LOCALISATION OF A HIGH AFFINITY BINDING SITE FOR COAT PROTEIN ON THE 3′-TERMINAL PART OF RNA 4 OF ALFALFA MOSAIC VIRUS

Karel STOKER, Ellen C. KOPER-ZWARTHOFF, John F. BOL and E. M. J. JASPARS
Department of Biochemistry, State University, PO Box 9505, 2300 RA Leiden, The Netherlands

Received 2 September 1980
Revised version received 25 September 1980

1. Introduction

The genome of alfalfa mosaic virus (AMV) consists of 3 single stranded RNA molecules of messenger polarity: RNA 1, RNA 2 and RNA 3. However, the bare genome RNAs are not infectious unless a catalytic amount of the coat protein of the virus or its messenger, the subgenomic RNA 4, is added (reviewed [1]). For infectivity all 3 genome RNAs need some coat protein bound [2,3]. The affinity of the RNAs for coat protein is so high that they remove protein from the coat of intact virions [4,5].

The 4 RNAs have a homology of ⩾83% in the sequence of the last 150 3′-terminal nucleotides [6–8]. Protein binding is likely to take place preferentially near the 3′-ends and probably serves the recognition by the replicase [9].

3′-Terminal fragments of 88 and 94 nucleotides of RNA 4, obtained by partial digestion of this RNA with ribonuclease T1 and called 29B and 29C, respectively, have been shown indirectly to interact with virus particles. In contrast to larger 3′-fragments, they do not remove protein subunits from virions. However, when a partial digest of RNA 4 is incubated with virus particles, the amount of 29B and 29C as free fragments is strongly reduced. This has been interpreted as sticking of these small fragments to the coat of the particles [9].

Here we show by means of gradient centrifugation that the sticking takes place, and that it is a specific process. Subfragments of fragment 29C were also tested for sticking to particles and it was shown that 36 but not 50 nucleotides could be removed from the 3′-end of fragment 29C without the sticking property being affected.

2. Materials and methods

2.1. *Materials*

T4 polynucleotide kinase was from Boehringer Mannheim. [γ-^{32}P]ATP (spec. act. 3 Ci/μM) was obtained from the Radiochemical Centre (Amersham); RNase T1 from Calbiochem. Bottom component virions (94 S) of AMV strain 425 were prepared according to [10] and turnip yellow mosaic virus (TYMV) according to [11].

2.2. *Preparation, labelling and subfragmentation of fragment 29C of RNA 4*

A mixture of fragments 29B and 29C of RNA 4 (strain 425) was isolated according to [12] and 5′-end labelled with T4 polynucleotide kinase (4 units) and [γ-^{32}P]ATP as in [13]. In each experiment ~0.7 μg RNA was labelled with 0.2–0.5 mCi [γ-^{32}P]ATP; the fragments were separated on 20% polyacrylamide slab gels as in [12] and eluted as in [14]. Partial digestion of fragment 29C with RNase T1 was according to [15], using 0.1 unit enzyme and 15 μg carrier RNA (tRNA from *Escherichia coli*) at 50°C for 15 min. Purification and elution of subfragments was as for fragments 29B and 29C. In addition to the expected G(5′)p-hits also some aspecific cleavage was found probably resulting from traces of nuclease contamination in the kinase preparation. The lengths of the fragments were determined by comparing the electrophoretic pattern resulting from the T1 digestion with that of an alkaline hydrolysate as shown in [12]. The subfragments are indicated by the position of their 3′-terminal nucleotide counted from the 3′-terminus of RNA 4. A fragment of 68 nucleotides from the cistron region of RNA 4 (nucleotides 302–370 from the 5′-end) [16] was a gift from F. Th. Brederode.

2.3. *Virion-binding assay*

The end-labelled RNA fragments and subfragments to be tested were ethanol precipitated, dried and dissolved in 50 μl 0.01 M NaH_2PO_4, 0.001 M EDTA (pH 7.0) (PE-buffer). After a short heat treatment (3 min, 60°C) to abolish aggregation, 40 μl virion suspension (4.8 μg bottom component/μl in PE-buffer suppleted with 1 mM NaN_3) were added and the mixtures were incubated at 4°C. Finally they were loaded onto sucrose gradients (10–40%, PE-buffer (pH 7.0)) centrifuged in a Beckman SW 27.1 rotor (22×10^3 rev./min, 12 h, 4°C) and fractionated with continuous A_{260} recording. Čerenkov and liquid scintillation counting was performed in a Mark II (Nuclear Chicago). The virion-binding ratio was determined by dividing the fast sedimenting counts by the total radioactivity in the gradient, both corrected for background counts.

