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Small Reovirus-Specific Particle with Polycytidylate-Dependent RNA Polymerase Activity

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We previously reported that virus-specific particles with polycytidylate [poly(C)]-dependent RNA polymerase activity accumulated at 30°C in reovirus-infected cells. These particles sedimented heterogeneously from 300 to 550S and traversed through a 40% glycerol cushion to the pellet in 3 h at 190,000 × *g*. In the present report, we found that smaller particles with poly(C)-dependent RNA polymerase activity remained in the glycerol cushion. These smaller, enzymatically active particles, when purified, sedimented at 15 to 16S. They were spherical or triangular with a diameter of 11 to 12 nm. They were comprised mostly, and likely solely, of one reovirus protein, sigma NS. No particles with poly(C)-dependent RNA polymerase activity were found in mock-infected cells. Chromatography on the cation exchanger, CM-Sephadex, ascertained that sigma NS was the poly(C)-dependent RNA polymerase and showed its existence in two forms. In one form, it was enzymatically active and eluted from the column at 0.5 M KCl. In the enzymatically inactive state, it did not bind to the column. Our results suggest that the enzymatically active form of sigma NS carries a greater net positive charge than the inactive form. They also suggest that both forms of sigma NS are associated with a particle which has poly(C)-dependent RNA polymerase activity.

We reported previously that virus-specific particles accumulated at 30°C in reovirus-infected cells which responded in vitro to polycytidylate [poly(C)] to yield the double-stranded (ds) poly(C)·polyguanylate [poly(G)] (8). The particles with poly(C)-dependent RNA polymerase were heterogeneous in size, but most sedimented from 300 to 550S, with the peak fraction sedimenting at 456S. We presumed that the enzyme responding to poly(C) was the replicase because the templates for both enzymes were single-stranded (ss) RNAs and the products of both were ds RNAs. The size of the poly(C)·polyriboguanylate synthesized in vitro was dependent on the size of the poly(C) used as template. The whole template was copied into a complementary strand of similar size. A nearest-neighbor analysis of the product labeled with [^{32}P]GMP indicated that the synthesis of the poly(G) strand in vitro did not proceed by end addition to the poly(C) template, but in fact proceeded on a separate strand.

The particles with poly(C)-dependent RNA polymerase activity described above were in the pellet obtained by centrifuging cytoplasmic extracts through a 40% glycerol cushion at 39,000 rpm for 3 h. It was possible that smaller particles with such activity were not recovered in this manner. In this report, we find that smaller components with poly(C)-dependent RNA polymerase activity remained in the glycerol cushion after the 3-h centrifugation and that they were essentially comprised of one reovirus protein.

MATERIALS AND METHODS

Cells and virus. L cells, strain L929, a continuous cell line derived from mouse fibroblasts, were grown at 37°C in suspension in Eagle Spinner medium (5) supplemented with 7% fetal bovine serum. BHK-21 cells, clone 13, were grown as monolayers in roller bottles. The harvest from passage 2 of the Dearing strain of reovirus (type 3, cloned [7, 9]) was used as stock virus in all experiments described in this report. The procedures for infection and incubation of cells at 30°C in the presence of actinomycin D (0.15 μg/ml), for growth of labeled virus and plaque assay, and for purification of cell-associated virus were as described previously (7-9, 12).

Preparation of extracts from infected cells. For labeling viral components with [^{3}H]adenosine or with [^{35}S]methionine, actinomycin D at 0.15 μg/ml was present throughout infection, including the viral adsorption period. After adsorption, when [^{3}H]adenosine was the precursor used, Eagle medium or Eagle Spinner medium supplemented with 7% fetal bovine serum was added. When [^{35}S]methionine was the labeled precursor, Eagle medium minus methionine or Eagle Spinner medium containing 33% of normal complement of amino acids, both supplemented with 7% fetal bovine serum, was added. After 12 to 13 h at 30 to 31°C, either [^{3}H]adenosine at 10 μCi/ml or [^{35}S]me-

thionine at 2 to 13 μCi/ml was added. The labeled precursor was present until harvest.

As reported previously (8), the L cells were pelleted at time of harvest, washed with 1× ST buffer (0.1 M Tris-hydrochloride [pH 8.1], 0.01 M NaCl), resuspended at a concentration of 7×10^7 to 10×10^7 cells per ml in 1× ST buffer containing 1% Triton X-100 and 1% sodium deoxycholate, and disrupted in a Dounce homogenizer. The BHK cells were scraped from monolayers into phosphate-buffered saline (4) and resuspended in the above detergent mixture for disruption in a Dounce homogenizer. After centrifugation for 8 min at $300 \times g$ in a PR2 International centrifuge, the supernatant fluid containing the cytoplasmic extract was collected. The nuclear pellet was treated with 3 ml of the same detergent buffer per 1 ml of nuclear pellet, and the suspension was recentrifuged for 8 min at $300 \times g$. The supernatant fluid was added to the main cytoplasmic extract.

An 8-ml portion of cytoplasmic extract was layered over a 4-ml 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, there were four bands of visible material (Fig. 1): lipid-like material which had floated to the top of the aqueous layer; a band at the top of the glycerol cushion, region 1 (R-1); and two very close bands, region 2 (R-2), about 5 to 10 mm below the buffer-glycerol interface. The remaining glycerol layer, region 3 (R-3), which appeared clear or was only marginally turbid, contained no discernible bands of material. The material in R-1 and R-2 and that in the lower 80% of the glycerol cushion was centrifuged in an SW41 rotor at 4°C for 15 h at 40,000 rpm, and the pelleted material from each was taken separately for further analysis.

