Small Reovirus Particles Composed Solely of Sigma NS with Specificity for Binding Different Nucleic Acids

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We reported previously that polycytidylate [poly(C)]-dependent RNA polymerase activity was a property of small spherical or triangular reovirus-specific particles which sedimented at 13 to 19S and were composed solely of the reovirus protein, sigma NS. Depending on the fraction of cellular extracts from which they were obtained, these particles exhibited marked differences in stability. Most 13 to 19S particles from a particular fraction repeatedly disaggregated into smaller 4 to 5S subunits with no enzymatic activity. Disruption of many particles could be prevented and polymerase activity retained after these particles had bound different single-stranded (ss) RNAs. Our previous results indicated that there was heterogeneity among the 13 to 19S particles in that possession of poly(C)-dependent RNA polymerase activity was a property of only some. Support for this heterogeneity was derived from the demonstration in this report that there were at least three types of binding sites present within particles in any purified preparation: (i) those binding only poly(C); (ii) those binding only reovirus ss RNAs; and (iii) those binding one or the other, but not both at the same time. It is suggested that only those particles able to bind either poly(C) or reovirus ss RNAs had poly(C)-dependent RNA polymerase activity, as reovirus ss RNAs markedly inhibited the polymerase activity. All three size classes of reovirus ss RNAs were equally effective in binding, but once bound, they were not copied. It is possible that heterogeneity in binding capacity of different particles comprised of only one protein, sigma NS, could result from the ability of subunits containing this protein to assemble into slightly different 13 to 19S particles with specificity of binding or polymerase activity conferred by the configuration of the assembled particles. The high capacity of sigma NS to bind many different nucleic acids with some specificity suggests that these particles may act during infection as condensing agents to bring together 10 reovirus ss RNA templates in preparation for double-stranded RNA synthesis.

We previously reported the purification of small reovirus-specific particles with polycytidylate [poly(C)]-dependent RNA polymerase activity from extracts of infected cells (7). These particles were spherical or triangular with a diameter of 11 to 12 nm, and they sedimented from 13 to 19S. They were composed solely of the reovirus protein, sigma NS. No such particles with poly(C)-dependent RNA polymerase activity were found in mock-infected cells. Chromatography on the cation exchanger CM-Sephadex revealed that there were two forms of sigma NS in preparations of these particles. In its enzymatically active state, sigma NS eluted from the column only with high salt concentrations, whereas in its inactive state, it did not bind to the column or was eluted with low salt concentrations. Our results suggested heterogeneity within the population of particles of sigma NS, as possession of active poly(C)-dependent RNA

polymerase was a property of only some.

Heterogeneity was further demonstrated by the finding that these particles which copurified with a particular cellular fraction repeatedly exhibited a remarkable fragility in contrast to similar but more stable particles in other cellular fractions. Although sigma NS had been found in a nucleoprotein complex with reovirus single-stranded (ss) RNAs in extracts from infected cells (9), association with these ss RNAs could not be the sole basis for the differences in stability of these particles copurifying with the various cellular fractions. This conclusion was derived from the fact that we found this variation in stability of particles even though all had been effectively stripped of their association with reovirus ss RNAs during the cesium chloride density gradient centrifugation used in purification.

In this report, we determined that the fragile

particles of sigma NS indeed did bind different ss RNAs and that their disruption into smaller subunits was prevented thereby. Moreover, we established that the particles of sigma NS had different binding affinities for various ss RNAs.

MATERIALS AND METHODS

Cells and virus. L cells, strain L929, a continuous cell line, were grown at 37°C in Eagle Spinner medium (3) supplemented with 7% fetal bovine serum. The harvest from passage 2 of the Dearing strain of reovirus (type 3, cloned [5, 8]) was used as stock virus in all experiments described here. The procedures for infection and incubation of cells at 30°C in the presence of actinomycin D (0.15 µg/ml) were as described before (5-7). For labeling of reovirus proteins, Eagle Spinner medium containing 33% of the normal complement of amino acids supplemented with 7% fetal bovine serum was used. After 12 to 13 h at 30 to 31°C, [³⁵S]methionine at 3.8 µCi/ml was added. The labeled precursor was present until harvest at 23 h, when cytoplasmic extracts were prepared (6, 7).

Purification of 13 to 19S particles with poly(C)-dependent RNA polymerase activity composed only of sigma NS. Purification of cytoplasmic extracts of infected cells was described previously (7). Because of its importance to this report, a brief account is given here. An 8-ml portion of cytoplasmic extract was layered over a cushion of 40% glycerol (wt/wt) in TTX buffer (0.001 M Tris-hydrochloride [pH 8.1] and 0.02% Triton X-100) and centrifuged in an SW41 rotor at 4°C for 3 h at 39,000 rpm. After centrifugation, the material in the glycerol cushion was divided into three regions. Visible material at the top of the glycerol cushion was considered in region 1 (R-1). Region 2 (R-2) extended 5 to 10 mm below the buffer-glycerol interface and contained two very close bands. The remaining glycerol layer, region 3 (R-3), which appeared clear or was only marginally turbid, contained no discernible bands of material. Region 4 (R-4) contained all material which had pelleted during the 3-h centrifugation.

