Sarma, R. H., Lee, C.-H., Evans, F. E., Yathindra, N., & Sundaralingam, M. (1974) *J. Am. Chem. Soc. 96*, 7337-7348.
Schaller, H., Weimann, G., Lerch, B., & Khorana, H. G. (1963) *J. Am. Chem. Soc. 85*, 3821-3827.
Sussman, J. L., Barzilay, I., Keren-Zur, M., & Lapidot, Y. (1973) *Biochim. Biophys. Acta 308*, 189-197.
Tavale, S. S., & Sobell, H. M. (1970) *J. Mol. Biol. 48*, 109-123.
Uesugi, S., Shida, T., & Ikehara, M. (1981) *Chem. Pharm. Bull. 29*, 3573-3585.
Wang, A. H.-J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., van Boom, J. H., van der Marel, G., & Rich, A. (1979) *Nature (London) 282*, 680-686.
Wang, A. H.-J., Quigley, G. J., Kolpak, F. J., van der Marel, G., van Boom, J. H., & Rich, A. (1981) *Science (Washington, D.C.) 211*, 171-176.
Warshaw, M. M. (1975) in *Handbook of Biochemistry and Molecular Biology, Nucleic Acids* (Fasman, G. D., Ed.) Vol. I, p 597, CRC Press, Cleveland, OH.
Young, M. A., & Krugh, T. R. (1975) *Biochemistry 14*, 4841-4847.

Protein Binding Sites in Nucleation Complexes of Alfalfa Mosaic Virus RNA 4†

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ABSTRACT: The subgenomic coat protein messenger RNA 4 of alfalfa mosaic virus forms complexes with one and three coat protein dimers, which are designated complexes I and III, respectively. These complexes were separated, subjected to digestion with ribonuclease T_1, and filtered onto Millipore filters. Phenol extracts of the filters contained specific fragments of RNA 4, which were sequenced after electrophoretic separation on nondenaturing and denaturing polyacrylamide gels. Complex I yielded only a 68-nucleotide fragment including the 3′ terminus [fragment 814–881 according to the numbering of Brederode, F. Th., Koper-Zwarthoff, E. C., & Bol, J. F. (1980) *Nucleic Acids Res. 8*, 2213–2223]. Complex III yielded in addition to the former fragment also other, mostly extracistronic, fragments from the 3′-terminal region, as well as fragments from an intracistronic region, comprising positions 425–474, in the middle of RNA 4. The 3′-terminal region was subdivided by small gaps into three coat protein binding sites: 799–881, 759–787, and 667–753, designated sites 1, 2, and 3, respectively, and possibly representing the sites occupied by the three coat protein dimers. A similarity may exist between the secondary structure of sites 1 and 3, which both may have three hairpins, two of which flanked at their 3′ side by an AUGC sequence. Furthermore, a complementarity was noted between the loop of a large hairpin which can be drawn in the intracistronic site and the upper part of one of the three hairpins in the 3′-terminal site 1. These binding features have been combined in a model structure for the complex of RNA 4 with three coat protein dimers.

Recently evidence has been obtained that the genome of alfalfa mosaic virus becomes infectious only after each of the three genome parts (viz., the RNA species 1, 2, and 3) has bound a few coat protein molecules (Smit et al., 1981). Binding studies with specific fragments of RNA 4 (the subgenomic coat protein messenger RNA of alfalfa mosaic virus, which is homologous with the 3′ half of the smallest genome part, RNA 3) have strongly suggested that high-affinity binding sites for the coat protein are located close to the 3′ terminus of the molecule (Houwing & Jaspars, 1978; Stoker et al., 1980). In a preceding study we have isolated complexes of RNA 4 with one and three coat protein dimers and designated them complex I and complex III, respectively (Houwing & Jaspars, 1980). It was assumed that the coat protein in these complexes was bound specifically to the high-affinity sites of RNA 4, since in the complex forming reaction the coat protein was donated by virions. In this report we describe the sites on RNA 4 which are protected in complexes I and III against degradation by ribonuclease T_1. It appeared that these sites are not exclusively located in the 3′-terminal region of the molecule.

Materials and Methods

RNA 4, coat protein, and RNA 4/coat protein complexes were prepared as described previously (Houwing & Jaspars, 1980, and references therein). Immediately before ribonuclease digestion, complex I was found by analytical gel electrophoresis to contain about 30% free RNA 4; complex III contained about 35% free RNA 4 and lower complexes. The quantities of complexes are expressed in micrograms of their RNA contents.

Other Materials. Ribonucleases T_1 and U_2 were from Sankyo (via Calbiochem). T4 polynucleotide kinase was obtained from Boehringer Mannheim. [γ-^{32}P]ATP (3000 Ci/mmol) was from The Radiochemical Centre, Amersham, England. Dextran T 500 was obtained from Pharmacia, and 24-mm HAMK filters were from the Millipore Corp. Ultrapure urea was purchased from Schwarz/Mann and 2 times crystallized p.A. acrylamide from Serva (Heidelberg). All other chemicals were reagent grade. The concentration of glycerol refers to glycerol as supplied, with no correction for water contamination (about 13%).