3. Results

3.1. *Virion-binding of fragments 29B and 29C; binding specificity*

Fig.1A shows that a large part of a fragment 29C preparation cosediments with AMV virions after 6 h incubation. Under similar conditions no cosedimentation was found with fragment 29C and TYMV virions (fig.1B) nor with an RNA 4 cistron fragment (nucleotides 302–370 from the 5′-end; see section 2) and AMV virions (fig.1C). After 4–5 h the binding ratio reached its maximum value (0.4–0.5) and did not decrease within 24 h.

Kinetic studies revealed a slight difference in complexation rate during the first 2 h, in favour of fragment 29B (fig.2).

3.2. *Virion-binding of subfragments of fragment 29C*

Subfragments of different lengths having the 5′-terminus of fragment 29C were assayed for binding to virions. Digests differed in the amount of each of the subfragments so that the set of subfragments that could be assayed in successive experiments was different. In 2 expt. subfragment 55 resulting from an aspecific hit was tested. Incubation time was 6 h. The results are shown in table 1. A sharp transition between the binding ratios of binding and non-binding subfragments can be observed. Obviously the smallest binding fragment is 37. The nucleotide sequence and a possible secondary structure of this and other subfragments is shown in fig.3, according to [12].

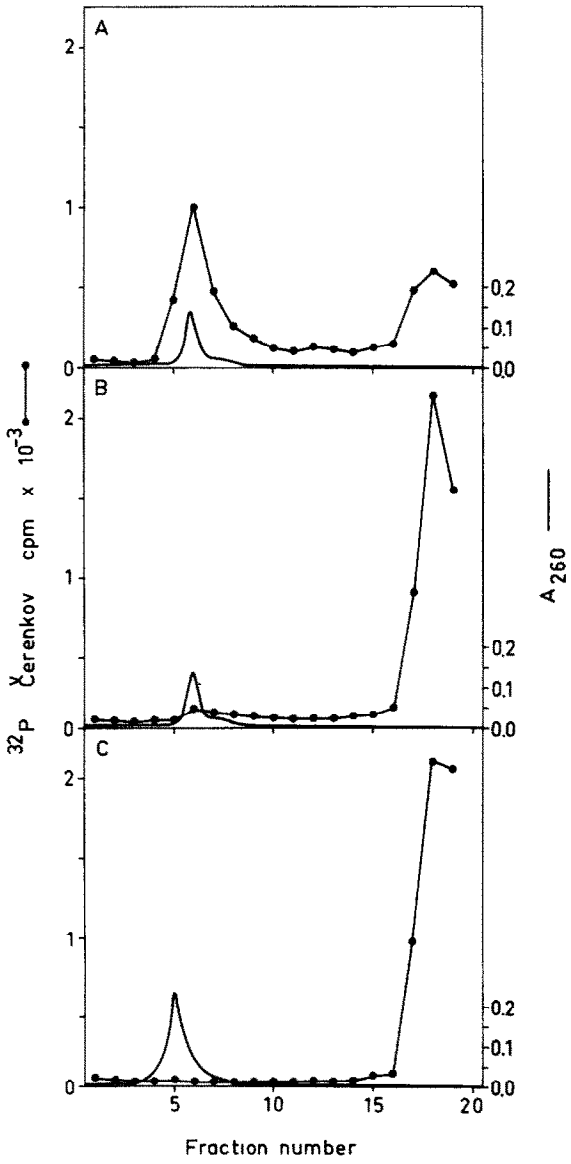

Fig.1. Distribution of radioactivity (o——o) and absorbance (——) in a sucrose gradient of an incubation mixture of RNA 4 fragments with virions. Incubation was for 6 h. (A) Fragment 29C with AMV virions; binding ratio 0.53. (B) Fragment 29C with TYMV. (C) RNA 4 cistron fragment (nucleotides 302–370 from the 5′ end; see section 2) with AMV virions. Each gradient was loaded with ~6000 Čerenkov cpm (RNA) and 200 μg protein.

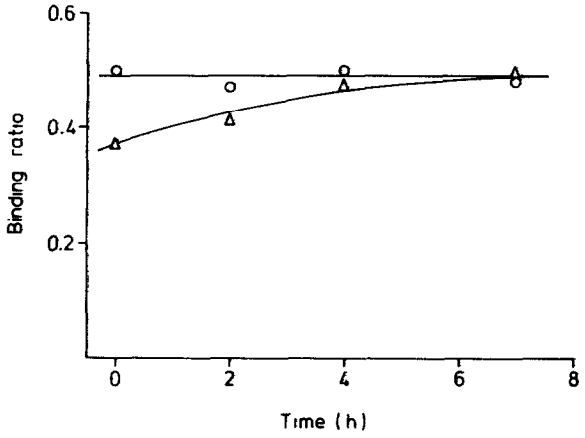

Fig.2. Kinetics of the binding of fragments 29B (o——o) and 29C (——) to virions. Only 1/6th of the usual amount of virions was added in these experiments. Samples taken at zero time could still react during loading and running of the gradients.