In this report, only the material in R-1 and R-3 was pelleted, suspended, and sonicated. Thereafter, it was centrifuged through a CsCl density gradient in an SW41 rotor at 4°C for 5 h at 40,000 rpm. Equal fractions were collected from above. The material with poly(C)-dependent RNA polymerase activity was identified in the gradient in a white band which coincided with the peak of ³⁵S-labeled reovirus proteins. This material with peak enzymatic activity was layered over a 20 to 40% glycerol gradient in TTX buffer and centrifuged at 4°C for 38 h at 27,000 rpm in an SW28 rotor. Equal fractions were collected from above, and a portion of each was analyzed for poly(C)-dependent RNA polymerase activity. Fractions containing particles with maximal poly(C)-dependent RNA polymerase activity sedimenting from 13 to 19S were pooled for use in all experiments and called 13 to 19S particles (Fig. 1). Analysis of these particles by polyacrylamide gel electrophoresis (7) confirmed that sigma NS was the only labeled or unlabeled protein present.

Assay for poly(C)-dependent RNA polymer-

ase. The assay mixture was that described previously (6, 7). Briefly, the incubation was carried out in a final volume of 0.4 ml containing the enzyme, 12.5 µg of poly(C) per ml, 2.5 mM Mn²⁺, 7 mM mercaptoethanol, 6 µM GTP, 100 mM Tris-hydrochloride (pH 8.1), and 3 to 5 µCi of [³²P]GTP. After incubation for 23 min at 39°C, 200 µg of bovine plasma albumin was added to each reaction mixture, and the reaction was terminated by addition of 0.1 ml of saturated sodium pyrophosphate and 2 ml of 10% trichloroacetic acid. The precipitated RNA was washed as described previously and collected on glass fiber filters. The amount of radioactivity was determined in a Packard scintillation spectrometer with a toluene-based scintillation fluid.

Preparation of reovirus ss RNAs. ³²P-labeled and unlabeled capped and methylated reovirus ss RNAs were synthesized *in vitro* by reovirus cores as described (1), modified only to contain 0.02 mM S-adenosylmethionine. Purified reovirus was a generous

FIG. 1. Distribution in a 20 to 40% glycerol gradient of 13 to 19S particles from R-3 labeled with [³⁵S]methionine which synthesize poly(C)·poly(G) *in vitro* in response to poly(C). The steps for the isolation of these particles from reovirus-infected cells and the conditions for glycerol gradient centrifugation are described in the text. Fractions of 1 ml were collected from above, and a portion of each, equal to 1/20 of each fraction, was analyzed for ³⁵S content and for poly(C)-dependent RNA polymerase activity as described in the text. The remaining 95% of material in fractions 10 to 18, the shaded region called 13 to 19S particles, was pooled. A 0.5-ml portion of these pooled particles used in each experiment of Fig. 2, 3, and 4 contained 22,230 cpm of ³⁵S-labeled reovirus-specific sigma NS and incorporated 93,930 cpm of [³²P]GMP into the ds poly(C)·poly(G).

gift of A. J. Shatkin. That all three size classes of reovirus ss RNAs were synthesized *in vitro* was verified by sucrose density gradient centrifugation.

Analysis of RNA binding to sigma NS. (i) By sucrose density gradient centrifugation. A portion of the 13 to 19S particles comprised solely of sigma NS was mixed with the appropriate amount of nucleic acid(s) in binding buffer (0.01 M Tris-hydrochloride [pH 8.1], 0.02% Triton X-100, 2.5 mM $MnCl_2$, and 7 mM mercaptoethanol). After incubation at 5°C for 5 min, the mixture was layered over 15 to 30% (wt/vol) sucrose gradients in binding buffer and centrifuged in an SW41 rotor at 4°C at 40,000 rpm for the period indicated in the figure legends. Approximately 30 or 60 equal fractions were collected from above, and a sample was taken from alternate fractions to determine the amount of radioactivity and enzymatic activity.

(ii) On membrane filters. Schleicher & Schuell 13-mm BA85 membrane filters were presoaked for 1 h in binding buffer. The 13 to 19S particles and nucleic acid(s) in a final volume of 0.4 ml of binding buffer were incubated at 5°C for 5 min. Thereafter, duplicate 0.15-ml portions were applied to separate filters, washed with 0.1 ml of binding buffer, and dried. The amount of radioactivity was determined as described above. All values reported are the average of duplicate determinations and were corrected for the minimal nonspecific binding of template RNAs in absence of divalent cation.