Ribonuclease Digestion. RNA and ribonucleoprotein complexes I and III were digested at a concentration of 5–15

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μg/mL with 2.7 units of ribonuclease T_1 per μg of RNA in a diluted McIlvaine buffer (21.4 mM Na_2HPO_4 and 9.3 mM citric acid) with 10% (v/v) glycerol, pH 6.8, for 30 min at 25 °C.

Filtration onto Millipore Filters. Digests were filtered at room temperature essentially according to Riggs et al. (1970). Filters were subsequently shaken on a rotating tube mixer with 1 mL of 5 times diluted Loening buffer, 0.5% sodium dodecyl sulfate, and 1 mL of water-saturated phenol for 20 min at room temperature, followed by incubation for 3 min at 50 °C. The water layer obtained after centrifugation was shaken again for 5 min with phenol and extracted 4 times with ether. Finally, traces of ether were removed with a stream of nitrogen gas.

Preparative electrophoresis of RNA fragments in nondenaturing cylindrical gels was performed in buffer according to Loening (1967) as reported previously (Houwing & Jaspars, 1978). The elution rate was 3.2 mL/h, and fractions of 0.7 mL were collected.

End Labeling and Purification of Coat Protein Binding RNA Fragments. Fractions from a preparative electrophoresis run were precipitated with 2 volumes of ethanol in conical polypropylene Eppendorf tubes. For facilitation of precipitation, solutions of dextran and sodium acetate, pH 5, were added to final concentrations of 25 μg/mL and 0.2 M, respectively, and the mixture was left for at least 2 h in ice and then centrifuged. Residual ethanol was removed from the pellet under vacuum. It was then dissolved in 8 μL of distilled water, heated for 6 min at 65 °C, and 5′ end labeled with T4 polynucleotide kinase (2 units) and [γ-^{32}P]ATP (125 μCi). Amounts of RNA varied from 5 to 50 ng. End labeling and subsequent electrophoresis on denaturing polyacrylamide slab gels were performed essentially as described by Donis-Keller et al. (1977). The labeled RNA fragments were eluted from the gel according to De Wachter & Fiers (1972) and precipitated with ethanol in the presence of 0.1 M sodium acetate and 10 μg of crude top a fraction RNA of alfalfa mosaic virus.

Determination of the sequence of G and A residues was performed by partial digestion with OH^- and with ribonucleases T_1 and U_2, followed by electrophoresis on 0.35-mm polyacrylamide slab gels and autoradiography essentially according to Donis-Keller et al. (1977), except that the conditions for the digestion with ribonuclease U_2 were as described by Krupp & Gross (1979) and that a buffer according to Peacock & Dingman (1968) was used for electrophoresis.

Radioactivity measurements were performed in a Nuclear Chicago Mark II scintillation counter. Samples from fractions obtained by preparative electrophoresis were mixed with 10 times their volume of Hydroluma; dried filters were put in 10 mL of Lumac. Both scintillation liquids were from Lumac Systems A.G., Basel, Switzerland. ^{32}P label in gel pieces was determined by Čerenkov counting.

Results

Coat Protein Affinity of RNA Digested with Increasing Amounts of Ribonuclease. We investigated what ribonuclease concentration had to be used to get a digest with the minimum of coat protein affinity. At this concentration of ribonuclease, the protection of specific sites by the coat protein would be most evident. Coat protein was added in a ratio of six subunits per RNA molecule. It appeared that a plateau was reached at about 1 unit of ribonuclease T_1 per μg of RNA (Figure 1). The plateau was at about 4% of the input RNA.

Nucleotide Sequences from Ribonuclease Digests of Complexes I and III Bound onto Millipore Filters. (A) Preparative Electrophoresis in Cylindrical Nondenaturing Gels. Com-

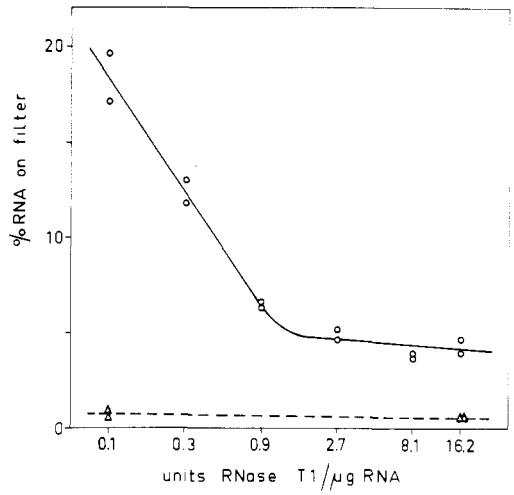

FIGURE 1: Millipore filter binding by coat protein of RNA 4 digested with different amounts of ribonuclease T_1. Amounts of 3.1 μg of [^{3}H]RNA 4 were digested with increasing amounts of ribonuclease T_1 (Sankyo units). Incubation was continued at 25 °C for 30 min in the presence (○) (six subunits per RNA molecule) and in the absence (△) of coat protein. The mixtures were then filtered onto Millipore filters, and the radioactivity of the dried filters was determined and expressed as a percentage of the input (68 000 cpm as measured on filters).