Density and velocity gradient centrifugations. The pelleted samples were resuspended and sonicated for 3 min, layered on CsCl gradients (1.20 to 1.40 g/ml in TTX buffer), and centrifuged in an SW41 rotor at 4°C for 5 h at 40,000 rpm. The gradients were unloaded by collecting approximately 40 equal fractions from above. A portion of each fraction was sampled to determine the amount of radioactive material, and the poly(C)-dependent RNA polymerase was identified by assaying a portion of individual gradient fractions after dialysis against TTX buffer or chromatography through Sephadex G-50 Fine. The 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 or for 16 h at 40,000 rpm in an SW41 rotor. After centrifugation, approximately 40 equal fractions were collected from above, and a sample was taken from each to determine the amount of radioactivity and enzymatic activity. After identification of the fractions containing enzymatic activity, the material was applied to CM-Sephadex columns or was dialyzed against TTX buffer and taken to dryness by lyophilization in preparation for gel analysis. Alternatively, the material in the active fractions was centrifuged at 4°C for 17 h at 45,000 rpm in an SW50.1 rotor to obtain a pellet for visualization in an electron microscope.

Column chromatography. The cation exchanger CM-Sephadex C-25 (Pharmacia Fine Chemicals, Piscataway, N.J.) was swollen in 0.001 M HEPES buffer (*N*-2-hydroxymethylpiperazine-*N'*-2-ethanesulfonic acid, pH 6.8) containing 0.1 M KCl and 5% glycerol. A 16- by 1.5-cm column was poured in the same solution. It was thereafter washed with two column volumes of 0.001 M HEPES buffer with 5% glycerol but lacking KCl, after which the column height increased about 1 cm. Chromatography was at 4°C.

Electron microscopy. The pelleted material, resuspended in 25 to 50 μl of phosphate-buffered saline, pH 7.2, was placed on a microscopic grid covered with a carbon-coated Formvar film, stained with uranyl acetate (20), and air dried. All specimens were examined at 80 kV with a Philips-300 electron microscope equipped with a specimen cooling device.

Electrophoresis of polypeptides in 10% sodium dodecyl sulfate-polyacrylamide slab gels in discontinuous Tris-glycine buffer systems. Conditions for preparation of 10% slab gels, solubilization of samples, and running buffers used were those described by Maizel (2, 3, 15, 18) or alternatively those described by Laemmli (13). After electrophoresis for 11 h at 100 V, the gel slabs were fixed and stained for 1.5 h in a 50% methanol solution containing 0.2% Coomassie blue (Schwarz/Mann) to which a final concentration of 7% acetic acid was added before use. The gels were destained in 7% acetic acid containing 20% methanol. Subsequently, the gels were treated with dimethylsulfoxide and PPO (2,5-diphenyloxazole) (1), dried under vacuum, and processed for radioautography on Cronex-4 Medical X-ray film (Dupont-de-Nemours and Co.). Where indicated, the gels or films were scanned at 525 nm with a Quick-Scan Jr. microdensitometer (Helena Laboratories, Beaumont, Tex.).

Assay for poly(C)-dependent RNA polymerase. The assay mixture for poly(C)-dependent RNA polymerase has been described previously (8), but will be repeated because of its importance to the present report. The reaction mixture contained the experimental sample in a final volume of 0.80 ml with 80 μmol of Tris-hydrochloride, pH 8.1, 1.9 μmol of manganese chloride, 5.5 μmol of 2-mercaptoethanol, 10 μg of poly(C), 0.005 μmol of GTP, and 3 to 5 μCi of [α-^{32}P]GTP. Since guanylyl (3' to 5') guanosine was not necessary for synthesis when Mn^{2+} was the divalent cation (8), it was omitted from the assay mixture. After incubation for 20 to 25 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 (7). It was collected on glass fiber filters, and the amount of radioactivity was determined in a Packard scintillation spectrometer with a toluene-based scintillation fluid.

Materials. Translation grade [^{35}S]methionine and the α-^{32}P-labeled GTP were obtained from New England Nuclear Corp., Boston, Mass. Unlabeled ribonucleoside triphosphates and poly(C) ($s_{20,w}$ of 8 to 13) were purchased from P-L Biochemicals, Milwaukee, Wis.; actinomycin D was from Merck, Sharp, and Dohme, West Point, Pa.; and sodium deoxycholate was from Schwarz-Mann, Orangeburg, N.Y. Triton X-100 and HEPES were from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Purification of small particles with poly(C)-dependent RNA polymerase activity. We previously found that particles with poly(C)-dependent RNA polymerase activity in cytoplasmic extracts of reovirus-infected L929 mouse fibroblasts (8) or BHK-21 cells sedimented through a 40% glycerol cushion and collected in the pellet, namely in region 4 (R-4) of Fig. 1, after centrifugation for 3 h at 39,000 rpm. To determine whether smaller particles with such activity existed, in the present report we analyzed material from extracts of the two cell lines which remained at the glycerol-aqueous interface and in the glycerol cushion after the above centrifugation. Our approach for identification of smaller particles with poly(C)-dependent RNA polymerase activity was to label their proteins with [^{35}S]methionine and any bound RNA with [^{3}H]adenosine.

After the 3-h centrifugation, there was a visible band of material at the glycerol-aqueous interface, region 1 (R-1), and two close bands about 5 to 10 mm below the interface, region 2 (R-2). Region 3 (R-3), the remaining glycerol layer below R-2, was visibly clear or at most only slightly cloudy. When cells were labeled during infection with [^{3}H]adenosine in the presence of actinomycin D, all three size classes of reovirus ss RNAs (7) were found in material from R-1, R-2, and R-3, with most labeled RNAs found in R-3 (Fig. 2). No ds RNAs were detected in material from these three regions, and all of the ds RNA was in material which had pelleted in 3 h, namely in R-4.