Materials. Translation-grade [^{35}S]methionine and [α - ^{32}P]GTP were obtained from New England Nuclear Corp., Boston, Mass. Unlabeled ribonucleoside triphosphates, poly(C) ($s_{20,w}$ of 7.8), polyuridylylate [poly(U); $s_{20,w}$ of 6.1], and polyadenylate [poly(A); $s_{20,w}$ of 7.2] were purchased from P-L Biochemicals, Milwaukee, Wis. 3H -labeled poly(C) ($s_{20,w}$ of 11.1) was obtained from Miles Laboratories, Inc., Elkhart, Ind. Brome mosaic virus (BMV) RNA 3 and avian myeloblastosis (AMV) 70S RNA were kind gifts of R. M. Krug and M. J. Modak, respectively. Density gradient sucrose was purchased from Schwarz/Mann, Orangeburg, N.Y. All other chemicals were the best grade available.

RESULTS

Reovirus-specific particles with poly(C)-dependent RNA polymerase activity which sedimented at 13 to 19S were purified from several fractions obtained from extracts of infected L929 mouse fibroblasts. The stability of these particles differed depending on the region of the original glycerol cushion from which they were obtained, whether from R-1, R-2, or R-3 (Materials and Methods; 7). When the purified particles from R-1 were pelleted, more than 90% of enzymatic activity and ^{35}S -labeled proteins sedimented to the pellet, with the particles remaining intact. In contrast, only 30% of particles from R-3 sedimented to the pellet under the same conditions of centrifugation, and many appeared to be in a state of disaggregation into smaller subunits (7). Yet, the fragile 13 to 19S particles

from R-3 seemed more suitable candidates for a putative biological role, since (i) their total polymerase activity was equal to that of particles from R-1, but at a four- to fivefold higher specific activity, and (ii) most reovirus ss RNAs were in the same cellular fraction (R-3) from which the fragile 13 to 19S particles were obtained.

To quantitate the extent of fragility within the population of 13 to 19S particles purified from R-3, we determined the percentage of particles remaining intact on centrifugation through 15 to 30% sucrose gradients. As the fragile particles of R-3 copied poly(C) only when intact and not when disaggregated, it was possible that association with nucleic acids could promote their stabilization. Thus, a comparative study of binding of various synthetic and natural RNAs to these particles was undertaken. Two criteria were used to monitor binding: (i) cosedimentation of the nucleic acid with the 13 to 19S particles and (ii) retention of poly(C)-dependent RNA polymerase activity in the complex, a property which requires that added poly(C) displace the bound heterologous RNA from the template-binding site. The nucleic acids used were: (i) poly(C), as the particles had poly(C)-dependent RNA polymerase activity; (ii) reovirus ss RNAs, which were likely to be the natural RNAs bound intracellularly to these particles; and (iii) BMV RNA 3, an RNA from a plant virus whose primary sequence is likely to be available, and which has a cap 0 structure which differs from the cap 1 or cap 2 structure naturally found on reovirus ss RNAs. Moreover, as seen below, BMV RNA 3 did not inhibit the ability of the polymerase to copy poly(C).

Source of fragile 13 to 19S particles with poly(C)-dependent RNA polymerase activity. As a source of fragile particles, we pooled the 13 to 19S particles with poly(C)-dependent RNA polymerase activity purified from R-3 through the step of glycerol gradient centrifugation (shaded area in Fig. 1). We reported previously (7) and confirmed in this report that sigma NS was the only protein in these 13 to 19S particles from R-3. A 0.5-ml portion of the pooled 13 to 19S particles contained 22,230 cpm of ^{35}S -labeled reovirus sigma NS and synthesized *in vitro* in 23 min the double-stranded (ds) product, poly(C)-polyguanidylylate [poly(G)], containing 93,900 cpm incorporated *in vitro* from [α - ^{32}P]GTP. This 0.5-ml portion was committed to each gradient of Fig. 2 and 3 for analysis of disaggregation of particles in the absence of nucleic acids and of the stabilization of particles when poly(C) or reovirus ss RNAs were present.

Extent of fragility. To quantitate the extent of disruption of the 13 to 19S particles from R-3, the pooled particles in the 0.5-ml portion were

FIG. 2. Distribution in 15 to 30% sucrose gradients of ^{35}S -labeled 13 to 19S particles when recentrifuged (A) with no added poly(C) and (B) after binding poly(C). A 0.5-ml portion of the 13 to 19S particles of sigma NS from Fig. 1 was incubated at 5°C for 5 min in binding buffer without added template (panel A) and with $100\text{ }\mu\text{g}$ of poly(C) (panel B). Centrifugation was at 4°C in an SW41 rotor for 16 h at 40,000 rpm. The gradients were unloaded from above by collection of 30 equal fractions of 0.4 ml. (A) One-half of each even fraction was assayed for poly(C)-dependent RNA polymerase activity with poly(C) added to $25\text{ }\mu\text{g}/\text{ml}$ in the assay mixture. (B) One-half of each odd fraction was tested for polymerase activity with no poly(C) added for assay. Thus, under this condition, the only template present was that which was bound initially in the RNA-protein complex before centrifugation. The poly(C) used as template for binding and assay had an $s_{20,w}$ of 7.8. Under these conditions of centrifugation, most free poly(C) was in fraction 19.

