic completely inhibited TMV RNA-directed leucine incorporation at 0.1 mM (not shown). Furthermore, binding to ribosomes of a mixture of reovirus [*methyl*-^{3}H]RNAs yielded only 80S complexes even at low (2.2 μg/ml) RNA concentration (Fig. 2*C*). Two possible mechanisms could lead to the formation of disomes consisting of TMV RNA and two ribosomes: (*i*) apart from binding one ribosome at the AUG initiation codon in position 69–71 (ref. 12), TMV RNA also binds a second ribosome at an internal–natural or artifactual–initiation site; (*ii*) TMV RNA with one ribosome at AUG 69–71 has a 5′-terminal tail of about 55 nucleotides [80S ribosomes protect about 12 nucleotides upstream of the AUG codon (15)] available for interaction with a second ribosome.

To distinguish between these two possibilities, we took advantage of the fact that the 5′-terminal leader sequence of TMV RNA is resistant to digestion by RNase T1, because the 5′-terminal m^7GpppG-U is separated from AUG 69–71 by 66 nucleotides devoid of G residues (11). Moreover when the 5′-

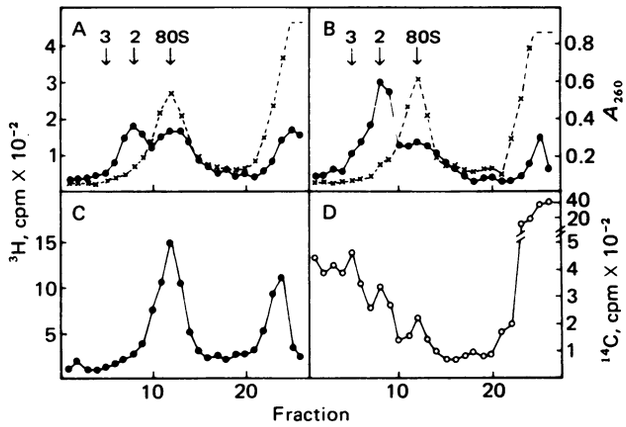

FIG. 2. Sedimentation profiles of complexes between TMV [*methyl*-^{3}H]RNA or reovirus [*methyl*-^{3}H]RNA and wheat germ ribosomes. TMV RNA concentration was 12 μg/ml (*A*) or 6 μg/ml (*B*); reovirus mRNA was 2.2 μg/ml (*C*). In *B*, binding assay was 150 μl. Monosome (80S), disome (2) and trisome (3) markers were derived by centrifugation in a parallel gradient (*D*) of [^{14}C]leucine-labeled polysomes obtained by translating rabbit globin mRNA in a wheat germ extract for 40 min at 30°C as described (17) and measuring the hot trichloroacetic acid-precipitable radioactivity in each fraction after centrifugation. ●, ^{3}H radioactivity; ○, ^{14}C radioactivity; ×, A_{260}.

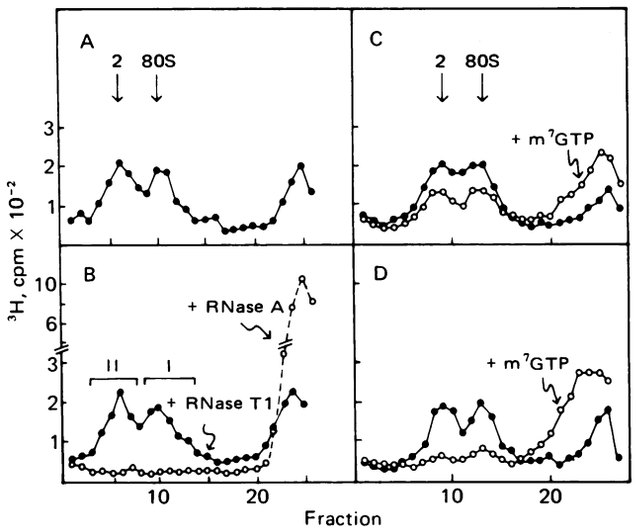

FIG. 3. Effect of nuclease treatment (*A* and *B*) and of m^{7}GTP (*C* and *D*) on TMV [*methyl*-^{3}H]RNA·ribosome complexes. (*A*) Sedimentation of TMV RNA·ribosome complexes without nuclease treatment; (*B*) sedimentation after RNase A (○) or T1 (●) treatment (brackets indicate fractions pooled for analysis of the RNase T1-resistant fragments shown in Fig. 4); (*C*) formation of TMV RNA·ribosome complexes at 50 mM K$^+$ in the absence (●) or presence (○) of 0.2 mM m^{7}GTP; (*D*) as *C* except that K$^+$ was 100 mM. Positions of markers as in Fig. 2.

