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Reovirus-Specific Enzyme(s) Associated with Subviral Particles Responds In Vitro to Polyribocytidylate to Yield Double-Stranded Polyribocytidylate · Polyriboguanylate

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In reovirus-infected cells, virus-specific particles accumulate that have associated with them a polyribocytidylate [poly(C)]-dependent polymerase. This enzyme copies in vitro poly(C) to yield the double-stranded poly(C) · polyriboguanylate [poly(G)]. The particles with poly(C)-dependent polymerase were heterogeneous in size, with most sedimenting from 300*S* to 550*S*. Exponential increase in these particles began at 23 h, and maximal amounts were present by 31 h, the time of onset of exponential growth of virus at 30°C. Maximal amounts of particles with active transcriptase and replicase were present at 15 and 18 h after infection. Thereafter, there was a marked decrease in particles with active transcriptase and replicase until base line levels were reached at 31 h. Thus, the increase in poly(C)-responding particles occurred coincident with the decrease in particles with active transcriptase and replicase. The requirement for poly(C) as template was specific because no RNA was synthesized in vitro in response to any other homopolymer, including 2′-*O*-methyl-poly(C). Synthesis was optimal in the presence of Mn^{2+} as the divalent cation, and no primer was necessary for synthesis. In contrast, the dinucleotide GpG markedly stimulated synthesis in the presence of 8 mM Mg^{2+}. The size of the poly(C) · poly(G) synthesized in vitro was dependent on the size of the poly(C) used as template. This suggested that the whole template was copied into a complementary strand of similar size. The T_m of the product was between 100 and 130°C. Hydrolysis of the product labeled in [^{32}P]GMP with alkali or RNase T2 yielded GMP as the only labeled mononucleotide. This does indicate that the synthesis of the poly(G) strand in vitro did not proceed by end addition to the poly(C) template, but proceeded on a separate strand.

Complete reovirus contains two protein shells as its coat, and within are the 10 segments of double-stranded RNA that are the viral genome (33, 37). The transcriptase that is present in the viral core (4, 32) uses as template the genome double-stranded RNAs. The replicase that catalyzes the synthesis of the genome double-stranded RNAs uses single-stranded RNAs of only one polarity as templates for synthesis of the complementary RNA strand (1, 27, 30) and has been shown to be associated with subviral precursor particles that are destined to become completed virus (25, 28, 40). During the maturation of virus, the replicase is either inactivated, modified, or removed (1, 41, 43).

The reovirus transcriptase in the viral core synthesizes in vitro and in the infected cell reovirus single-stranded RNAs of only one polarity (12, 16, 37, 42). These RNAs are the mRNA's used as templates for synthesis of viral proteins both in vivo and in vitro (6, 22). They are also templates for synthesis of double-stranded RNAs (27, 30). Five other enzymatic activities have been detected in the virus particle (36). Only one, the polyadenylic acid [poly(A)] polymerase (34, 38), is active when the virus is complete. The remaining four are manifest only in viral cores or subviral particles. Three are concerned with capping and methylation at the 5′ end of the newly synthesized RNA (10). The fourth is a phosphohydrolase (5, 18). The enzyme that catalyzes pyrophosphate exchange with ribonucleoside triphosphates may be the transcriptase acting in the reverse reaction of polymerization (39).

Our experiments have been designed to characterize the replicase by demonstrating that there are components in reovirus-infected cells that can catalyze the synthesis in vitro of RNA in response to exogenously added homopolymers. We presume that the replicase will use

single-stranded homopolymers as templates for synthesis of double-stranded RNA. Here, we report on the accumulation in reovirus-infected cells of a virus-specific particle that responds to added polyribocytidylate [poly(C)] to synthesize double-stranded RNA in vitro.

MATERIALS AND METHODS

Cells and virus. L cells, strain 929, a continuous cell line derived from mouse fibroblasts, were grown at 37°C in suspension in Eagle Spinner medium supplemented with 5% fetal bovine serum (9). The harvest from passage 2 of Dearing strain of reovirus type 3, cloned, was used as stock virus in all the experiments described in this paper. The procedures for growth of labeled virus, plaque assay, and purification of cell-associated virus have been described previously (15, 19).

Preparation of extract from infected cells. L cells grown at 37°C were harvested by centrifugation and infected with stock virus at high multiplicity in the presence of actinomycin D at 0.15 μg/ml of inoculum virus. After adsorption for 3 h at 30°C, the cell suspension was washed once with Eagle Spinner medium and then was resuspended in Eagle Spinner medium containing 7% fetal bovine serum and actinomycin D at 0.15 μg/ml. The infected cells were maintained for specified times in suspension at 30°C. [^{3}H]uridine at 0.5 μCi/ml was added to obtain virus and precursor particles labeled in RNA, or ^{3}H-labeled L-amino acid mixture at 1 μCi/ml was added to label viral proteins. During the labeling of viral proteins, the amino acid content of the growth medium was reduced to 33% of its normal complement. The radioactive labeled precursor was added after viral adsorption.