centrifuged through sucrose gradients. After centrifugation, equal fractions were collected and analyzed for ^{35}S content and for material with polymerase activity. Of all ^{35}S -labeled particles committed for centrifugation, 24% remained intact and sedimented from 13 to 19S (fractions 8 to 20 of Fig. 2A and Table 1). These intact particles synthesized poly(C)·poly(G) at 12% of the capacity of particles originally applied to the gradient. The remaining particles disrupted into smaller 4 to 5S subunits with no enzymatic activity. The smaller subunits were observed only after resedimentation of the 13 to 19S particles, and thus had to be derived from the 13 to 19S particles. The reproducibility of these results, namely, that 20 to 24% of the particles remained intact with retention of 12 to 15% of the original enzymatic activity, indicated that the breakdown was not random and that any stabilization of these fragile particles after binding nucleic acids could be quantitated.

7.8S poly(C) in complex. For analysis of complex formation, $100\text{ }\mu\text{g}$ of poly(C) was incubated with the 0.5-ml portion of pooled 13 to 19S particles before centrifugation through the 15 to 30% sucrose density gradient. The number of 13 to 19S particles remaining intact after prior exposure to poly(C) increased from 24 to 61% of that originally applied to the gradient (Fig. 2B and Table 1). A portion of alternate fractions was analyzed for polymerase activity without additional template added in the assay mixture to confirm that poly(C) indeed was bound to the particles of sigma NS and could be copied. With no additional poly(C), the stabilized particles demonstrated polymerase activity equal to 30% of the total originally applied to the gradient, up from the 12% when no poly(C) was added to the pooled particles before centrifugation.

Even though saturating amounts of poly(C) had been used in binding, the amount of product, poly(C)·poly(G), synthesized by fractions 2 to 18 of Fig. 2B could be increased to 46 and 52% of total polymerizing capacity applied to the gradient if poly(C) was added to 25 and $250\text{ }\mu\text{g}/\text{ml}$, respectively, for assay of each fraction (Table 1). The reason for the increased polymerase activity remains to be determined, but we have ascertained that the sizes of the product synthesized under these conditions, namely, with or without additional poly(C) added for assay, were identical. Thus, the increased incorporation did not reflect slippage of the polymerase on the template, but in fact it indicated synthesis of an increased amount of poly(C)·poly(G).

24, 19, and 14S reovirus ss RNAs in complex. In vitro-synthesized reovirus ss RNAs were incubated with a 0.5-ml portion of the pooled 13 to 19S particles, and an analysis sim-

FIG. 3. Distribution in 15 to 30% sucrose gradients of complex formed between 13 to 19S particles of sigma NS and ^{32}P -labeled reovirus ss RNAs previously synthesized *in vitro* by viral cores. A 0.5-ml portion of the 13 to 19S particles described in Fig. 1 was incubated in binding buffer at 5°C for 5 min with 25 μg of *in vitro*-synthesized reovirus ss RNAs (A) without Mn^{2+} present and (B and C) with Mn^{2+} present. Centrifugation was for 7 h at 4°C in an SW41 rotor at 40,000 rpm. Equal fractions were collected from above, and a portion of each was taken to determine the amounts of ^{35}S -labeled sigma NS and of ^{32}P -labeled reovirus ss RNAs in each individual fraction (A and B). (C) The polymerase activity is for material in fractions of (B), but for clarity of presentation is shown in a separate panel. The re-

TABLE 1. Stabilization of fragile 13 to 19S particles purified from R-3 after binding ss RNAs

RNA bound before centrifugation	^{35}S -sigma NS in stable particles	% of total applied to gradients ^a		
		Polymerase activity retained in stable particles		
		None ^b	25 μg	250 μg
None	24	0	12	15
Poly(C)	61	30	46	52
Reovirus ss RNAs	59	0	22	44

^a The data were derived from Fig. 2 for no template or poly(C) added before centrifugation and from Fig. 3B and 3C for stabilization by reovirus ss RNAs. The 100% is based on the 22,230 cpm of ^{35}S -labeled sigma NS in the 0.5-ml portion of 13 to 19S particles committed to each gradient. These particles synthesized in 23 min an amount of poly(C)·poly(G) containing 94,900 cpm incorporated *in vitro* from [^{32}P]GTP with poly(C) as template at 25 $\mu\text{g}/\text{ml}$.