penultimate G is methylated, the resulting Gm-U linkage becomes insensitive to RNase T1. Hence it should be expected that if a ribosome binds at AUG 69–71, the labeled 5′ terminus will not be removed by RNase T1. As seen in Fig. 3 *A* and *B*, the labeled 5′ end of TMV RNA was indeed protected in the 80S and disome complexes from RNase T1 digestion, whereas no protection was observed against RNase A.‡ Consequently, the nucleotide sequence separating the two ribosomes within the disome complex is RNase T1 resistant, thereby strongly suggesting that the second ribosome is bound to the 5′-terminal guanosine-free sequence of TMV RNA.

However, the possibility that the second ribosome in the disome was located to the 3′ side of the AUG initiator region still remained. Such a disome might be RNase T1 resistant if the two ribosomes were positioned close to one another; in such a situation, the length of the RNase T1-resistant fragment isolated from disomes should be longer by at least 30 nucleotides—the average length of the ribosome protected sequence (15)—than the corresponding fragment isolated from the 80S complex. We therefore characterized by polyacrylamide gel electrophoresis the length of the TMV RNA fragments protected within the 80S and disome complexes.

Jonard *et al.* (12) found two main TMV RNA fragments in the ribosome-bound material after treatment of TMV [^{32}P]-RNA·80S complexes with RNase T1, corresponding to regions 2–71 and 2–83 of the 5′-terminal sequence in TMV RNA. Two similar fragments were recovered from both the 80S and the disome complexes described here (Fig. 4 *A* and *B*). The shorter fragment (a) coelectrophoresed with the labeled 5′-terminal polynucleotide obtained by total digestion of TMV [*methyl*-^{3}H]RNA with RNase T1 (Fig. 4*C*); it extends from the m^{7}G cap to G residue 71. The second fragment (b) was about 10 nucleotides longer and probably extended from the m^{7}G to G residue 83. No longer RNA fragments specific to disomes were detected. These experiments demonstrate that the second ribosome of the disome complex is bound at the 5′-terminal guanosine-free tail of TMV RNA. This is confirmed by the finding that the isolated 5′-terminal fragment of TMV RNA extending from the m^{7}G cap to residue 71 also forms disome complexes (Fig. 4*C*, *inset*).

‡ In control experiments it was found, in agreement with other authors (16), that wheat germ 80S ribosomes conferred 28% and 16% protection to the labeled 5′ termini of reovirus [*methyl*-^{3}H]mRNAs against digestion by RNase T1 and A, respectively.

