RIBOSOME BINDING OF CAPPED SATELLITE TOBACCO NECROSIS VIRUS RNA

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1. Introduction

Most eucaryotic cellular and viral messenger RNAs (mRNAs) contain a unique 5′-terminal structure $m^7G(5')ppp(5')\cdots$. This 'cap' serves to protect the mRNA against inactivation by exonucleases and participates in mRNA-dependent initiation of translation [1]. Studies using reovirus and vesicular stomatitis virus mRNAs have demonstrated, for these mRNAs, a complete dependence on the presence of a 5′ 'cap' for in vitro translation in a wheat germ cell free extract and for interaction with ribosomes to form an mRNA–ribosome initiation complex [2,3]. In addition, chemical removal of the $m^7G(5')ppp$ terminus from a number of 'capped' mRNAs by periodate oxidation and aniline mediated β-elimination resulted in a strong curtailing of messenger function [4]. In contrast with these data, however, is the evidence that the RNAs of several viruses, e.g., poliovirus, encephelomyocarditis virus and satellite tobacco necrosis virus (STNV) all lacking the 5′ cap, [5–9] are fully functional in protein synthesis [6,10–13]. A possible explanation of these results is that there are two intrinsically different types of mRNA, one type utilizing a 5′ 'cap' to facilitate its attachment to ribosomes and another type that utilizes solely an internal initiating site. To examine this question we have used a crude extract from vaccinia virus which has been demonstrated to contain 'capping' activity [14], to synthesize in vitro a 5′-terminal cap on STNV RNA. This has allowed a direct comparison of the in vitro initiation activity of capped and noncapped STNV RNA. In addition we have compared the relative in vitro rates of initiation of the capped RNA from tobacco mosaic virus (TMV) with the capped and noncapped STNV RNAs.

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2. Materials and methods

2.1. *Preparation of ^{14}C internally-labeled STNV RNA*

STNV RNA (200 μg) [15], was uniformly labeled by incubating for 45 min at 37°C with 4 μl $(^{14}CH_3)_2SO_4$ [6.25 mCi/ml; spec. act. 13.3 μCi/mol] in 0.1 ml 0.1 M EDTA, pH 7. The RNA was freed of unreacted $(^{14}CH_3)_2SO_4$ on Sephadex G 25 (in 0.2 M KCl, 20 mM Tris–chloride, pH 7.6) and precipitated with 2.5 vol. ethanol. The precipitate was washed with 70% ethanol, 30 mM potassium acetate, dried under N_2 and dissolved in water. The RNA was recovered intact (see fig.1A) in 80–90% yield at a specific radioactivity of 1600–2200 cpm/μg.

2.2. *Preparation of radioactive capped STNV RNA, guanylated STNV RNA and methylated TMV RNA*

Unlabeled STNV RNA, TMV RNA, or ^{14}C internally-labeled STNV RNA were incubated for 20 min at 37°C in a 1 ml reaction mixture containing 50 mM Tris chloride pH 7.6, 2 mM GTP, 1 mM dithiothreitol, 2 mM $MgCl_2$, 1 μCi *S*-adenosyl [methyl-^{3}H]methionine (8.9 Ci/mmol) and 50 μl vaccinia enzyme extract [14]. Wheat tRNA [16], 50 μg, were added and the reaction mixture was extracted twice with EDTA-washed phenol and precipitated with 2.5 vol. ethanol. The STNV RNA preparations were then fractionated by centrifuging through a 5–21% sucrose gradient in 100

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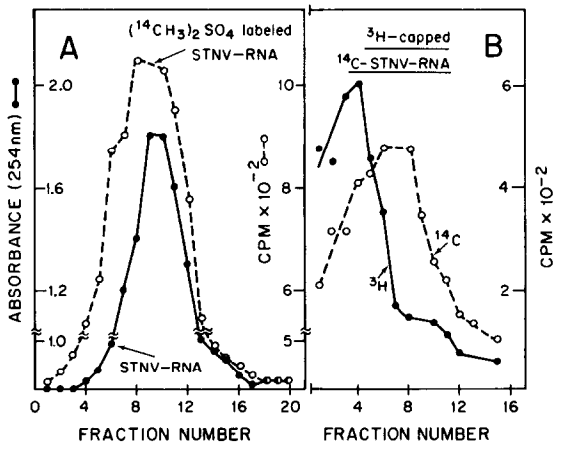