Cesium chloride density gradient centrifugation. The material from R-1, R-2, and R-3 which sedimented to a pellet at 40,000 rpm in an SW41 rotor was separately resuspended in TTX buffer, overlaid on preformed CsCl density gradients (1.20 to 1.40 g/ml), and centrifuged at 4°C at 40,000 rpm in an SW41 rotor. After this centrifugation, most of the visible material in R-1, R-2, and R-3 was in the top third of the CsCl density gradient. Figure 3 shows this portion of the gradient after centrifugation of an R-3 fraction from mock-infected cells and from infected cells harvested at 16 and 27 h after infection. Band A is a yellowish band which is present in all three samples. In contrast, band B is present in R-3 material only from infected cells.

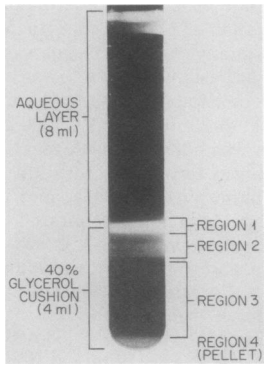

FIG. 1. *Distribution in 40% glycerol cushion (4 ml) of material from cytoplasmic extract of reovirus-infected L929 mouse fibroblasts or BHK-21 cells after centrifugation at 4°C in an SW41 rotor at 39,000 rpm for 3 h. Particles from the four regions, R-1 through R-4, were separated by collection from above. Details of the preparation of cytoplasmic extracts are given in the text under Materials and Methods.*

When the gradients after centrifugation of material in R-1, R-2, and R-3 from infected cells were collected into 40 equal fractions and each was analyzed for its content of ^{35}S-labeled reovirus proteins, it was found that the radioactive proteins in material from all three regions distributed similarly in the density gradients with the peak of radioactive proteins in particles in fractions 30 to 32 (Fig. 4). It was in these very fractions, 30 to 32, that the material of band B of Fig. 3 was collected. In contrast to the accumulation of the protein-containing structures in the top third of the CsCl density gradient, the majority of the reovirus ss RNAs in material from R-1, R-2, and R-3 (shown in Fig. 2) accumulated in the pellet and in fractions of the lower one-half of the CsCl density gradients. The density gradient centrifugation thus served the very important function of separating the labeled reovirus proteins from the reovirus ss RNAs.

CsCl was removed from individual fractions of the density gradient either by dialysis or by chromatography through Sephadex G-50, and the material in each fraction was assayed for poly(C)-dependent RNA polymerase activity. It was found that most particles with poly(C)-dependent RNA polymerase activity in material in R-1, R-2, and R-3 from infected cells were present in fractions 30 to 32 of each gradient coincident with the visible band B of Fig. 3. The distribution of enzymatic activity is shown only for material in R-3 from infected cells (shaded area of Fig. 4).

When cells were mock-infected for 27 h, some labeled proteins were found in fractions 30 to 32 of the CsCl density gradients (shown only for R-3 in the lowest panel of Fig. 4), and no poly(C)-dependent RNA polymerase was found in any of the gradient fractions of material from R-1, R-2, or R-3 of mock-infected cells. The kinetics of appearance of particles with poly(C)-dependent RNA polymerase activity in material of regions R-1, R-2, and R-3 were such that by 16 h

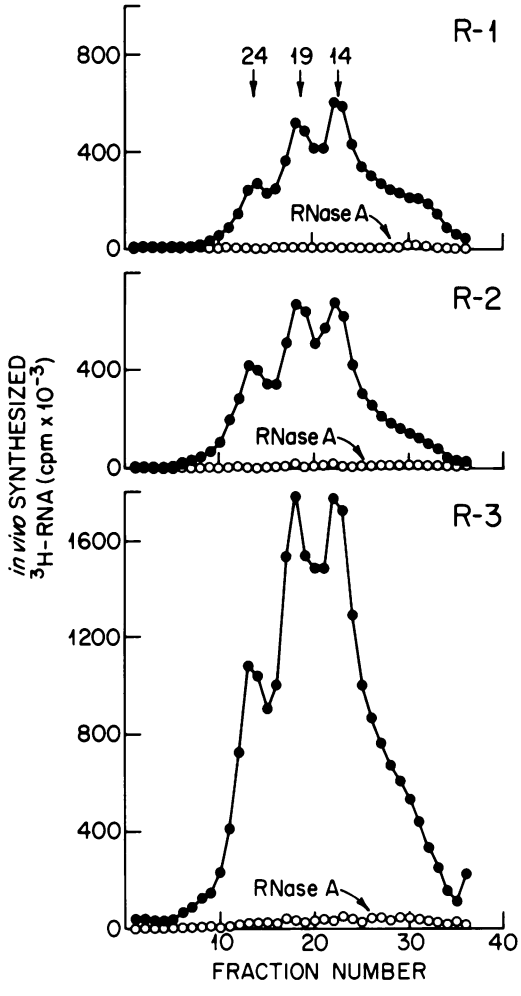

FIG. 2. *Distribution in 15 to 30% sucrose gradients of in vivo-labeled reovirus RNAs present in material from R-1, R-2, and R-3 of Fig. 1. BHK-21 cells were infected at 30 to 31°C with reovirus in the presence of actinomycin D (0.15 μg/ml) and labeled with [^{3}H]adenosine at 10 μCi/ml from 12 to 26 h when the cells were harvested. Details are given in the text. The cytoplasmic extract was overlaid on a 40% glycerol cushion. After centrifugation at 4°C in an SW41 rotor at 39,000 rpm for 3 h, the material in R-1, R-2, and R-3 was collected from above, and a portion from each of the three regions was treated with sodium dodecyl sulfate, Brij 58, and EDTA at final concentrations of 1.95%, 0.5%, and 0.0005 M, respectively. Centrifugation in density gradients of 15 to 30% sucrose in TNE buffer (0.01 M Tris-hydrochloride–0.1 M NaCl–0.001 M EDTA, pH 7.4) was at 18°C in an SW27 rotor at 26,000 rpm for 18 h. Equal fractions of 1 ml were collected from below and divided into two sets. In the first set, the total acid-insoluble radioactivity was determined after addition of 100 μg of bovine serum albumin as described in the text. Fractions in the second set were brought to 1× RSB (0.01 M Tris-hydrochloride–0.01 M NaCl–0.0015 M MgCl$_2$,*