^b Amount of poly(C) added for assay.

ilar to that done for the sigma NS-poly(C) complex was performed on fractions of the sucrose gradient containing the sigma NS-reovirus ss RNAs complex. As was the case but not shown for binding of poly(C), a divalent cation was absolutely necessary for complex formation. Mn^{2+} was the preferred divalent cation (Fig. 3A). In its absence, the ^{32}P -labeled reovirus ss RNAs and the 13 to 19S particles appeared to sediment as separate components. In its presence, the sedimentation of reovirus ss RNAs and of the 13 to 19S particles of sigma NS increased markedly, and both sedimented together and faster than either alone (Fig. 3B). As was found with poly(C) as bound RNA, about 60% of particles were stabilized and prevented from disruption by reovirus ss RNAs. When assayed with the usual 25 μg of poly(C) per ml present, these stabilized particles supported synthesis of poly(C)·poly(G) at 22% of the total polymerizing capacity of material applied to the gradient (Table 1). If the poly(C) added for assay was increased to 250 $\mu\text{g}/\text{ml}$, the amount of polymerizing capacity increased to 44%, a value similar to that obtained when the poly(C)-sigma NS complex was tested with poly(C) present in the assay mixture at 25 $\mu\text{g}/\text{ml}$ (Fig. 3C and Table 1). Demonstration of

maining material in each fraction of (B) was assayed for poly(C)-dependent RNA polymerase activity with poly(C) added to each fraction at 250 $\mu\text{g}/\text{ml}$, 10-fold the amount usually used for assay. At this higher concentration, the poly(C) added to each fraction was template for the poly(C)-dependent RNA polymerase in that fraction by displacing the already bound reovirus ss RNAs. [^3H]GTP was used as the labeled precursor for incorporation into poly(C)·poly(G).

poly(C)-dependent RNA polymerase activity required that if reovirus ss RNAs were bound to the poly(C) template-binding site, they could be replaced by poly(C) before synthesis of the product, poly(C)·poly(G). Requirement of poly(C) in more than usual amounts used for demonstration of full activity suggested that reovirus ss RNAs may be inhibitory to the polymerase activity. This conclusion was further supported by the finding that when material in fractions similar to those of Fig. 2A, which were derived from centrifugation of the pooled particles without template added before centrifugation, was assayed with poly(C) added to 250 $\mu\text{g}/\text{ml}$, the polymerase activity increased but minimally (Table 1).

Inhibition of poly(C)-dependent RNA polymerase activity by heterologous ss RNAs. As a similar amount of 13 to 19S particles of sigma NS (about 60% of total analyzed) was preserved intact by poly(C) and by reovirus ss RNAs, yet the amount of demonstrable enzymatic activity differed, we explored the possibility that poly(C) competed with reovirus ss RNAs and other ss RNAs for the poly(C)-binding site on the enzymatically active particles. If this were the case, larger amounts of poly(C) than ordinarily used would be necessary to demonstrate poly(C)-dependent RNA polymerase activity in the presence of these competing ss RNAs. We included in our analysis the homopolymers poly(U) and poly(A) and the heteropolymers AMV 70S RNA and BMV RNA 3. Copying of poly(C) by sigma NS to yield the ds poly(C)·poly(G) was in fact markedly inhibited by poly(U) and reovirus ss RNAs (Fig. 4). It was not inhibited by BMV RNA 3, and poly(A) and AMV 70S RNA had an intermediate inhibitory effect.

Direct examination of binding of reovirus ss RNAs (Fig. 3B) or poly(U) [data not shown for poly(U)] revealed that both formed complexes with sigma NS, and both were strong inhibitors of the poly(C)-dependent RNA polymerase activity (Fig. 4). The possibility thus existed that poly(U) could be copied by sigma NS in the presence of ATP and that reovirus ss RNAs could be copied in the presence of the four nucleoside triphosphates, as was poly(C) in the presence of GTP. But in experiments similar to those shown in Fig. 2 and 3, neither template was in fact copied. It made no difference if Mn^{2+} or Mg^{2+} was the divalent cation used for binding or for assay, or whether the primer oligoadenylate was present with poly(U) as template or the primer GpG or GpA was present with reovirus ss RNAs. We conclude that even though these two templates competed effectively with poly(C) for the enzymatically active particles,

FIG. 4. Effect of competing ss RNAs on poly(C)-dependent RNA polymerase activity of sigma NS in the 13 to 19S particles. Results presented are percentage of activity relative to the amount of product synthesized in the absence of competing RNA; 100% of activity corresponds to 4,550 cpm of [^{32}P]GMP incorporated by a 0.1-ml portion of the 13 to 19S particles when poly(C) was added for assay at 12.5 $\mu\text{g}/\text{ml}$. The pooled 13 to 19S particles were first mixed with poly(C) and the competing ss RNA before the assay mixture containing Mn^{2+} and mercaptoethanol was added. Thus, both templates had an equal opportunity to bind to sigma NS. The amount of poly(C)-dependent RNA polymerase activity was determined as described in the text.

these two templates were not copied.