plexes I and III, ^{3}H labeled in their RNA moiety, were digested in the presence and absence of a 5-fold excess of unlabeled RNA 4 and filtered onto Millipore filters. Preparative electrophoresis in nondenaturing polyacrylamide gels showed one single peak in the case of complex I (Figure 2C), whereas complex III yielded three prominent peaks (Figure 2E). Comparing parts D and F of Figure 2 with parts C and E of Figure 2, respectively, shows that these peaks do not decrease appreciably if the degradation is carried out in the presence of an excess of unlabeled RNA 4, with the exception of the slowest moving peak of the complex III pattern. This means that there is not an important redistribution of protein among RNA fragments during digestion and filtration.

Furthermore, it is evident that the above peaks result from protection by coat protein, since addition of five to six coat protein molecules per RNA 4 molecule (thus the same amount as is present in complex III) after the degradation has taken place yields only material that moves faster than the major peaks of complexes I and III (Figure 2B). Material in the latter mobility range has also been found in the complex III pattern, but not in the complex I pattern.

Samples of all major and minor peaks, indicated in Figure 2B,C,E (with solid circles), have been taken for further investigation.

(B) Electrophoresis in Denaturing Slab Gels. The material of the indicated fractions of Figure 2 was end labeled with ^{32}P and run on denaturing slab gels. Figure 3 shows part of the autoradiograms obtained in this way. It appeared that some samples were virtually homogeneous, whereas others were mixtures of several fragments. Complicated patterns were especially found among the material with higher mobility (fraction numbers lower than 40). Many fractions were analyzed in the higher mobility range of Figure 2E since the pattern did not consist of well-separated peaks. The prominent bands in Figure 3 were cut from the gels and Čerenkov counted. After elution the material of the bands was analyzed for its nucleotide sequence. Knowing the complete sequence of RNA 4 (Brederode et al., 1980), it was easy in most cases to identify the fragments in question on the basis of the sequence of their G and A residues. In some cases, when a

Table I: Percentage of Recovery on Millipore Filters of Protein Binding Fragments Generated by Ribonuclease T_1 Digestion of RNA 4 and of Its Complexes with One and Three Coat Protein Dimers

digested material	^{3}H cpm input ($\times 10^{-3}$)[a]	panel in Figure 2	fractions of peak in Figure 2[b] numbers	fractions of peak in Figure 2[b] total ^{3}H cpm	fraction of Figure 2 analyzed on denaturing gel[c]	fragment indication in Figure 3[d]	fragment position in sequence of RNA 4[e]	fragment ^{32}P cpm ($\times 10^{-3}$)	fragment recovery[f] (%)
RNA 4	245	B	19(1/2), 20, 21	2154	21	NS	702–724	1832	16.4
							846–867	68	0.6
							442–459	960	8.6
							425–441	516	4.6
							425–439	89	0.8
							288–301	71	0.6
			22–24, 25(1/2)	3310	22	B22a	702–724	2118	23.7
						B22b	442–459	973	10.9
						B22c	425–441	1301	14.5
			25(1/2), 26–28, 29(1/2)	1500	27	B27a	mixture	43	1.9
						B27b	317–349	99	4.3
						B27c	319–349	154	6.7
						B27d	mixture	171	7.4
			29(1/2), 30, 31, 32(1/2)	719	31	B31a	485–522	260	8.9
			32(1/2), 33–37	2883	35	B35a	302–349	72	1.2
						B35b	2–39	714	12.3
complex I	687	C	40(1/2), 41–46	16870	43	C43a	814–881	2317	33.3
complex III	210	E	20(1/2), 21, 22	824	22	NS	667–691	42	1.3
							702–724	109	3.2
							(442–459)	91	2.7
							425–441	255	7.5
							625–637	159	4.7
			23, 24(1/2)	621	23	E23a	667–691	294	4.7
						E23b	702–724	100	1.6
						E23c	425–441	213	3.4
						E23d	460–474	68	1.1
			24(1/2), 25, 26	1204	25 + 26	NS	(814–845)	53	0.5
							(759–787)	855	7.5
							(442–469)	91	0.8
							667–691	107	0.9
							731–753	533	4.7
							476–496	109	1.0
							457–474	126	1.1
							460–474	37	0.3
			27	641	27	E27a	(440–474)	256	2.3
						E27b	(759–787)	331	2.9
						E27c	731–753	327	2.9
			28, 29, 30(1/2)	952	28 + 29	NS	(440–475)	285	2.0
							(440–474)	932	6.5
							759–787	96	0.7
							731–753	196	1.4