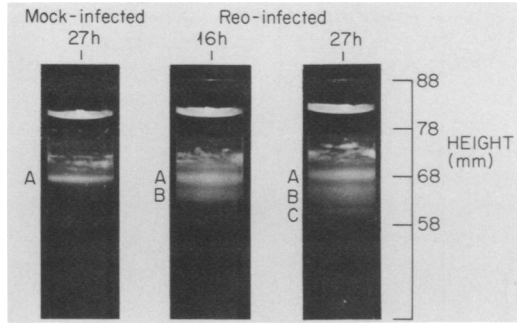

FIG. 3. *Distribution in CsCl gradient of material from R-3 of mock-infected L cells harvested at 27 h and reovirus-infected L cells harvested at 16 and 27 h. Equal amounts of cells were committed to the different samples. The height of the cellulose nitrate tubes used for the SW41 rotor was 88 mm. Conditions of the CsCl density gradient centrifugation are described in the text.*

after infection 40% of maximal amounts of enzymatically active particles were present. Maximal amounts were found at 19 h after infection, and the amount decreased at most by 10 to 20% during the next 8 h.

Glycerol gradient velocity centrifugation. If the particles containing the poly(C)-dependent RNA polymerase activity (fractions 30 to 32 of Fig. 4) were pelleted, further analysis was not possible because the pelleted material could not be disrupted. We were, however, able to analyze the material in the gradient fractions by diluting the CsCl and layering the diluted fraction on a 20 to 40% glycerol gradient in TTX buffer. After centrifugation at 4°C for 38 h at 27,000 rpm in an SW28 rotor, 40 equal fractions were collected. Each was analyzed for ^{35}S-labeled proteins and for poly(C)-dependent RNA polymerase activity by incorporation of [^{32}P]-GMP into poly(C)·poly(G).

Particles with poly(C)-dependent RNA polymerase activity from R-1, R-2, and R-3 sedimented at similar rates, with sedimentation values ranging from 13 to 19S and with peak values at 15 to 16S (Fig. 5). In contrast to the similar distribution of particles with enzymatic activity of R-1, R-2, and R-3, the distribution of ^{35}S-labeled reovirus proteins in the glycerol gradients differed (Fig. 5). In material from R-1, the labeled proteins and particles with active

pH 7.4) and 2× SSC (1× SSC = 0.15 M sodium chloride–0.015 M sodium citrate) and treated with 3 μg of RNase A per ml for 30 min at 37°C, and the remaining acid-insoluble radioactivity was determined after addition of 100 μg of bovine serum albumin. The total acid-insoluble RNAs in each of the three regions are presented in the figure.

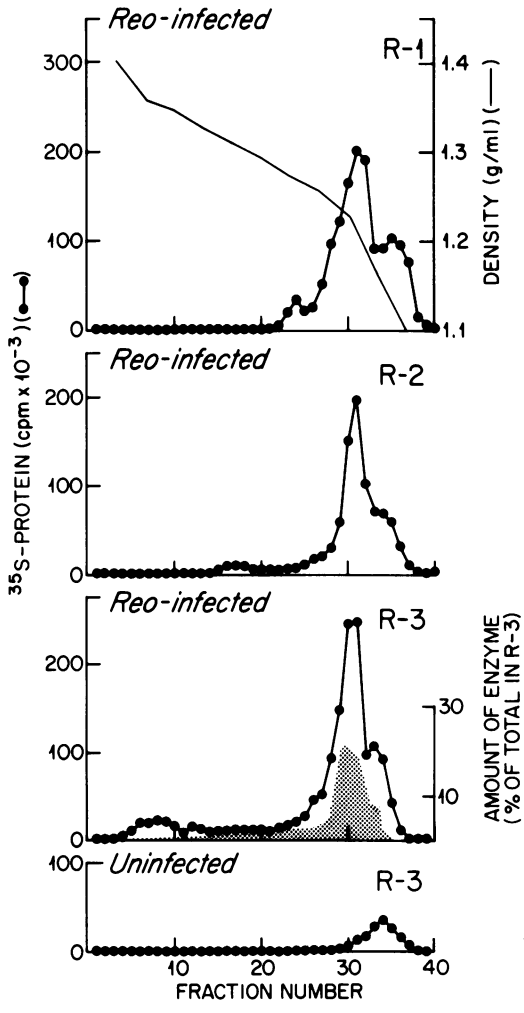

FIG. 4. *Distribution in CsCl density gradients of ^{35}S-labeled reovirus proteins in material from R-1, R-2, and R-3 and distribution of particles with poly(C)-dependent RNA polymerase in material from R-3 of infected L929 cells (shaded area). L929 cells infected with reovirus or mock-infected in the presence of actinomycin D at 0.15 μg/ml were labeled with [^{35}S]-methionine at 2.5 μCi/ml from 15 to 27 h when cells were harvested as described in the text. The cytoplasmic extracts from infected or mock-infected cells each represented the yield from 1.4 × 10^9 cells. The cytoplasmic extracts were overlaid on the 40% glycerol cushions for centrifugation for 3 h at 39,000 rpm as done for Fig. 1. The material in R-1, R-2, and R-3 was collected from above, diluted two- to sixfold with 1/10 × ST buffer, and centrifuged at 4°C in an SW41 rotor at 40,000 rpm for 15 h. The pelleted material was resuspended in 1 ml of TTX buffer, dounced, sonicated, and overlaid on a CsCl density gradient (1.20 to 1.40 g/ml in TTX buffer). The gradients were unloaded by collecting equal fractions of 0.32 ml from above. The radioactive profiles shown are for total radioactivity in a 5-μl portion of each fraction. The bottom panel shows the distribution in the gradient of labeled proteins in R-3 from mock-infected cells. Not shown is that the distribution of labeled proteins in R-2 and R-1 of mock-infected cells was similar to that of R-3 in the bottom panel. Individual gradient fractions were dialyzed to remove CsCl before assay for poly(C)-dependent RNA polymerase activity as described in the text. The shaded area shows the distribution of poly(C)-dependent RNA polymerase in R-3 of infected cells. A similar distribution of enzymatic activity was found in R-1 and R-2 of infected cells. No poly(C)-dependent RNA polymerase activity was found in any fraction from mock-infected cells.*