Fig.1A. STNV [^{14}C]RNA (10 500 cpm) 6 μg, labeled with $(^{14}CH_3)_2SO_4$ were mixed with 50 μg untreated STNV RNA and centrifuged through a 5–21% sucrose gradient in a Spinco SW50.1 rotor for 16 h at 35 000 rev/min. The gradient was monitored A_{254} and fractions were collected and analyzed for radioactivity. Fig.1B. STNV [^{14}C]RNA 8 μg, enzymatically capped with *S*-adenosyl[methyl-^{3}H]methionine (38 900 cpm ^{3}H and 11 400 cpm ^{14}C) were fractionated as in fig.1A.

mM NaCl, 10 mM Tris acetate, pH 7.6, 2 mM disodium EDTA for 16 h at 35 000 rev/min in a Spinco SW50.1 rotor. Gradients were collected as in fig.1B and the tubes containing ^{3}H radioactivity were pooled and precipitated with 2.5 vol. ethanol. These RNAs were used in all experiments except for those of table 1 where unfractionated capped RNA was used. The extent of capping of STNV RNA and of methylation of TMV RNA was determined by filtering aliquots of the initial reaction mixture on DEAE-filter discs and removing the nonadsorbed radioactivity by washing [14]. With STNV RNA at 30 μg/ml, the extent of capping was 70–80%, while at 50 μg/ml, 40–55% capping was obtained. With TMV RNA at 50 μg/ml, a lower level of enzyme extract (20 μl/ml) was added and 80% methylation was obtained. The theoretical level for 100% capping of STNV RNA (mol. wt 400 000) was determined on the basis of 2 mol. methyl/mol/RNA (11 200 cpm/pmol) and that for methylation of TMV RNA (mol wt 2×10^6) on the basis of 1 methyl/RNA (5600 cpm/pmol). For preparation of guanylated STNV RNA, *S*-adenosylmethionine was omitted, 4 units of yeast pyrophosphatase (Sigma) were added, and either [^{3}H]GTP (2 mCi at 3×10^{-7} M) with unlabeled STNV RNA or unlabeled GTP (2×10^{-6} M) with U-^{14}C-labeled STNV RNA, were added in the incubation. The extent of guanylation in the preparation with [^{3}H]GTP could not be determined directly on a DEAE-filter since a control incubation with RNA omitted gave very high ^{3}H retention. Instead, the guanylation incubation mixture was put through a column of Sephadex G 25 and the excluded volume was phenol extracted, fractionated on a gradient just as for the capped STNV RNA, and precipitated with ethanol. At this stage, one aliquot of the solution was counted in the usual manner on a DEAE-filter and gave total 132 200 cpm. A second aliquot treated similarly after incubation with P1 nuclease gave a total of 25 600 cpm. The amount of recovered STNV RNA was assumed to be 30 pmol (based on the average yield of a number of similar isolations of capped STNV RNA) giving a spec. act. 3560 cpm/pmol (i.e., 132 200–25 600/30) for the guanylated STNV-RNA. Direct counting of the [^{3}H]GTP used in the incubation mixture gave 6.7×10^3 cpm/pmol. The product obtained was therefore 53% guanylated (3560/6700).

2.3. *Ribosome binding assay*

The incubation mixture contained 20 mM KCl, 70 mM potassium acetate, 2.3 mM magnesium acetate, 3 mM dithiothreitol, 80 μM spermine, 30 μM L-methionine, 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, 40 μg/ml creatine phosphate kinase, 20 mM Hepes buffer, pH 7.6, 200 μM sparsomycin, 15 μl or 40 μl S23 [12], and radioactive mRNA in 100 μl. After the appropriate incubation, 150 μl 100 mM KCl, 20 mM Tris acetate, pH 7.6, 5 mM $MgAc_2$ were added and the solution was centrifuged through a 10–30% glycerol gradient in 100 mM KCl, 20 mM Tris acetate, pH 7.6, 5 mM Mg acetate in a Spinco SW50.1 rotor for 75 min at 49 000 rev/min. The gradient was monitored $A_{254\,nm}$ and the various fractions were filtered through cellulose acetate filters. The filters were washed with the gradient buffer and counted. The data presented are the sum of the radioactivity bound in the peak tubes corresponding to the 80 S ribosomes. With TMV[^{3}H]RNA (fig. 2B), radioactivity was found in tubes beyond the 80 S ribosome peak at longer times of incubation. Since this RNA may be dicistronic [17], this radioactivity was included in the data for 80 S ribosome binding. The input levels of mRNA in the