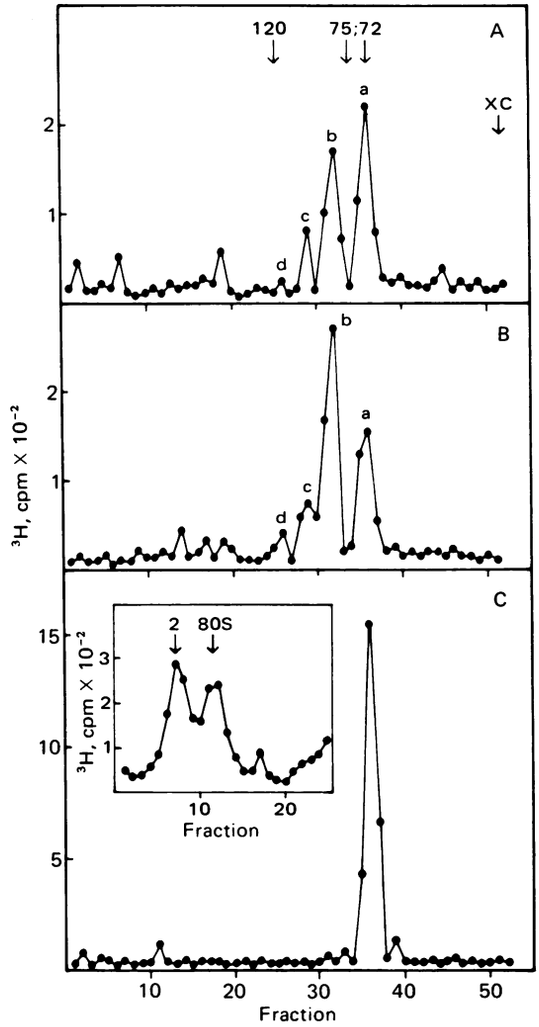

FIG. 4. Polyacrylamide gel electrophoresis of RNase T1-resistant TMV [*methyl*-^{3}H]RNA fragments recovered from 80S and disome complexes. Assays were performed in 150 μl. Gradient fractions corresponding to 80S and disome complexes (I and II of Fig. 3*B*) were pooled and precipitated with ethanol. The RNA extracted with phenol/sodium dodecyl sulfate and precipitated with ethanol was electrophoresed (see *Materials and Methods*). The 5′-terminal RNase T1-resistant fragment of TMV RNA was prepared by incubating 5 μg of TMV [*methyl*-^{3}H]RNA with 10 units of RNase T1 in 50 mM Tris·HCl (pH 7.5)/1 mM EDTA for 10 min at 22°C. The RNA was recovered by phenol extraction and ethanol precipitation and electrophoresed. The labeled fragment was eluted from the gel slices as described (16) and used (4000 cpm) for binding assays. The positions of *Escherichia coli* 5S RNA (120 nucleotides), beef liver tRNA$_i^{Met}$ (75 nucleotides, a gift of J. P. Waller, Paris), the RNase T1-resistant 5′-terminal fragment of TMV RNA (72 nucleotides), and xylene cyanol (XC) are indicated. Fragments a, b, c, and d were reproducibly recovered from 80S and disome complexes. (*A* and *B*) Fragments recovered from 80S and disome complexes, respectively; (*C*) 5′-terminal RNase T1-resistant fragment of TMV [*methyl*-^{3}H]RNA. (*Inset*) Ribosome binding to purified 5′-terminal fragment of TMV [*methyl*-^{3}H]RNA. Positions of markers as in Fig. 2.

To gain some insight into the mechanism of ribosome binding to the 5′-terminal sequence of TMV RNA, we studied the importance of the m^{7}G cap for the formation of 80S and disome

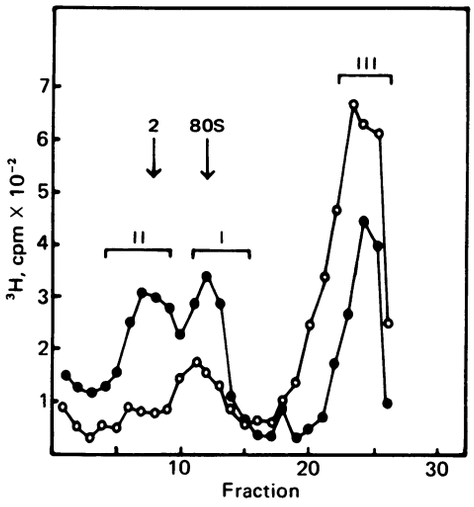

FIG. 5. Sedimentation of TYMV [*methyl*-^{3}H]RNA·ribosome complexes formed in the absence (●) or presence (○) of 0.2 mM m^{7}GTP. Brackets indicate fractions pooled for RNA analysis (Table 1). Positions of markers as in Fig. 2.

complexes. It was shown previously that cap analogs or enzymatic removal of the 5′-terminal m^{7}GMP from TMV RNA inhibit binding of the RNA to ribosomes and synthesis of large polypeptides coded by the 5′-proximal gene of TMV RNA (17, 24), and that the importance of the m^{7}G cap for TMV RNA translation increases with increasing K^+ concentration (17, 25). In the presence of 0.2 mM m^{7}GTP, formations of 80S and disome complexes with TMV RNA were equally affected (Fig. 3 *C* and *D*), the inhibition being much less pronounced at low (50 mM) than at higher (100 mM) K^+ concentration. Similar results were obtained with m^{7}GMP (0.5 mM) and m^{7}GpppGm (0.1 mM), whereas addition of GTP, GMP, or GpppGm had no effect (not shown). Furthermore, removal of the 5′-terminal m^{7}GMP by treatment of TMV [*methyl*-^{3}H]RNA with potato nucleotide pyrophosphatase (17, 19) strongly inhibited formation of both 80S and disome complexes (not shown).