poly(C)-dependent RNA polymerase cosedimented, and there were few ^{35}S-labeled proteins left at the top of the gradient. In material from R-3, some ^{35}S-labeled proteins also cosedimented with the enzymatically active particles, but most labeled proteins remained close to the top of the gradient, and these were enzymatically inactive. The labeled proteins from R-2 distributed in an intermediate fashion, about equally with the enzymatically active particles and the enzymatically inactive proteins at the top of the gradient. The patterns shown in Fig. 5 were for material from infected L929 fibroblasts, but the same distribution of enzyme and labeled proteins was obtained when similar fractions from infected BHK-21 cells were analyzed. The enzyme in the glycerol gradient fractions can be stored at −28°C for at least 8 months with little or no loss in activity.

Protein composition of particles with poly(C)-dependent RNA polymerase. The ^{35}S-labeled proteins in the enzymatically active fractions from R-1, R-2, and R-3 of Fig. 5 were analyzed in two different gel systems where particular reovirus proteins migrate differently with respect to each other (2, 3, 16–18). In the Laemmli system, the reovirus proteins sigma NS and sigma 2 migrate together, or sigma NS migrates marginally slower. In the Maizel gel system, the migration of sigma 2 is substantially retarded relative to migration of sigma 3, whereas that of sigma NS is not. Secondly, relative to mu 1C, the reovirus structural protein mu 2 migrates marginally slower in the Laemmli system, whereas mu 2 migrates slightly faster than mu 1C in the Maizel gel system. In both gel systems in our laboratory, the migration of mu NS was faster than that of mu 1C.

Whether the particles with poly(C)-dependent RNA polymerase activity were from R-1, R-2, or R-3 of L929 mouse fibroblasts or BHK-21 cells, the protein sigma NS comprised greater than 90% of the ^{35}S-labeled reovirus proteins (Fig. 6) and more than 85% of the proteins in R-2 and R-3 that could be detected by staining with Coomassie blue (Fig. 7). In addition to sigma

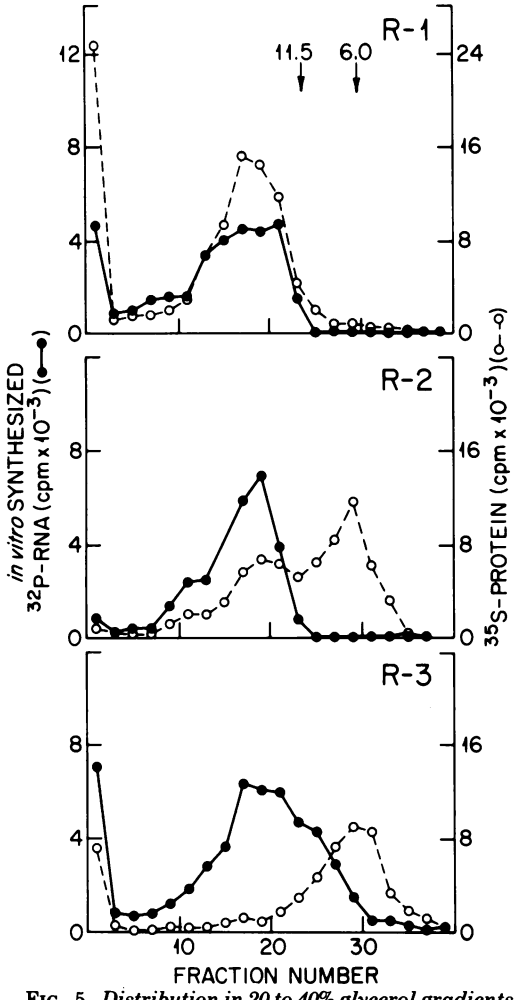

FIG. 5. *Distribution in 20 to 40% glycerol gradients of particles labeled with [^{35}S]methionine of fractions 30 to 32 of Fig. 4 which synthesize poly(C)·poly(G) in vitro in response to poly(C). The material in fractions 30 to 32 from the three regions shown in Fig. 4 was separately combined, diluted sevenfold with 1/10 × ST buffer, and layered over a 20 to 40% glycerol gradient in TTX buffer for centrifugation at 4°C in an SW28 rotor at 27,000 × g for 38 h. Equal fractions of 1 ml were collected from above, and a portion of each equal to 1/20 of each fraction was analyzed for ^{35}S content and for poly(C)-dependent RNA polymerase activity as described in the text. The sedimentation markers shown in the top panel were the two components of catalase centrifuged in a separate tube. M. G. Hamilton kindly performed a separate ultracentrifugal analysis of catalase from Pharmacia in TTX buffer. The analysis showed catalase to contain two components with sedimentation values of 11.5 and 6.0.*

NS, other proteins that were most often, but not always, present in enzymatically active fractions from all three regions of L929 fibroblasts and BHK-21 cells were as follows: (i) the viral structural proteins mu 2 and sigma 3 which were present in varying amounts in different experiments, from undetectable to 4% that of sigma NS, and (ii) a putative host protein, HP of Fig. 7, with a molecular weight of 55,000 to 60,000 which was in extracts from both L929 and BHK21 cells and was present in amounts from undetectable to 4% that of sigma NS. When R-3 glycerol gradient fractions from mock-infected cells equivalent to the enzymatically active fractions of infected cells were pooled, concentrated, and analyzed on gels, no labeled proteins were seen. The major proteins stained with Coomassie blue were the host protein of 55,000 to 60,000 daltons, a protein equivalent in mobility to sigma 3, and a protein of 28,000 daltons. In particular, no labeled or unlabeled protein the size of sigma NS was identified in the glycerol gradient fractions from mock-infected cells.