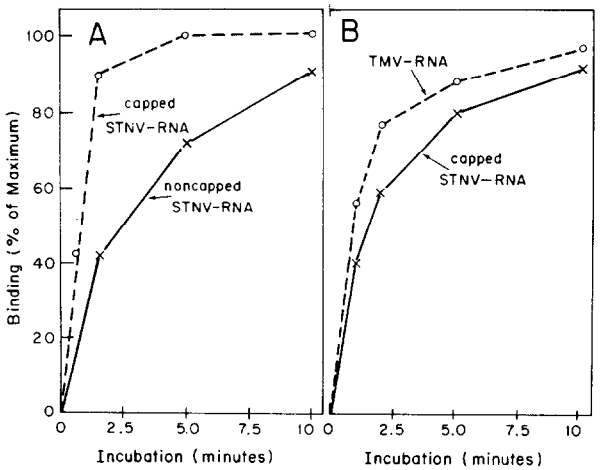

Fig.2A. The ribosome binding assay was carried out with 40 μl S23 with reaction mixtures transferred to ice at the times indicated. The capped STNV RNA preparation was a ^{14}C-labeled STNV RNA that was enzymatically capped in the usual manner with *S*-adenosyl [methyl-^{3}H]methionine to an extent of 30%. The uncapped STNV RNA was carried through a procedure identical to that of the capped STNV RNA except that *S*-adenosyl methionine was omitted (see text). The data for the capped STNV RNA are the ^{3}H radioactivity bound to 80 S ribosomes while that for the noncapped STNV RNA are the ^{14}C radioactivity bound. Since the capped STNV RNA also contained considerable noncapped STNV RNA, the binding of its ^{14}C radioactivity (data not shown) fell at points between the two curves. Fig.2B. The standard ribosome binding assay was carried out with 15 μl S23 and 0.5 pmol either of 56% capped STNV or 72% methylated TMV RNA.

different experiments refer to the particular labeled moiety rather than amounts of intact mRNA. This is explained more fully in the text. Much of the data is presented as percent maximum binding, the latter figure being routinely obtained from an incubation for 20 min at 25°C.

3. Result

3.1. *Ribosome binding of capped and noncapped STNV RNA*

Incubation of STNV RNA with *S*-adenosyl [methyl-^{3}H]methionine, GTP and crude extract from vaccinia virus results in the capping of the RNA [14]. Table 1 presents data showing that a substantial fraction of the capped STNV RNA can be bound to 80 S

Table 1
Binding of capped and uncapped STNV RNAs

	S23 (μl)	80 S radioactivity (% input)
^{3}H-Capped STNV RNA	15	20
3126 cpm	40	41
Noncapped-STNV [^{14}C]RNA	15	4
2100 cpm	40	16

Unfractionated capped STNV RNA (approx. 1 μg total STNV RNA – see Materials and methods and STNV [^{14}C]RNA (approx. 1 μg – not incubated with the vaccinia extract) were incubated in the standard ribosome binding assay for 10 min at 25°C.

ribosomes. With 40 μl wheat germ extract (S23), 16% of the noncapped STNV RNA and 41% of the capped STNV RNA are associated with an 80 S ribosome complex. Analysis of the capped product on a linear sucrose gradient (fig.1B) indicated that the RNA had undergone considerable cleavage. Since the radioactive methyl label is present only in the 5′-terminal nucleotide [14], and since 5′ fragments bind efficiently to ribosomes (table 1, ref. [18]) we used this preparation to study the effect of capping on the binding of the RNA to ribosomes. For quantitative comparison, the noncapped STNV RNA was incubated with the vaccinia enzyme exactly as for the capped RNA except that GTP and *S*-adenosyl methionine were omitted and an identical fraction corresponding to the ^{3}H peak (see fig.1B) was taken from both preparations. The data were determined as the relative rates of binding by ascertaining the binding at early time points in terms of the percent maximal binding. A kinetic analysis is shown in fig.2A. The binding of capped STNV RNA is 90% complete in 2 min whereas the reaction with the noncapped RNA is only 40% complete under the same conditions. Calculation of the initial rates from a number of such experiments resulted in the conclusion that capped STNV RNA binds at a rate 2.1–2.5-times that of the noncapped STNV RNA. Because of the low specific activity of the ^{14}C internally-labeled STNV RNA these experiments were performed using a saturating level of S23, i.e., 40 μl. In more limited experiments using a low level of S23, i.e., 15 μl, the relative rate of binding was also two times greater with the capped STNV RNA (see table 3).