One or more than one RNA per particle? For this analysis of RNAs per particle, the 13 to 19S particles had to bind two different nucleic acids to form complexes that could be distinguished from each other. Moreover, for demonstration of poly(C)-dependent RNA polymerase activity, poly(C) had to displace bound heterologous RNA. The two RNAs chosen were the 7.8S poly(C) and 14S BMV RNA 3, which did not inhibit the copying of poly(C) by the polymerase (Fig. 4). When analyzed separately, the complex of 13 to 19S particles of sigma NS with BMV RNA 3 (Fig. 5A) sedimented distinctly faster than that formed with poly(C) (Fig. 5B). When both RNAs were added to the 13 to 19S particles before centrifugation (Fig. 5C), about 90% of complexes formed, monitored by ^{35}S content and by polymerase activity, sedimented similarly to the complexes formed with poly(C) alone (Fig. 5B). Whereas no complexes containing poly(C) sedimented in fractions 15 to 25 (Fig. 5B), about 10% of total complexes were present in these fractions when both poly(C) and BMV

FIG. 5. Comparison in sucrose gradients of the size of complexes formed between the 13 to 19S particles of sigma NS and (A) BMV RNA 3 only, (B) poly(C) only, and (C) both RNAs present together before centrifugation. A 0.5-ml portion of the 13 to 19S particles was mixed with 25 μ g of BMV RNA 3 (panel A), 100 μ g of poly(C) (panel B), or 25 μ g of BMV RNA 3 together with 100 μ g of poly(C) (panel C) before addition of binding buffer containing Mn^{2+} for incubation for 5 min at 5°C. Centrifugation was for 6.75 h at 40,000 rpm at 4°C in an SW41 rotor. The gradients were unloaded from above into 30 equal fractions and analyzed for ^{35}S content and for poly(C)-dependent RNA polymerase activity as described in Materials and Methods. Poly(C) was added for assay of polymerase activity to 25 μ g/ml,

RNA 3 were mixed with the enzyme (Fig. 5C). This small number of complexes was similar in size to those complexes formed when BMV RNA 3 alone was mixed with the 13 to 19S particles (Fig. 5A). These results suggest that only one type of RNA is bound to a particle and that binding of one RNA excludes binding of a heterologous RNA.

Are nucleoprotein complexes with particles from R-1 and R-3 the same size? Our experiments could not distinguish whether the fragile particles of R-3 were stabilized by added RNA, or whether particles of the same 13 to 19S size were re-formed from the inactive 4 to 5S subunits. We could, however, distinguish whether the number of 4 to 5S subunits of sigma NS in the complexes was specific for a particular RNA, with the number dependent on the size or other feature of the RNA in the complex. For this, we took advantage of the 13 to 19S particles of R-1 which were stable to recentrifugation (7) and did not break down to smaller 4 to 5S subunits as was the case with particles from R-3. If the nucleoprotein complexes formed were the same size with particles from R-1 and R-3, it was likely that the nucleoprotein complex with particles or subunits from R-3 contained the same number of 4 to 5S protein subunits as were initially present in the 13 to 19S particles.

The size of the complex of BMV RNA 3 was the same with the 13 to 19S particles from R-1 and R-3 (Fig. 6A). Moreover, the size of the complex of poly(C) with particles from R-1 and R-3 was also the same (Fig. 6B). As would be expected from the smaller S value for poly(C), 7.8S versus 14S for BMV RNA 3, the complexes with poly(C) sedimented slower than those with BMV RNA 3. The complexes in Fig. 6A and B were monitored by the presence of poly(C)-dependent RNA polymerase activity. This was possible because bound BMV RNA 3 was displaced by poly(C) added for assay, and BMV RNA 3 was not inhibitory to polymerase activity (Fig. 4). Our results indicated that RNA added to the fragile particles of R-3 stabilized them or promoted their re-formation into 13 to 19S particles which were similar to the stable 13 to 19S particles purified from R-1.

Specificity of binding. As our results suggested that only one RNA was bound per particle, and we had previously shown that not all 13 to 19S particles of sigma NS had poly(C)-dependent RNA polymerase activity (7), it was possible that some 13 to 19S particles could differ from other 13 to 19S particles in their binding affinities for specific nucleic acids. If different binding specificities were indeed de-

as BMV RNA 3 did not inhibit the polymerase activity.