Morphology of particles with poly(C)-dependent RNA polymerase activity. The material in fractions of the glycerol gradients with maximal poly(C)-dependent RNA polymerase activity was pelleted at 4°C in the SW50.1 rotor at 45,000 rpm for 17 h. Under these conditions, the particles with poly(C)-dependent RNA polymerase activity from R-1, R-2, and R-3 exhibited different stabilities to the conditions of centrifugation. More than 90% of the enzymatic activity and ^{35}S-labeled reovirus proteins from R-1 sedimented to the pellet, whereas the corresponding recoveries in R-2 and R-3 were 50 and 30%, respectively. By electron microscopy, the material pelleted from R-1 of infected L929 mouse fibroblasts appeared homogeneous and contained nearly spherical particles 11 to 12 nm in diameter (Fig. 8A). The material pelleted from R-2 of infected L929 mouse fibroblasts (Fig. 8B) contained a few particles similar in morphology to those found in R-1, but was composed mostly of small granular material that could be interpreted to be subunits of the spherical particles of R-1 and aggregates of the subunits. The material in R-3 from infected L929 cells also contained some of the nearly spherical particles, but it consisted mostly of irregular structures resulting from trace amounts of Triton X-100 remaining in the preparation (Fig. 8C). In material pelleted from the enzymatically active fractions of the glycerol gradient from R-3 of infected BHK-21 cells, many particles again were seen, but these were slightly smaller and were triangular rather than spherical in shape (Fig. 8D).

The nearly spherical particles with an electron-luscent area from R-1 (Fig. 8A) appeared to have a morphology similar to ferritin. However, unlike ferritin, these particles could not be visualized without uranyl acetate staining, and

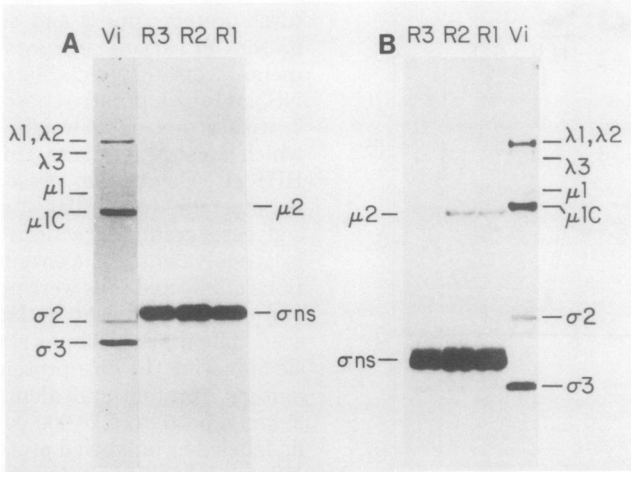

FIG. 6. *Analysis of the ^{35}S-labeled proteins of R-1, R-2, and R-3 from infected L929 mouse fibroblasts present in the glycerol gradient fractions of Fig. 5 with most poly(C)-dependent RNA polymerase activity. (A) Laemmli Tris-glycine system; (B) Maizel Tris-glycine system. The proteins shown represent 3% of total in fraction 19 from R-1, 3% of material in fractions 18 and 19 from R-2, and 5% of total in fractions 18 to 20 of R-3. Preparation of samples, solubilization, conditions for electrophoresis, and processing for fluorography were as described in the text. The ^{35}S-labeled proteins from purified reovirus were used as markers in the two gel systems. Duration of radioautography: 40 h.*

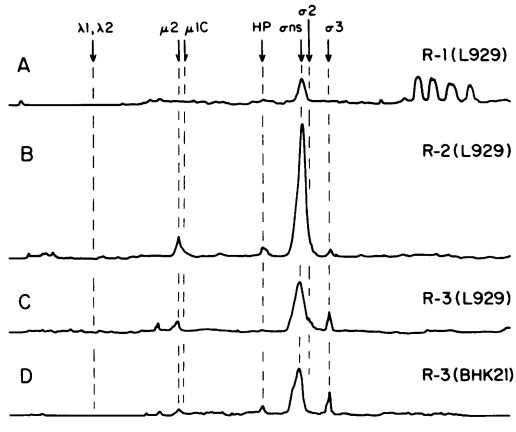

FIG. 7. *Densitometric tracing of migration in the Laemmli gel system of proteins in pooled glycerol gradient fractions with poly(C)-dependent RNA polymerase activity. Glycerol gradient fractions 16 to 24 from the R-1, R-2, and R-3 of L929 cells of Fig. 5 and from a similarly prepared R-3 of BHK-21 cells were separately pooled and centrifuged at 4°C in an SW 50.1 rotor at 45,000 rpm for 17 h. Greater than 90% of total enzymatic activity in R-1, 50% of that in R-2, and 30% of that in R-3 was recovered in the pelleted material. The pelleted material was divided into two unequal portions, with most committed to the electron microscopy shown in Fig. 8 and the remaining for gel analysis here. The conditions for solubilization and electrophoresis in the Laemmli gel system and for staining of gels with Coomassie blue were those described in the text. Densitometric tracings of the stained gels were made at 525 nm. Proteins present in particles with poly(C)-dependent RNA polymerase activity from: (A) R-1 (L929 cells); (B) R-2 (L929 cells); (C) R-3 (L929 cells); and (D) R-3 (BHK-21 cells).*

some particles had an electron-luscent channel extending from the periphery to the center. Moreover, we have determined that the particles do not react in immunodiffusion with known human, horse, or mouse antiferritin sera and that human, horse, and mouse ferritins do not have poly(C)-dependent RNA polymerase activity.