30(1/2), 31	307	31	E31a	692–730	115	2.5
32, 33, 34(1/2)	309	32 + 33	NS	(401–441)	58	1.5
				692–730	60	1.5
34(1/2), 35, 36	387	35 + 36	NS	(425–474)	153	1.4
				(302–349)	44	0.4
				(702–753)	49	0.4
				485–532	24	0.2
				2–39	32	0.3
37	357	37	E37a	702–753	127	2.3
38, 39, 40(1/2)	587	38 + 39	NS	485–545	45	0.5
				702–753	252	3.1
				2–39	18	0.2
40(1/2), 41–47	5008	44	E44a	814–881	1558	32.3
51–54, 55(1/2)	3390	53	E53a	803–881	1209	17.1
55(1/2), 56–60	3030	57	E57a	799–881	741	14.6

[a] Radioactivity of the digested material put on the filter. [b] Radioactivities of leading and trailing fractions of a peak were often halved. [c] Some fractions of panel E of Figure 2 were combined before analysis. [d] Part of the gels is not shown in Figure 3 (NS). [e] Some bands were mixtures and could not be sequenced; others, in parentheses, were contaminated, but the dominating fragment in the mixture could be identified. [f] Calculated as described in the text.

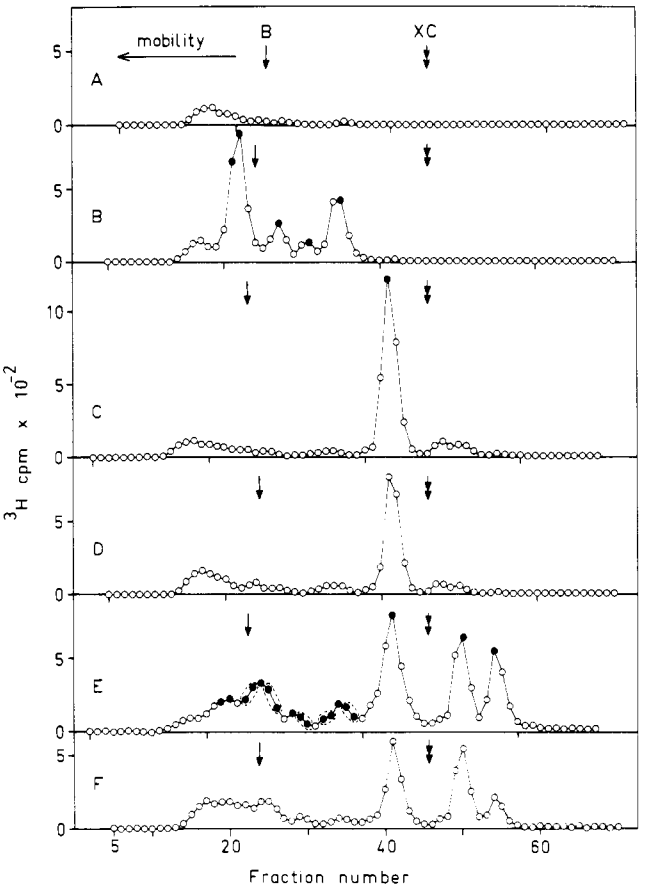

FIGURE 2: Preparative electrophoresis of RNA 4 fragments that were bound by coat protein onto Millipore filters. Free [^{3}H]RNA 4 or [^{3}H]RNA 4/coat protein complexes were digested and filtered onto Millipore filters. Phenol extracts of the filters were subjected to preparative electrophoresis in cylindrical nondenaturing polyacrylamide gels. Ribonuclease digestions were performed with 11 μg of [^{3}H]RNA 4 (A, B), 29 μg of ^{3}H-labeled complex I (C), 14 μg of ^{3}H-labeled complex I to which 70 μg of unlabeled RNA 4 was added (D), 10 μg of ^{3}H-labeled complex III (E), and 5 μg of ^{3}H-labeled complex III to which 25 μg of unlabeled RNA 4 was added (F). In the case of (B), 5 μg of coat protein was added after 30 min, and incubation was then continued for another 30 min. Volumes taken for radioactivity determination were 0.65 (A, D, F) or 0.325 mL (B, C, E). Radioactivity in the fractions of all gradients is converted to a basis of 5 μg of [^{3}H]RNA 4 (105 000 cpm) input. Fractions indicated by solid circles were taken for end labeling and subsequent electrophoresis in denaturing slab gels (Figure 3). In (E) fractions were combined as indicated. Single and double arrows indicate the position of the dye markers bromophenol blue and xylene cyanol FF, respectively. Electrophoresis patterns have been aligned with respect to the position of the latter dye.

number of G and A positions coincided and when one or more G and/or A positions did not correspond with bands of the alkaline degradation track, it was clear that we were dealing with a mixture of fragments. However, the identity of the dominating fragment in these mixtures could nearly always be established. The length of the dominating fragment could be derived from the alkaline degradation track, whereas its position in the sequence of RNA 4 followed usually from characteristic features in the pattern of the heaviest bands in the G and A tracks.