FIG. 6. Comparison of size in 15 to 30% sucrose gradients of the nucleoprotein complexes formed between 13 to 19S particles from R-1 or R-3 with BMV RNA 3 (panel A) or with poly(C) (panel B). The complexes formed with particles from R-1 or R-3, identified only by their polymerase activity, were analyzed in separate gradients, but are presented for best comparison in the same panel for each of the RNAs used. Symbols: ●, Complex with particles from R-1; ○, complex with particles from R-3. A 0.5-ml portion of 13 to 19S particles from R-1 and R-3 was separately incubated either with 25 μ g of BMV RNA 3 (panel A) or with 100 μ g of poly(C) (panel B) in binding buffer at 5°C for 5 min. Centrifugation was at 4°C for 6.75 h at 40,000 rpm in an SW41 rotor. The gradients were unloaded by collecting from above 30 equal fractions and analyzed for poly(C)-dependent RNA polymerase activity as described in Materials and Methods. Poly(C) was added to 25 μ g/ml for assay of polymerase.

monstrable, we would have an additional means for categorizing differences among 13 to 19S particles. Quantitation of competition between nucleic acids for binding sites on sigma NS was feasible, as about 90% of 35 S-labeled sigma NS bound to nitrocellulose filters in the presence or absence of RNA. In contrast, RNA was retained on filters only when bound to sigma NS, with Mn^{2+} being absolutely required for the binding. Thus, the capacity of 13 to 19S particles to bind a particular RNA was measured by binding the first RNA to sigma NS in buffer containing Mn^{2+} . Thereafter, different amounts of competing RNA were added to the individual assay mixtures. After filtration, the respective amount of first-bound RNA and competing RNA remaining in the complex with sigma NS on the filter was determined.

In our analysis, we used 32 P-labeled reovirus ss RNAs previously synthesized *in vitro* by viral cores and 3 H-labeled poly(C) to monitor competition of the templates for the binding site(s) on the 13 to 19S particles. The bound RNA was used at its 1 $\times$ level or in a greater amount. The 1 $\times$ level for an RNA was taken as the least

amount in micrograms necessary to ensure that 100% of possible binding sites on a fixed number of particles of sigma NS was occupied. At saturation, 0.21 μ g of poly(C) or 0.26 μ g of reovirus ss RNAs was bound to the number of particles routinely used for the binding studies (Table 2). We found that all three size classes of reovirus ss RNAs had similar binding capacities.

When 13 to 19S particles of sigma NS were mixed in binding buffer with both poly(C) and reovirus ss RNAs, each at their respective 1 $\times$ level to ensure that all binding sites for each were occupied, we found that poly(C) and reovirus ss RNAs were bound concomitantly to the particles of sigma NS. The order of addition of the RNAs, however, affected the results. When [3 H]poly(C) was the first RNA bound at its 1 $\times$ level, 89% remained bound after the 1 $\times$ level of reovirus ss RNAs was added, and 21% of reovirus ss RNAs was also bound (Table 2). A 20-fold increase in the amount of poly(C) added did not prevent the approximately 20% of reovirus ss RNAs from binding. When the 1 $\times$ level of poly(C) was added to the 13 to 19S particles already saturated in the presence of a 20 $\times$ level

TABLE 2. Competition between [^3H]poly(C) and ^{32}P -labeled reovirus ss RNAs for binding to sigma NS in 13 to 19S particles

Amt ^a		Activity (% of maximal binding)			
		[^3H]poly(C) bound first ^b		^{32}P -reovirus ss RNAs bound first	
[^3H]poly(C)	^{32}P -reovirus ss RNAs	[^3H]poly(C)	^{32}P -reovirus ss RNAs	[^3H]poly(C)	^{32}P -reovirus ss RNAs
1×	20×	75	84	50	98
1×	1×	89	21	78	54
20×	1×	100	17	96	44

^a The 1× level is defined in the text. At saturation, 0.21 μg of poly(C) containing 10,000 cpm or 0.26 μg of reovirus ss RNAs (4,000 cpm) was maximally bound to the particles of sigma NS.

^b Order of addition: (i) [^3H]poly(C) plus 13 to 19S particles; (ii) binding buffer; (iii) competing reovirus ss RNAs.

of reovirus ss RNAs, at most 2% of bound reovirus ss RNAs were displaced, yet simultaneously 50% of possible poly(C)-binding sites were occupied by [^3H]poly(C). These results taken together suggest that about 20% of the total reovirus ss RNA-binding sites were exclusive for reovirus ss RNAs and did not accept poly(C) and that, in turn, 50% of all poly(C)-binding sites were exclusive for poly(C) and did not accept reovirus ss RNAs.

As reovirus ss RNAs were strong inhibitors of the poly(C)-dependent RNA polymerase activity (Fig. 4) and chromatography through CM-Sephadex showed that the polymerase activity was a property of some 30% of particles, it was likely that particles with polymerase activity could bind either reovirus ss RNAs or poly(C), but not both at the same time. The results shown in Table 2 support this conclusion. When poly(C) was first bound at its 1× level, only particles with the exclusive reovirus ss RNA-binding site had bound reovirus ss RNAs. When, however, the amount of competing ^{32}P -labeled reovirus ss RNAs was increased 20-fold, some [^3H]poly(C) was displaced with a concomitant increase of bound ^{32}P -labeled reovirus ss RNAs to 84% of maximum. In turn, when reovirus ss RNAs were the first-bound RNAs, there was displacement of bound ^{32}P -labeled reovirus ss RNAs with concomitant binding of [^3H]poly(C) if poly(C) was added as competing RNA at the 20× level. We conclude that our results have demonstrated the existence within the population of 13 to 19S particles of three binding types: (i) with only poly(C)-binding capacity; (ii) with only reovirus ss RNA-binding capacity; and (iii) with capacity to bind either poly(C) or reovirus ss RNAs.