The gel scans in Fig. 7 were for proteins present in each material visualized in Fig. 8. The discrepancy between the amount of protein in the pelleted material from R-1 (Fig. 7A) and the number of particles seen in Fig. 8A is more apparent than real. Less of the pelleted material was available for gel analysis since most was used for comparison with ferritin. The spherical particles of R-1 of L929 cells have associated with them host proteins with molecular weights from 22,000 to 28,000 which were not present in material pelleted from R-2 and R-3 (Fig. 7). Because the material from R-2 and R-3 had some of the spherical structures, it is likely that the major protein of particles with this morphology was sigma NS. What is certain is that the major protein of the granular and triangular structures observed in R-2 from L929 cells and R-3 of BHK-21 cells was sigma NS.

Column chromatography. To establish more firmly that sigma NS and the protein with poly(C)-dependent RNA polymerase activity were identical, we took advantage of a property of both, dependent on characteristics differing from those responsible for sedimentation behavior. The method chosen for further purification was affinity chromatography on the cation ex-

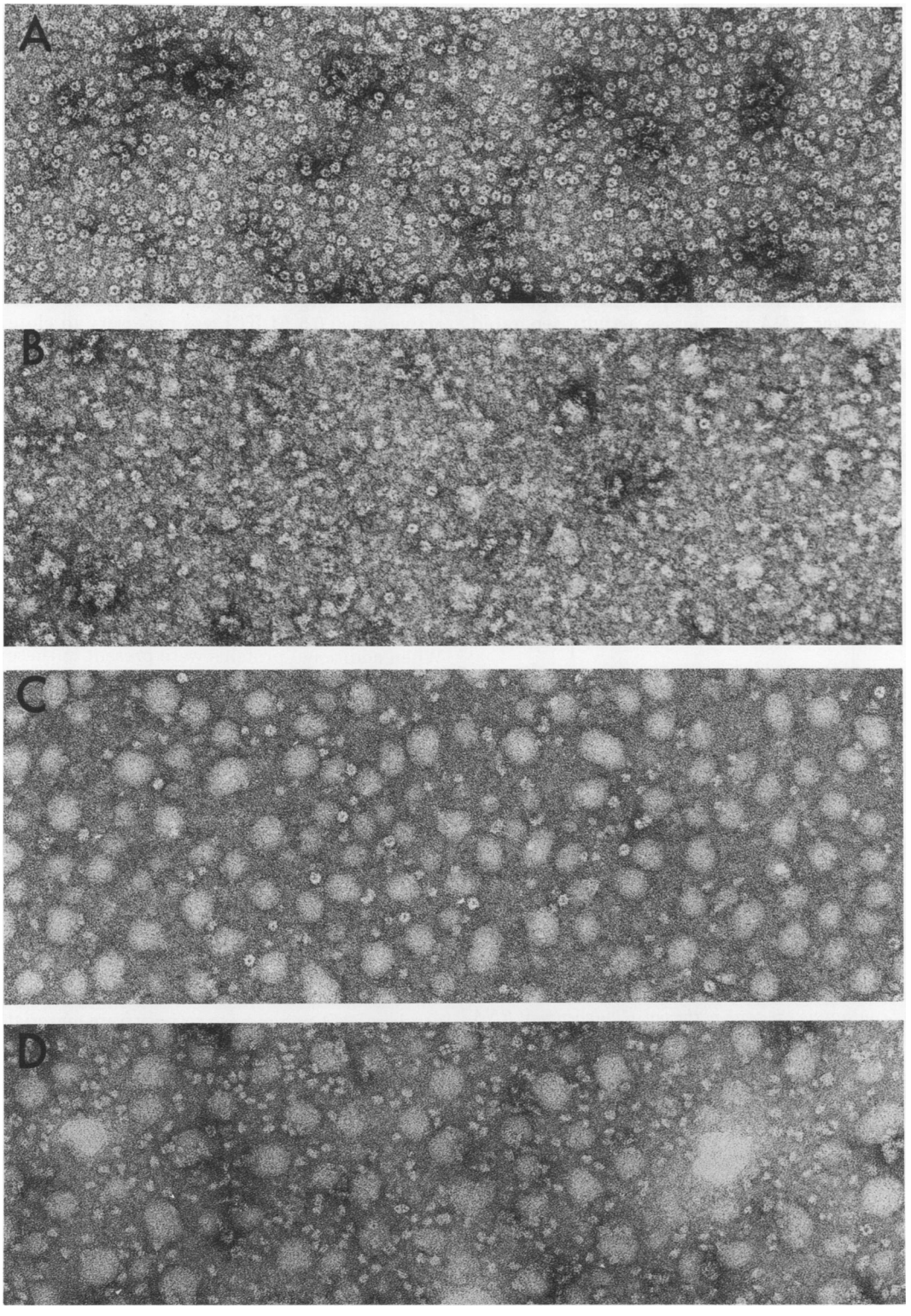

FIG. 8. *Electron micrographs of material with poly(C)-dependent RNA polymerase activity analyzed in Fig. 7. (A) Pelleted from glycerol gradient fractions with enzymatic activity from R-1 (L929 cells); (B) from R-2 (L929 cells); (C) from R-3 (L929 cells); and (D) from R-3 (BHK-21 cells).*

changer CM-Sephadex C-25. Results obtained during chromatography were reproducible, and greater than 90% of ^{35}S-labeled proteins and enzymatic activity was recovered in fractions eluted from the column. Since sigma NS represented more than 90% of the labeled protein in the poly(C)-dependent RNA polymerase purified through the glycerol gradient step, similar behavior of both during chromatography would support the conclusion that sigma NS had poly(C)-dependent RNA polymerase activity. If during chromatography a protein with poly(C)-dependent RNA polymerase activity separated from ^{35}S-labeled sigma NS, the two proteins were clearly different.