Ribosome binding to TYMV RNAs

RNA extracted from TYMV consists of two major populations: the genome RNA with the 5′-terminal sequence m^{7}GpppG and the coat protein mRNA with the 5′ terminus m^{7}GpppA, separated from the AUG initiators by ≥86 and 18 nucleotides, respectively (13, 14). Low amounts of intermediate-sized RNAs also found in TYMV probably bear the same 5′-terminal sequences as genome RNA (ref. 26 and our unpublished results).

Total TYMV [*methyl*-^{3}H]RNA efficiently formed 80S and disome complexes with wheat germ ribosomes (Fig. 5). Formation of both kinds of complexes was inhibited by m^{7}GTP (Fig. 5) but not by GTP (not shown). We determined which of the two RNA populations was involved in formation of 80S and disome complexes. Analysis of the labeled material recovered from the TYMV RNA·80S complex revealed that 66% of the radioactivity corresponded to Am (2′-*O*-methyladenosine) and 34% to Gm (2′-*O*-methylguanosine) (Table 1). This ratio was reversed when TYMV RNA isolated from the disome region was analyzed: as much as 87% of the radioactivity chromatographed with the Gm marker and only 13% with Am. When corrections are made for cross-contamination of the 80S and disome complexes, it is clear that virtually only genome RNA, a species having a long leader sequence, binds two ribosomes, whereas 80S complexes are predominantly formed with coat protein mRNA. The observation that translation of coat protein mRNA in the wheat germ system is more resistant to inhibition by cap analogs than that of genome RNA (J. R. Mellema, C. W. A. Pleij, and L. Bosch, personal communication) may explain why m^{7}GTP inhibited binding of TYMV RNA to 80S complexes less strongly than to disomes (Fig. 5).

Table 1. Digestion products of TYMV [*methyl*-^{3}H]RNA associated with 80S and disome complexes

Origin of TYMV [*methyl*-^{3}H]RNA	[^{3}H]methyl nucleosides, %	
	Gm	Am
80S complexes	34	66
Disomes	87	13
Unbound	74	26

Binding of RNA to ribosomes was performed in 0.3 ml. Fractions containing RNA associated with 80S complexes and disomes, or unbound RNA, were pooled as indicated in Fig. 5 (I, II, and III, respectively). The RNA isolated as described in Fig. 4 was digested and the resulting [^{3}H]methyl-labeled nucleosides were analyzed with Gm and Am markers. Input RNA was 63% Gm and 37% Am.

DISCUSSION

The results presented here indicate that, with wheat germ ribosomes, mRNAs with a long leader sequence (≥69 nucleotides, such as genome TMV and TYMV RNAs) can form disomes in the presence of sparsomycin, whereas those with short leader sequences (≤32 nucleotides, such as reovirus mRNAs and TYMV coat protein mRNA) cannot. In TMV RNA, the second ribosome of the disome complex is bound in the 5′-terminal leader sequence, upstream of the 80S particle presumably occupying the AUG-containing initiation site. Furthermore, the isolated 5′-terminal fragment of TMV RNA extending from the m^{7}G cap to G residue 71 can also accommodate two ribosomes in the presence (Fig. 4*C*) and in the absence (unpublished results) of sparsomycin. Disomes are also formed between TMV or TYMV [*methyl*-^{3}H]RNAs and rabbit reticulocyte ribosomes (unpublished results).