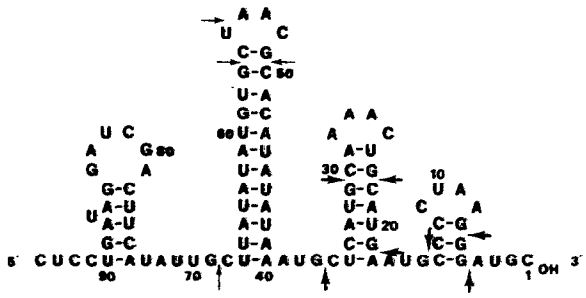

Fig.3. Nucleotide sequence and a possible secondary structure of fragment 29C, according to [12] (revised). T1 specific and non-specific hits gave rise to binding (thick arrows) and non-binding (thin arrows) 5'-end labelled subfragments. The subfragment ending in nucleotide 37 is the shortest binding subfragment.

4. Discussion

From the above it is evident that the specific virion-binding site of 3'-terminal fragments of AMV RNA 4 is located upstream from nucleotide 36 from the 3'-end. Fragment 29C binds less rapidly than fragment 29B, although 6 nucleotides longer. This is in accordance with the difference in binding between these fragments found in [9]. Fragment 29C may contain a weak hairpin at its 5'-terminus (fig.3; $G^\circ = -0.8$ kcal/mol), which will be absent in fragment 29B. Apparently this hairpin counteracts the binding to some extent. In the structure of the complete RNA 4 this hairpin is supposedly absent [6]. Taken together the results suggest that the stable hairpin ($G^\circ = -18.6$ kcal/mol) in which nucleotides 40–67 are supposed to be involved [12] (fig.3) plays an important role in the binding. This is in agreement with the finding that a 3'-terminal fragment of ~35 nucleotides did not bind to virions (C. J. Houwing, E. M. J. J., unpublished). The region of the stable hairpin is also present in the genome RNAs. The small sequence differences found between the RNAs would not affect the proposed base-paired structure [6–8]. Thus, in the activation of the AMV genome by a few coat protein molecules coat protein interaction at this site could be an essential step.

Table 1
Virion-binding ratios of subfragments of fragment 29C

Subfragment[a]	Binding ratio[b]				
	1	2	3	4	Mean
1 (=29C)			0.47		0.47
5	0.31	0.42		0.52	0.42
7	0.52	0.52			0.52
15		0.66			0.66
19		0.48			0.48
23	0.36			0.22	0.29
31	0.40	0.43		0.34	0.39
37	0.39	0.53	0.53	0.46	0.48
51				0.03	0.03
55	0.03			0.01	0.02
57	0.08				0.08
69	0.09			0.00	0.05

[a] The subfragments are indicated by the position of their 3'-terminal nucleotide counted from the 3' terminus of RNA 4. Thus, subfragment 37 contains nucleotides 37–94
[b] Four successive experiments were performed

Acknowledgements

This work was sponsored in part by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

References

[1] Van Vloten-Doting, L. and Jaspars, E. M. J. (1977) in: Compr. Virol. 11, 1–53.
[2] Smit, C. H. and Jaspars, E. M. J. (1980) Virology in press.

[3] Smit, C. H., Van Vloten-Doting, L. and Jaspars, E. M. J. (1980) in preparation.
[4] Verhagen, W., Van Boxsel, J. A. M., Bol, J. F., Van Vloten-Doting, L. and Jaspars, E. M. J. (1976) Ann. Microbiol. (Paris) 127A, 165–172.
[5] Van Boxsel, J. A. M. (1976) PhD Thesis, University of Leiden.
[6] Koper-Zwarthoff, E. C., Brederode, F. Th., Walstra, P. and Bol, J. F. (1979) Nucleic Acids Res. 7, 1887–1900.
[7] Pinck, L. and Pinck, M. (1979) FEBS Lett. 107, 61–65.
[8] Gunn, M. R. and Symons, R. H. (1980) FEBS Lett. 109, 145–150.
[9] Houwing, C. J. and Jaspars, E. M. J. (1978) Biochemistry 17, 2927–2933.
[10] Bol, J. F. and Van Vloten-Doting, L. (1973) Virology 51, 102–108.
[11] Dunn, D. B. and Hitchborn, J. H. (1965) Virology 25, 171–192.
[12] Koper-Zwarthoff, E. C. and Bol, J. F. (1979) Proc. Natl. Acad. Sci. USA 76, 1114–1117.
[13] Richardson, C. C. (1965) Proc. Natl. Acad. Sci. USA 70, 1209–1213.
[14] De Wachter, R. and Fiers, W. (1972) Anal. Biochem. 49, 184–197.
[15] Donis-Keller, H., Maxam, A. M. and Gilbert, W. (1977) Nucleic Acids Res. 4, 2527–2538.
[16] Brederode, F. Th., Koper-Zwarthoff, E. C. and Bol, J. F. (1980) Nucleic Acids Res. 8, 2213–2223.