3.2. *Comparison of the binding of capped STNV RNA and TMV RNA*

In addition to adding the $m^7Gppp \cdots$ cap at the 5′ end of STNV RNA, the vaccinia extract also methylates the ribose attached to the penultimate base resulting in two methyl groups introduced for each modified STNV RNA [14]. With an RNA that is already capped such as TMV RNA [19,20], methylation still occurs at the penultimate position [14]. We therefore prepared capped (and methylated) STNV RNA and methylated TMV RNA, both methyl-labeled only at the 5′-terminus, and compared the time course of their binding to ribosomes. The data (fig.2b) show that the rate of binding of TMV RNA is only 1.3 times that of capped STNV RNA. Since the experiment is done with a limiting level of S23 (15 μl), these results represent a maximal difference between the two capped mRNAs. Capping of STNV RNA apparently brings its potential for ribosome binding to almost the same level as that of an RNA that is normally capped.

As an alternative approach to ascertain the contribution of the 5′ cap to the binding of STNV RNA, we studied the effect of the analog, pm^7G [10,12,21] on the formation of the 80 S complex. Table 2 shows that binding of capped STNV RNA is inhibited 64% by the analogue in the presence of 40 μl S23 and 78% with 15 μl S23. Binding of TMV RNA is inhibited to essentially the same extent as that of capped STNV RNA.

3.3. *Ribosome binding of guanylated STNV-RNA*

Since the vaccinia extract introduces both a $Gppp \cdots$ as well as two methyl groups at the 5′ end of STNV RNA [14], it was of interest to ascertain whether guanylation per se might result in an increased rate of ribosome binding. Table 3, exp. 1, presents

Tabel 2
Inhibition of the binding of capped STNV RNA and TMV RNA by pm^7G

	80 S radioactivity (cpm)		Inhibition
	Control	pm^7G (0.5 mM)	(%)
1. 40 μl S23:			
^{3}H-Capped STNV RNA 0.75 pmol – 8350 cpm	3058	1102	64
Noncapped-STNV [^{14}C]RNA 1.7 pmol – 1425 cpm	136	92	33
TMV-[^{3}H]RNA 0.5 pmol – 2000 cpm	1160	368	69
2. 15 μl S23:			
^{3}H-Capped STNV RNA 0.38 pmol – 4225 cpm	631	138	78
[^{3}H]Guanylated STNV RNA 2.1 pmol – 7462 cpm	176	137	22
TMV-[^{3}H]RNA 0.48 pmol – 1920 cpm	442	87	80

The incubations were 2 min at 25°C in exp 1 and 2 min at 20°C in exp. 2. The capped STNV RNA was 70% capped giving 1.05 pmol and 0.54 pmol total STNV RNA in the two experiments. The [^{3}H]guanylated STNV RNA was 53% guanylated and thus this incubation contained 4.0 pmol total STNV RNA. In another experiment, with a 1/3 level of the [^{3}H]guanylated RNA (0.67 pmol guanylated, total 1.3 pmol) and 40 μl S23, similar results (20% inhibition) were obtained. The assumption that guanylated STNV RNA is equivalent to noncapped STNV RNA is documented in the data of table 3

Table 3
Relative rates of binding of capped, guanylated and unmodified STNV RNAs

	80 S Radioactivity (cpm)		% at 2 min
	2 min 20°C	20 min 25°C	
1. 15 μl S23:			
^{3}H-Capped STNV RNA			
0.75 pmol – 8400 cpm	1939	3312	59
3.0 pmol – 33 600 cpm	4914	11 560	43
[^{3}H]Guanylated STNV RNA			
0.48 pmol – 1700 cpm	91	348	26
1.9 pmol – 6800 cpm	196	719	27
2. 15 μl S23:			
Unmodified STNV[^{14}C]RNA	89	282	31
Guanylated STNV[^{14}C]RNA	91	264	34
2b.40 μl S23:			
Unmodified STNV[^{14}C]RNA	196	492	40
Guanylated STNV[^{14}C]RNA	206	468	44