DISCUSSION

In this report, we have determined the capacity of a fragile 13 to 19S particle comprised solely of a multimer of the viral protein, sigma NS, to bind and copy different nucleic acids. Different

nucleic acids, by binding to these 13 to 19S particles, rendered them stable. Our previous studies showed that one stable morphology assumed by these particles was that of a sphere, diameter of 11 to 12 nm with an electron-lucent center and an electron-lucent channel extending from periphery to center (7). These particles could also assume a triangular configuration. This observed variation in their conformation suggested that smaller subunits comprised of sigma NS could assemble to form 13 to 19S particles of more than one shape. Whether spherical or triangular, they were comprised solely of sigma NS, and some particles had poly(C)-dependent RNA polymerase activity.

When recentrifuged in the absence of added template, only about 25% of the 13 to 19S particles from R-3 remained intact. These intact particles retained 12 to 15% of the polymerase activity originally present in the sample analyzed. The remaining particles disrupted into 4 to 5S subunits comprised of sigma NS with no demonstrable poly(C)-dependent RNA polymerase activity. Binding of poly(C), reovirus ss RNAs, BMV RNA 3, and poly(U) to these fragile particles before centrifugation rendered stability to additional particles so that about 60% of particles remained intact upon recentrifugation. Even though a similar number of particles were stabilized by each RNA, the amount of poly(C)-dependent RNA polymerase activity retained by these stabilized particles appeared to differ. The highest recovery of polymerase activity was when the particles were stabilized by poly(C) or BMV RNA 3. When the particles were in the complex with either of these two RNAs, they possessed about 50% of the polymerase activity originally present in the total sample under analysis. Only 22% of the original polymerase activity was recovered in particles stabilized by reovirus ss RNAs. Yet, the difference in recovered activity was more apparent than real, for if the amount of poly(C) added for assay was not sufficient to displace the already

bound reovirus ss RNAs, no polymerase activity would be detected. Such was in fact the case (Table 1). When 10-fold the amount of poly(C) customarily used for assay was added to the particles stabilized by the bound reovirus ss RNAs, the polymerase activity increased from 22 to 44%, a value close to the 50% recovered when the bound RNAs were poly(C) or BMV RNA 3.

We showed previously that chromatography on CM-Sephadex separated the 13 to 19S particles into two populations (7). The first only weakly bound to the column and was enzymatically inactive. The second, about 40% of total particles but with all of the poly(C)-dependent RNA polymerase activity originally present, eluted only at 0.5 M salt. This 40% figure supports the finding of the present report that less than half of all particles were available to be occupied by either poly(C) or reovirus ss RNAs, but not both. These particles were those with poly(C)-dependent RNA polymerase activity, as indicated by the marked inhibition of the enzymatic activity by reovirus ss RNAs. Our findings determined that there was selectivity in the binding capacities for poly(C) and reovirus ss RNAs among the remaining particles with no demonstrable poly(C)-dependent RNA polymerase activity. Some particles had a poly(C)-binding site, but not a reovirus ss RNA-binding site. Others had the opposite capacity. We suggest that such heterogeneity in binding capacity of different particles composed of only sigma NS could result from an ability of subunits composed of sigma NS to assemble into slightly different 13 to 19S particles with specificity of binding or activity conferred by configuration of assembled particles.

The high capacity of sigma NS to bind many different nucleic acids and in fact to select which nucleic acid can complex to a particular particle suggests that this protein may act during infection as a condensing agent to bring together 10 reovirus ss RNA templates in preparation for ds RNA synthesis. Such a hypothesis is supported by a number of previous reports. Sigma NS was found in complexes with the three size classes of reovirus ss RNAs in extracts from infected cells (9). It was one of the four proteins synthesized early in infected cells (11, 15) and, in fact, supplied an early function (4, 10). The mutant group E, defective in the segment coding for sigma NS, is ds RNA negative (12-14). Together with our finding that bound reovirus ss RNAs were not copied by the 13 to 19S particles of sigma NS, it seems that even though sigma NS does have polymerizing capacity, it alone is not sufficient to copy bound reovirus ss RNAs. Thus, proteins whose functions were detected by the two other reovirus ds RNA-negative mutant groups, alone

or together with sigma NS, are likely to be the integral components of the reovirus replicase.

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