The procedure for obtaining the cytoplasmic extract and its separation into components followed that described previously by Zweerink (40). At the specified time after infection at 30°C, the cells were pelleted and washed once with ST buffer (0.1 M Tris-hydrochloride–0.01 M NaCl, pH 8.0). The cells were resuspended at a concentration of 7×10^7 to 10×10^7 cells/ml in ST buffer containing 1% Triton X-100 and 1% sodium deoxycholate, and they were ruptured with 25 strokes 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 sediment was treated with 3 ml of the same detergent buffer per 1 ml of nuclear sediment, 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 amount of the cytoplasmic extract was layered over a 4-ml cushion of 40% glycerol (wt/wt in 0.001 M Tris-hydrochloride, pH 8.0) and centrifuged at 4°C in an SW41 rotor for 3 h at 39,000 rpm or for 15 h at 20,000 rpm. The pelleted material was resuspended by homogenizing in ST buffer, glycerol was added to a final concentration of 50%, and the suspension was stored unfrozen at −28°C.

To demonstrate the activities of transcriptase, replicase, and poly(C)-dependent polymerase, the material pelleted above and stored in 50% glycerol was diluted with ST buffer to a final concentration of 12% glycerol. The suspension was sonically treated at 0°C for 3 min at 0.9 A in a Raytheon sonic oscillator, layered over a glycerol gradient, 20 to 40% in 0.001 M Tris-hydrochloride, pH 8.0, and spun for 1 h at 30,000 rpm at 4°C in an SW41 rotor. Equal fractions containing 0.34 ml were collected from below, and each was assayed for enzymatic activity.

Assay for transcriptase and replicase. Both enzymes were assayed in the same reaction mixture, which contained the following components in a final volume of 0.75 ml: 0.34 ml of the glycerol gradient fraction, 0.34 ml of 2× ST buffer, 2.7 μmol of magnesium chloride, 2.7 μmol of 2-mercaptoethanol, 0.3 μg of actinomycin D, 0.3 μmol of the three unlabeled ribonucleoside triphosphates, 0.006 μmol of the fourth ribonucleoside triphosphate, and 3 to 5 μCi of this fourth nucleoside triphosphate labeled with ^{32}P in the alpha position. The reaction mixture was incubated for 1 h at 39°C, after which 0.1 ml of a mixture containing 0.5 μmol of the fourth ribonucleoside triphosphate and 130 μmol of sodium chloride was added and the sample was divided into two equal fractions. One half was incubated for an additional 30 min at 39°C, and the second half was incubated for the same time period in the presence of 20 μg of RNase A. After the second incubation at 39°C, 200 μg of bovine plasma albumin was added to each glycerol gradient fraction, and the reaction was terminated by the addition of 0.1 ml of saturated sodium pyrophosphate and 2 ml of 10% trichloroacetic acid. The precipitated RNA was washed as described previously (12). It was collected on membrane filters (Millipore Corp.), and the amount of radioactivity was determined in a Packard scintillation spectrometer, using a toluene-based scintillation fluid. The amount of double-stranded RNA synthesized in vitro was taken as that amount of RNA synthesized that was resistant to 20 μg of RNase A. The amount of single-stranded RNA synthesized was the difference between total RNA synthesized in vitro and that which was RNase resistant.

Assay for poly(C)-dependent polymerase. The reaction mixture contained the following in a final volume of 0.80 ml: 0.34 ml of the glycerol gradient fraction, 80 μmol of Tris-hydrochloride, pH 8.1, 1.9 μmol of manganese chloride unless otherwise specified, 5.5 μmol of 2-mercaptoethanol, 10 μg of poly(C), 50 μg of guanylyl (3′ to 5′)-guanosine (GpG), 0.005 μmol of GTP, and 3 to 5 μCi of [α-^{32}P]GTP. After incubation for 1 h at 39°C, 0.1 ml of a solution containing 0.5 μmol of GTP and 130 μmol of NaCl was added, and the reaction mixture was incubated for an additional 30 min at 39°C. The reaction was terminated, and the amount of acid-insoluble RNA synthesized was determined as described above. For uniform labeling of the product, GTP at 0.2 μmol in the reaction mixture and of known specific activity was present from the beginning of incubation at 39°C; NaCl was not added.

Analysis of the RNA synthesized in vitro by the poly(C)-dependent polymerase. After synthesis, the glycerol gradient fractions containing the in vitro synthesized product were pooled and brought to 0.3

M in sodium acetate and 0.0001 M in EDTA, and 2 volumes of ethanol were added. After 15 h at −28°C, the precipitate was collected by centrifugation at −10°C for 30 min at 35,000 × *g* in an RC2-B Sorvall centrifuge. The pellet was resuspended in water, extracted with phenol, and chromatographed through a 12-cm column of Sephadex G-50 in water. The material eluting in the void volume was lyophilized and used as a source for analysis of size of product and its sensitivity to nucleases.