The glycerol gradient fractions with active poly(C)-dependent RNA polymerase from R-3 of infected cells were pooled (e.g., those in Fig. 5) and applied to the cation exchanger in the absence of salt. All proteins with enzymatically active poly(C)-dependent RNA polymerase bound to the column and eluted with 0.5 M KCl along with about 40% of the ^{35}S-labeled sigma NS (Fig. 9). The remaining sigma NS either did not bind to the column (40%) or was eluted with 0.1 M KCl (20%), and was enzymatically inactive or minimally active. When analyzed on gels, the mobility and width of the sigma NS band eluting at 0.5 M or 0.1 M KCl or not binding to the cation exchanger were the same (data not shown, but similar to those of Fig. 6).

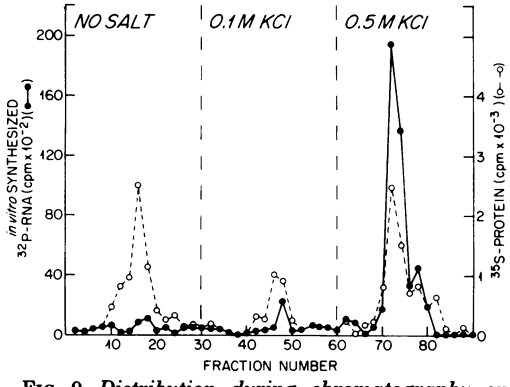

FIG. 9. *Distribution during chromatography on the cation exchanger CM-Sephadex of ^{35}S-labeled sigma NS and protein with poly(C)-dependent RNA polymerase activity. Glycerol gradient fractions 15 to 22 containing the peak of the poly(C)-dependent RNA polymerase activity of material in R-3 prepared similarly to that described for Fig. 5 were pooled and applied to the column equilibrated with 0.001 M HEPES buffer, pH 6.8, containing 5% glycerol and 0.02% Triton X-100 and washed in with the same buffer. Equal fractions of 1 ml were collected at 4°C. Elution was achieved as described in the figure. Flow rate was about 20 ml/h. The eluate fractions were assayed for ^{35}S-labeled reovirus protein and for poly(C)-dependent RNA polymerase activity. When salt was present in the eluate fraction, it was removed before assay for enzymatic activity by chromatography through Sephadex G-50 Fine. The ^{35}S-labeled proteins not binding to the column and eluting at the two salt concentrations were separately pooled, dialyzed against TTX buffer, lyophilized, and analyzed by gel electrophoresis.*

Our results suggest that the reovirus protein sigma NS exists in two states. In its enzymatically active state as poly(C)-dependent RNA polymerase, it is modified to behave as more positively charged than when enzymatically inactive, since 0.5 M KCl was necessary to elute the active sigma NS from the negatively charged CM-Sephadex. The inactive sigma NS only weakly bound or did not bind at all.

DISCUSSION

In this report, we described the isolation of a reovirus-coded polypeptide, sigma NS, in a near homogeneous state in which it represented more than 90% of the protein present. This protein was in a particle sedimenting from 13 to 19S which had poly(C)-dependent RNA polymerase activity, utilizing poly(C) as the template and yielding the ds poly(C)·poly(G) as the product. When additional proteins were present in these particles in glycerol gradients, they were most often two other reovirus proteins, mu 2 and sigma 3, and a host protein of 55,000 to 60,000 daltons. We do not know the configuration of the particles within the glycerol gradient, but when pelleted they formed triangular or spherical particles with a diameter of 11 to 12 nm. Particles with poly(C)-dependent RNA polymerase were not found in mock-infected cells, and they were maximally present by 19 h after infection at 30°C. We had reported previously that the activities of reovirus transcriptase and replicase were maximal at 15 to 18 h after infection (8).

We purified the particles with poly(C)-dependent RNA polymerase activity from material in extracts of infected L929 mouse fibroblasts and BHK-21 cells which had not sedimented through a 40% glycerol cushion to the pellet in 3 h at 190,000 × *g*, but remained at the interface or had penetrated into the cushion. By collecting separately into a pellet the material in different regions of the glycerol cushion, a large amount of soluble host and viral protein was left back in the supernatant fraction. The reovirus ss RNAs and ribosomal components were separated from the enzymatically active particles by CsCl density gradient centrifugation. The subsequent glycerol gradient centrifugation removed a large amount of reovirus proteins which had sedimented to the same region of the CsCl density

gradient. The final step in purification, chromatography on a cation exchanger, separated sigma NS in two states: one enzymatically active in which it bound to the column, and an enzymatically inactive form in which it weakly bound, or did not bind at all, to the column material. Our results suggest that in its enzymatically active state, sigma NS carries a greater net positive charge than when inactive and, moreover, that active and inactive forms of sigma NS can be present in the same particle which is enzymatically active. Thus, inactive sigma NS does not inhibit the enzymatic capacity of the active form of sigma NS.

What else is known about sigma NS? It is the translation product of the reovirus ss RNA segment, s3 (16, 17). The mutant group E, which is ds RNA negative, is mutated in the ds RNA segment from which s3 is transcribed (19). This mutant group is defective in an early function (6). Shortly after adsorption, shift-up of cultures to nonpermissive temperature did not prevent continued synthesis of ds RNA (11). Sigma NS appears to be complexed with the three size classes of reovirus ss RNAs in infected cell extracts (10). When purified, it bound in vitro to reovirus ss RNA, and it also bound to columns to which ribohomopolymers were covalently linked. Finally, sigma NS is one of the four proteins synthesized early in infected cells (14, 21, 22).

Since sigma NS can bind reovirus ss RNAs and we have shown in this report that it has the capacity to copy poly(C) to yield the ds poly(C)·poly(G), it would be tempting to speculate that this protein can also copy bound reovirus ss RNAs and yield ds RNAs.

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