In exp. 1, the STNV RNA was 68% capped and thus the two incubations had 1.1 pmol and 4.4 pmol, respectively, of total STNV pmol RNA. The [^{3}H]guanylated STNV RNA was 53% guanylated giving a total of 0.9 pmol and 3.6 pmol, respectively. In exp. 2, 5 pmol STNV [^{14}C]RNA (3270 cpm) were added in all the incubations. The unmodified STNV [^{14}C]RNA was carried through a mock guanylation procedure except that GTP was omitted (see text and Materials and methods). The standard binding assays were carried out for 2 min at 20°C and for 20 min at 25°C, the latter point serving as a measure of the maximal binding of the particular RNA

ribosome-binding data for capped and guanylated STNV RNAs under linear (2 min, 20°C) and maximal conditions (20 min, 25°C). The data show that capped STNV RNA binds at a rate at least twice that of guanylated STNV RNA. A further demonstration that guanylation per se does not affect the reactivity of STNV RNA was obtained in an experiment in which STNV[^{14}C]RNA was incubated with vaccinia extract in the presence and absence of 2×10^{-6} M unlabeled GTP. Although the extent of guanylation was not determined, it almost certainly exceeded the 53% obtained in the preparation in which [^{3}H]GTP was added at 0.3×10^{-6} M. Binding of the two STNV[^{14}C]-RNAs was compared at 15 μl S23 and 40 μl S23 and found to be essentially identical (table 3, exp. 2). Guanylation of STNV RNA thus has no effect on binding capacity.

3.4. *Extent of binding of capped and uncapped STNV RNAs*

To ascertain whether there are specific sites on the ribosome which recognize only capped STNV RNA, we determined the binding (10 min, 25°C) of ^{3}H-capped STNV RNA in reaction mixtures that had been preincubated for 5 min at 25°C with different levels of noncapped STNV RNA. The control incubation (15 μl S23, no RNA added during the preincubation) incorporated into the 80 S complex 1275 cpm from an input of 0.26 μg (7240 cpm) of 60% ^{3}H-capped STNV RNA (0.43 μg total STNV RNA). If 2 μg or 4 μg noncapped STNV RNA were added in the preincubation, the subsequent binding of the ^{3}H-capped STNV RNA was reduced to 212 cpm and 110 cpm, respectively. We conclude that the ribosome binding sites are equivalent, with no specificity for capped or noncapped RNA.

4. Discussion

The demonstration that capped STNV RNA binds to ribosomes at a rate 2–2.5 times that of noncapped

STNV RNA (fig.2A, table 3) and the considerably greater inhibition by the analog, pm^7G, of the binding of capped STNV RNA, clearly indicate that STNV RNA does not differ qualitatively from other mRNAs that are normally found in the capped form. Generalizing from this situation, we tentatively conclude that the uniqueness of mRNAs that function in vivo without a cap is not due either to their inability to serve as substrates for the capping enzymes, nor to structural differences that would result in an unfavorable positioning of the cap relative to the ribosome. Why such RNAs are not capped in vivo remains an interesting question.

The analogue, pm^7G, inhibits the ribosome binding of both capped STNV RNA and TMV RNA to 70–80%. Assuming that the residual binding of these mRNAs is largely a measure of their activity in the absence of a cap, the data suggest that noncapped TMV RNA would function at a rate 20–30% that of capped TMV RNA. In experiments comparing the translational activity of chemically uncapped (periodate oxidized and β-eliminated) TMV RNA with an appropriate control, we obtained similar results, i.e., a translational rate 20–30% that of the control (R. Roman, unpublished observation). We therefore conclude that TMV RNA and STNV RNA, like BMV RNA 4 [22], have considerable translational activity in the absence of the 5′ cap and thus differ from reovirus and vesicular stomatitis virus RNAs [23]. It has been suggested [23] that the increased dependence of these latter mRNAs on the 5′ cap is related to the use of the heterologous wheat germ system for translation. We have found, however, that translation of poly A(+) RNAs from wheat embryo polyribosomes is inhibited by 95% in the presence of 0.5 mM $m^7G^{5'}p$ (D. Herson, unpublished observation), indicating that the homologous mRNAs are strongly dependent on the 5′ cap. The difference between the mRNAs is therefore probably related to the accessibility of internal mRNA binding sites to the initiating system.

Several studies [23,24] have indicated that capped and noncapped mRNA differ not only in the rate of binding to ribosomes but also in the extent of maximum binding of the mRNAs. In the present study (table 3, exp. 1) we note similarly that the maximum binding of noncapped STNV RNA is 50% that of capped STNV RNA. That this conclusion is not correct is evident from the experiment in which the binding of ^{3}H-capped STNV RNA was almost completely inhibited by prebinding excess noncapped STNV RNA. We suggest that the discrepancy is a technical consequence of the use of limiting levels of mRNA in the time course assays. Diminished maximal binding would result from the reduced rate of binding of the noncapped form coupled with the continual breakdown of the mRNA.

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