The positions of the fragments and their radioactivities are summarized in Table I. Also the recoveries of the RNA fragments on the filter with respect to the input RNA have been listed. These recoveries could be calculated in the following way. From the ^{32}P label of the RNA fragments their molar ratio in a given fraction could be determined. We assumed that this molar ratio was also representative for the

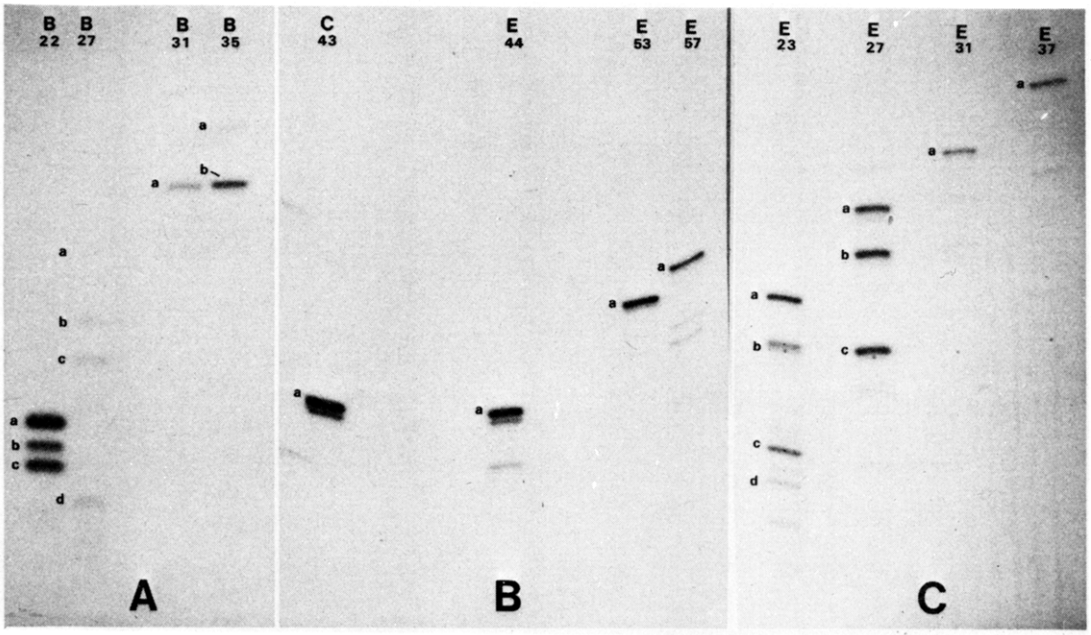

FIGURE 3: Reelectrophoresis under denaturing conditions of RNA 4 fragments bound by coat protein onto Millipore filters. Material of selected fractions from the preparative electrophoresis runs of Figure 2, indicated with the letter of the gel run and the number of the fraction, was end labeled as described under Materials and Methods and rerun on denaturing gels of 12% (A, B) and 20% (C) polyacrylamide. Bands indicated with small letters were cut out and eluted for sequence determination. Note that reelectrophoresis of fraction B23 and of the combined fractions of gel run E of Figure 2 is not shown.

neighboring fractions in Figure 2. So that the ^3H label in each RNA fragment could be known, a correction had to be made for its uridylic acid content since [^3H]uridine was used to label the RNA. The recoveries are minimum values since it was assumed that the RNA 4/protein complexes were pure. On the other hand, some minor fragments were neglected, which increases the relative contribution of the investigated fragments. Also the contribution of contaminated fragments is overestimated. A factor with an unknown influence on the recoveries is the efficiency of the ^{32}P end labeling which is not necessarily the same for all fragments.

The picture that emerges from these data is given in Figure 4. In complex I a single sequence of 68 nucleotides at the 3′ terminus is protected and is recovered for 33% on the filter (Figure 4B). With complex III, recovery of this terminal sequence increases until 65%, whereas other parts of the 3′-terminal region become also protected, though with a recovery of only 15% or less (Figure 4C). Two small pieces of the sequence, viz., residues 754–758 and 788–798, were not found on the filter. In addition to the 3′-terminal region which has only a small extension into the cistron, there is a separate protected region in the middle of the cistron. A recovery of about 15% is found from residue 425–474, with a peak of 25% at residues 440 and 441.