The exact nature of the additional particle present in the disomes is not obvious as yet. This particle probably corresponds to an 80S ribosome [rather than a 40S particle, which might be expected because the leader sequence contains no AUG required for 60S joining (27, 28)] because the TMV RNA disome complex always cosediments with dimer markers analyzed in parallel (Fig. 2) or in the same gradients. However, the possibility that the disome corresponds to an 80S + 40S structure or to a complex composed of one 80S and two 40S subunits cannot be excluded. Of these two possibilities the latter seems less likely because it is known that, at least with reovirus mRNAs, one wheat germ 40S subunit protects about 60 nucleotides (10). Interestingly, Hunter *et al.* (22) have also observed binding of two 80S ribosomes to TMV RNA in the presence of sparsomycin; although different interpretations were given for this observation (22, 23), such disomes may be similar to the ones described here.

Several groups using other mRNAs (21, 25, 29) have observed various levels of disome formation in the presence of elongation inhibitors. In some cases one of the ribosomes of the disome complex was possibly bound to the long leader sequence of the mRNA. Brome mosaic virus RNA 4 with a leader sequence of 9 nucleotides (30) forms only 80S complexes in the presence of sparsomycin, whereas RNAs 1 + 2 and 3 lead to 80S complexes and disomes (W. Zagorski, D. S. Shih, and P. Kaesberg, personal communication), suggesting that the leader sequence in these RNAs is rather long.

Although the detailed mechanism of ribosome binding to the leader sequence is unclear, disome formation may reflect the physiological events occurring at initiation of translation (31,

32). The steps leading to disome formation with TMV RNA could be as follows. The first ribosome having reached AUG 69–71 remains there because elongation is hindered; the free 5′-terminal RNA leader sequence of about 55 nucleotides is sufficiently long to allow entry of a new complex of a 40S ribosomal subunit (40S) with Met-tRNA. Because this 40S cannot reach the AUG codon, it might choose within the leader sequence a region containing another codon to which Met-tRNA could base-pair; at this point, 60S subunit binding would complete the formation of a second 80S ribosome. The 40S·Met-tRNA complex is thought to be the active intermediate that first interacts with mRNA (31–33) in a process facilitated by the m^7G cap (2, 34). Although we have no evidence for the existence of transient intermediates during disome assembly, the requirement of a cap for efficient disome formation suggests that binding of the second ribosome follows the sequence of events involving cap-dependent 40S binding to the RNA.

Because the leader sequence in TMV RNA contains no AUG codon, the location of the ribosome within this sequence poses an interesting problem. If this ribosome is located in a specific site, a region containing a codon related to AUG could be a likely candidate. There are several AUU codons that could interact with the Met-tRNA anticodon when entry of a 40S·Met-tRNA complex to the true initiation site is prevented. It is postulated and has been documented in certain instances that the first two codon nucleotides may suffice for codon–anticodon interaction (35). Interestingly, Hunter *et al.* (22) observed that the two dipeptides synthesized in conditions of disome formation with TMV RNA correspond to Met-Ala and Met-Thr. Whereas the former arises most likely from true initiation at AUG 69–71, the latter could result from the reading of the sequence A-U-U-A-C-C 15–20. For steric reasons and because of cap protection studies (Fig. 3*B*), AUU 15–17 would be the most likely candidate for such a codon–anticodon interaction. Indeed, wheat germ ribosomes can form 80S complexes with capped poly(A,U): Both *et al.* (36) reported that among a variety of synthetic polymers tested, only those of the $(A_2,U_2,G)_n$ and $(A,U)_n$ type led to 80S complex formation. Moreover, with $(A,U)_n$ polymers, disomes were also observed; they may have been formed by a mechanism similar to the one envisaged above.

Although the postulated assembly of a second 80S ribosome within the disome presumably results from impairment of 40S movement to the initiation site, the attractive possibility remains that joining of the 60S particle may occur physiologically before the 40S·Met-tRNA complex reaches the AUG codon. Irrespective of the nature of the additional particle within the disome and of the mechanism of its assembly, the structures described here lend strong support to the "scanning model" of eukaryotic initiation outlined by Kozak and Shatkin (8–10). Moreover, our results also imply that initial binding of ribosomes to the 5′ end of mRNAs—at least to those bearing a long leader sequence—is an AUG-independent process; the entry of a second ribosome to form a disome would take place when the AUG-containing sequence is already occupied by an 80S particle.

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