When RNA 4 is first maximally digested with ribonuclease T_1 and then incubated with coat protein and filtered onto Millipore filters, not much of the coat protein binding capacity of the 3′-terminal region is preserved, with the exception of that localized in fragment 702–724. Apparently, the three G residues in this fragment have been protected by secondary structure. From the internal region two contiguous coat protein binding fragments, 425–441 and 442–459, were preserved with a somewhat higher recovery than in complex III. Also these fragments have several G residues.

Still other coat protein binding fragments were found in the digest (2–39, 317–349, 319–349, and 485–522), but these were not significantly represented as sequences protected by coat protein in the complexes. This demonstrates again that little,

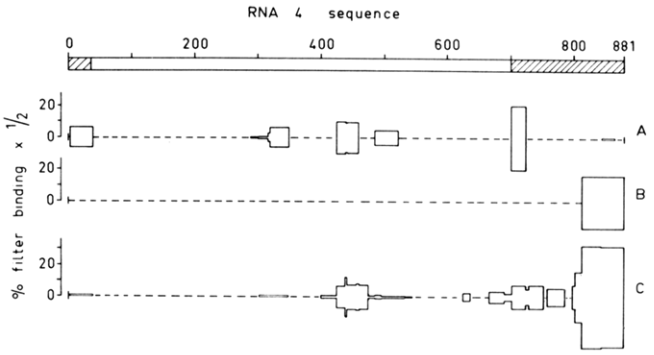

FIGURE 4: Histograms showing the position and the recovery on Millipore filters of sequences in RNA 4 with affinity for coat protein. RNA 4 (A) and complexes I (B) and III (C) were digested with ribonuclease T_1. The RNA 4 digest was incubated before filtration with 5 mol of coat protein per mol of RNA. Filter binding is expressed as percentage recovery with respect to the input RNA. Note that the recovery of a given sequence may be composed of the recoveries of several overlapping coat protein binding fragments and, thus, that the sequences distinguished in the histograms do not necessarily correspond with coat protein binding fragments. The latter have been listed in Table I. The hatched portions in the scale at the top represent extracistronic sequences.

if any, redistribution of coat protein subunits took place after the degradation of the complexes.

Discussion

The first coat protein dimer binding to RNA 4 protects the 3′-terminal sequence from residue 814 onward. Many G residues in this region are thought to be in the stem of hairpins (Koper-Zwarthoff & Bol, 1980), but some occur in single-stranded regions between the hairpins (Figure 5) and would be susceptible to ribonuclease T_1 attack if there was no protection by the protein. The presence of a minor band close to the 68-nucleotide band a in C43 and E44 of Figure 3 suggests that the last phosphodiester bond is sometimes hydrolyzed in the presence of coat protein. However, we did not sequence this minor band.

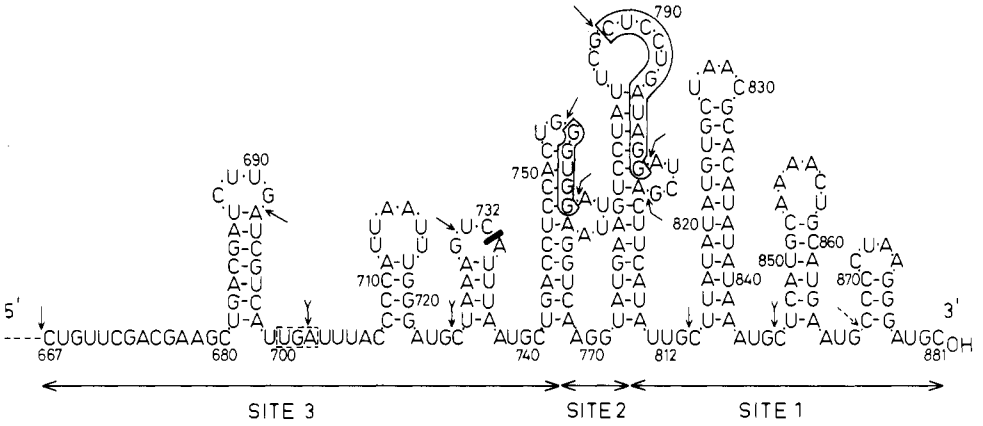

FIGURE 5: Nucleotide sequence of the 3′-terminal coat protein binding region of RNA 4. This is part of the total sequence as given by Brederode et al. (1980). The region can be subdivided into three sites, numbered 1–3 starting from the 3′ end, and separated by two short sequences (encircled with uninterrupted line) which were not found on the Millipore filters. Arrows indicate the positions of hits made by ribonuclease T_1, which border coat protein binding fragments found in digests of nucleoprotein complexes exclusively (→), in digests of both nucleoprotein complexes and free RNA (≫→), and in free RNA exclusively (⇢). The thick bar indicates the end of the 3′-terminal region homologous in all RNA species of the virus. The encircled UGA at positions 700–702 is the termination codon of the coat protein cistron.

If two more protein dimers are attached to the RNA, the 68-nucleotide terminal sequence becomes more protected, and the protection extends to the G residues at positions 813 and 802. Other parts of the 3′-terminal extracistronic region and of the last part of the cistron are protected to a significant degree as well. However, two fragments encircled in Figure 5 (754–758 and 788–798) were never found on the filters. They engender the 3′-terminal coat protein binding region to fall apart into three separate sites, numbered 1–3 inward. For sites 1 and 3, secondary structures can be drawn which bear a remarkable resemblance. There not only is a similar pattern of three hairpins but also are two of these hairpins of each site flanked at their 3′ side by the sequence AUGC, as was noticed already by Koper-Zwarthoff & Bol (1980). In contrast to sites 1 and 2, site 3 was never found as a contiguous piece, unless a lower ribonuclease T_1 concentration was used (result not shown). It must be noted that the recovery of site 2 in Figure 4 is overestimated because of its isolation as impure fragments from the denaturing gel (see fragments 759–787 in Table I).

It is interesting at this point to recall earlier experiments which concerned the binding of large RNA fragments resulting from partial degradation with ribonuclease T_1 to alfalfa mosaic virions (Stoker et al., 1980). It was found that fragments 787–881 and 794–881 (at that time designated fragments 29C and 29B, respectively), which completely overlap site 1, could bind specifically to alfalfa mosaic virions. Degradation by ribonuclease T_1 of fragment 787–881 from the 3′ end inward had no effect on the virion binding activity until G residue 831 was reached. Thus, it seemed that integrity of the dominating hairpin of site 1 is a prerequisite for virion and thus, presumably, for coat protein binding. However, in this work it appears that from a complete ribonuclease T_1 digest of RNA 4, which must contain this hairpin, it is not picked up by coat protein (Figure 4A). So, it is likely that the sequence stream up from the hairpin is also important for binding.

The middle hairpin of site 3 with its flanking sequences was recovered for 40% from the complete digest, indicating that it was present as such, and that it had retained its affinity for the coat protein.

The internal sequence protected in complex III is represented in Figure 6. It is seldom found as a contiguous fragment. A large hairpin can be drawn with the remarkable property that its loop could base pair with the loop and upper part of the dominating hairpin of site 1, yielding a negative free-energy change of 3.3 kcal/mol. The encircled CG sequence in the loop of the hairpin of Figure 6 has the highest recovery of the internal coat protein binding region. Both sides of the stem of the hairpin are equally recovered, which suggests that they form a base-paired structure in complex III. However, there is some uncertainty here since the recovery of several fragments starting at residue 440 or 442 is overestimated because of contamination (see Table I). Equal recovery of the sides of the stem is also found with the complete digest to which coat protein was added. Apparently, the coat protein recognizes the stem of the hairpin rather than the loop, since the loop is never found intact on the filter.

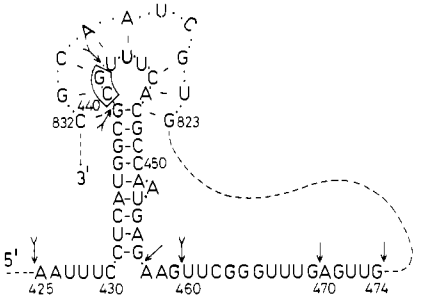

FIGURE 6: Nucleotide sequence of the internal coat protein binding region of RNA 4 and of a partially complementary region present in the 3′-terminal coat protein binding site 1 of the same RNA molecule. These sequences are parts of the total sequence as given by Brederode et al. (1980). The encircled CG at positions 440 and 441 has by far the highest recovery of the entire internal sequence. For the meaning of the arrows see the legend to Figure 5.

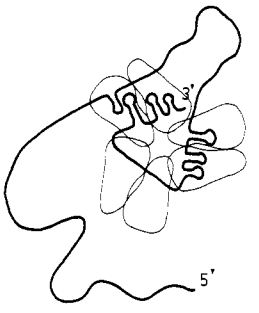

FIGURE 7: Tentative representation of nucleoprotein complex III, consisting of one molecule of RNA 4 and three coat protein dimers covering the 3′-terminal region of the RNA as well as an internal site.

Figure 7 summarizes most of the above findings in a model for complex III. Sites 1 and 3 have been drawn in a com-

parable position with respect to the dimers that protect them. The parts of the 3'-terminal sequence that were never found on the filter were positioned in the spaces between the first and second dimer and between the second and third dimer, thus being thought susceptible to ribonuclease attack. Site 2 has been drawn melted out in order to make spanning of the second dimer possible. This is not purely speculative since addition of a few coat protein subunits to RNA 4 results in a considerable decrease of ethidium bromide binding capacity (Srinivasan & Jaspars, 1982). It is quite possible that sites 1 and 3 have also lost most of their secondary structure upon binding of coat protein, thus permitting a long-range interaction with the internal coat protein binding site.

Since we know that infection by alfalfa mosaic virus only takes place if each of the three genome RNAs has bound some coat protein molecules (Smit et al., 1981), it is of much interest to investigate complex formation with these RNAs, too. If well-defined complexes are formed and can be separated, the problem of the minimum number of coat protein dimers sufficient for biological activation can be solved.

A priori the four alfalfa mosaic virus RNAs seem to have a common basis for complex formation, since the sequence of 150 nucleotides at their 3' termini is largely homologous (Pinck & Pinck, 1979; Koper-Zwarthoff et al., 1979; Gunn & Symons, 1980). Indeed it appeared that the coat protein protects part of this sequence in the three genome RNAs against ribonuclease degradation. A further similarity with the protein binding by RNA 4 is that certain internal sites of the genome RNAs are protected as well (D. Zuidema et al., unpublished results).

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References

Brederode, F. Th., Koper-Zwarthoff, E. C., & Bol, J. F. (1980) *Nucleic Acids Res. 8*, 2213-2223.
De Wachter, R., & Fiers, W. (1972) *Anal. Biochem. 49*, 184-197.
Donis-Keller, H., Maxam, A. M., & Gilbert, W. (1977) *Nucleic Acids Res. 4*, 2527-2538.
Gunn, M. R., & Symons, R. H. (1980) *FEBS Lett. 109*, 145-150.
Houwing, C. J., & Jaspars, E. M. J. (1978) *Biochemistry 17*, 2927-2933.
Houwing, C. J., & Jaspars, E. M. J. (1980) *Biochemistry 19*, 5255-5260.
Koper-Zwarthoff, E. C., & Bol, J. F. (1980) *Nucleic Acids Res. 8*, 3307-3318.
Koper-Zwarthoff, E. C., Brederode, F. Th., Walstra, P., & Bol, J. F. (1979) *Nucleic Acids Res. 7*, 1887-1900.
Krupp, G., & Gross, H. J. (1979) *Nucleic Acids Res. 6*, 3481-3490.
Loening, U. E. (1967) *Biochem. J. 102*, 251-257.
Peacock, A. C., & Dingman, C. W. (1968) *Biochemistry 7*, 668-674.
Pinck, L., & Pinck, M. (1979) *FEBS Lett. 107*, 61-65.
Riggs, A. D., Suzuki, H., & Bourgeois, S. (1970) *J. Mol. Biol. 48*, 67-83.
Smit, C. H., Roosien, J., Van Vloten-Doting, L., & Jaspars, E. M. J. (1981) *Virology 112*, 169-173.
Srinivasan, S., & Jaspars, E. M. J. (1982) *Biochim. Biophys. Acta 696*, 260-266.
Stoker, K., Koper-Zwarthoff, E. C., Bol, J. F., & Jaspars, E. M. J. (1980) *FEBS Lett. 121*, 123-126.

Nuclease S_1 Sensitive Sites in Parental Deoxyribonucleic Acid of Cold- and Temperature-Sensitive Mammalian Cells†

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ABSTRACT: Temperature-sensitive mutants of 3T3 cells (H6-15) express the transformed phenotype at 33 °C and the normal phenotype at 39 °C. Cold-sensitive mutants of Chinese hamster ovary cells (cs4-D3) express the transformed phenotype at 39 °C and the normal phenotype, along with a G_1 block, at 33 °C. When either cell type is under conditions such that it is normal and in a G_0 state, the number of S_1-sensitive sites in purified DNA, labeled in parental chains only, is zero. When the normal cells are stimulated by 10% serum, the number of S_1 sites per 10^5 base pairs increases slightly, to 0.7 in cs4-D3 and 1.1 in H6-15. Under conditions permitting the expression of the transformed phenotype, but not proliferation, the maximum number of S_1 sites per 10^5 base pairs is 5 in cs4-D3 and 44 in H6-15. When the stationary transformed cells are stimulated by 10% serum, the number of S_1 sites per 10^5 base pairs increases to 6 in cs4-D3 and 43 in H6-15. Furthermore, the DNA from the stimulated transformed H6-15 cells contains at least twice as many S_1 sites as the total number of breaks (nicks plus gaps), raising the possibility of the acquisition of stable looped or cruciform structures as the cells are stimulated.

Single-stranded regions in DNA have been demonstrated in numerous systems, but their nature and function are unknown at present (Painter & Schaefer, 1969; Habener et al., 1970; Schlegel & Thomas, 1972; Tan & Lerner, 1972; Collins, 1974, 1977, 1979; Case & Barker, 1975; Hoffman & Collins, 1976; Collins et al., 1977; Henson, 1978). Crick (1971) has proposed a model for their involvement as conformation-dependent regulatory signals. During replication, nucleosomal DNA is more sensitive to DNase I (85%) than when in bulk nucleosomes, suggesting a destabilized conformation (Seale, 1977). All of the current models of replication predict various structural changes in DNA such as nicks, unwound regions,

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