Determination of base composition. To remove the small amounts of GDP and GTP associated with the product in the void volume from the chromatography through Sephadex, the sample was resuspended in water. It was layered over a 15 to 30% sucrose density gradient and centrifuged in an SW27 rotor at 4°C for 22 h at 26,000 rpm. The fractions containing the product sedimenting from 8 to 12*S* were pooled for precipitation with 2 volumes of ethanol. The precipitated RNA was rechromatographed through Sephadex G-50 in the presence of water.

The product eluting in the void volume was lyophilized, dissolved in 0.2 ml of 0.3 M KOH, and hydrolyzed for 18 h at 37°C. Potassium ions were removed by chromatography on CM-82 paper (12, 29), and the eluate, to which carrier mononucleotides were added, was spotted on Whatman 3MM paper and subjected to electrophoresis for 4.5 h at 68 V/cm. The buffer used during electrophoresis was 20% acetic acid adjusted to pH 3.5 with concentrated ammonium hydroxide. Digestion with RNase T2 at 10 U/ml was at 37°C for 18 h in 0.05 M ammonium acetate, pH 4.5, and 0.005 M EDTA.

Treatment of the product of the poly(C)-dependent polymerase with nucleases. The heated or unheated product in a final volume of 1 ml of 0.01 M Tris-hydrochloride, pH 7.4, 0.001 M EDTA, and 100 μg of yeast RNA was treated with 10 μg of RNase A and/or 100 U of RNase T1 for 1 h at 39°C. For nuclease treatment in the presence of "high salt," the sample was brought to 0.3 M NaCl before treatment with nuclease.

Density gradient centrifugation. Stock solutions of 15 and 30% (wt/wt) RNase-free sucrose in TNE (0.01 M Tris-hydrochloride–0.1 M NaCl–0.001 M EDTA, pH 7.4) were treated with a coarse magnesium-bentonite fraction (26), and linear gradients were made. The sample was layered over the gradient, and it was centrifuged for 20 to 22 h at 18°C in an SW27 rotor at 26,000 rpm. Fractions containing 1 ml were collected from below, and the amount of acid-soluble radioactivity was determined in each fraction.

Chemical analysis. Protein was determined by the method of Lowry et al. (21) with crystalline bovine plasma albumin used as the standard.

Materials. [^{3}H]uridine, ^{3}H-labeled L-amino acid mixture, and the α-^{32}P-labeled ribonucleoside triphosphates were obtained from New England Nuclear Corp., Boston, Mass. Unlabeled ribonucleoside triphosphates, poly(C) (S_{20} of 8 to 13), and poly-2′-*O*-methylcytidylic acid, potassium, were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. [^{3}H]poly(C) (S_{20} of 4.9) was purchased from Miles Laboratories, Elkhart, Ind. GpG (ammonium salt, A grade), RNase T1 (B grade), and RNase T2 were obtained from Calbiochem, San Diego, Calif. Alpha-chymotrypsin (3× crystallized) and RNase A were obtained from Worthington Biochemicals Corp., Freehold, N.J. Sodium dodecyl sulfate was recrystallized by the method of Mandel (23). Actinomycin D was purchased from Merck Sharp and Dohme, West Point, Pa., sodium deoxycholate was from Schwarz/Mann, Orangeburg, N.Y., Triton X-100 was from Sigma Chemical Co., St. Louis, Mo., and Brij 58 was from Atlas Chemical Industries, Wilmington, Del.

RESULTS

Enzymes associated with virus or precursor particles. As a basis for our study of particles responding to added template, we determined the distribution in glycerol gradients of particles labeled in their RNA during infection and those that contained active replicase, transcriptase, and polyriboadenylic acid [poly(A)] polymerase. Material from cytoplasmic extracts of reovirus-infected cells that pelleted in the ultracentrifuge was sonically treated and fractionated into its components by centrifugation for 1 h in 20 to 40% glycerol gradients. The infected cells had been labeled with [^{3}H]uridine added after adsorption and present throughout the infection at 30°C until harvest at 18 h. As markers for the size of the different particles, we used purified reovirus labeled in its proteins with ^{3}H-amino acids and subviral particles similarly labeled, obtained by treatment of purified virus with chymotrypsin in the presence of phosphate-buffered saline (8) (Fig. 1A). Purified virus sedimented at 630*S* (14), and the subviral particles sedimented to the same position as the top component from infected cells, namely 470*S* (data not shown; 24). The subviral particles produced were active in synthesis in vitro of single-stranded RNAs (17, 31).

Figure 1C shows that most particles labeled with [^{3}H]uridine sedimented to the region of the gradient that encompassed an *S* value of 630*S*. The distribution of these particles in the gradient was broader than that of purified virus (Fig. 1A and C). This would indicate that nearly completed or immature virus particles were most likely present in those fractions in addition to completed virus.

The capacity of particles in each gradient fraction to synthesize RNA in vitro in the presence of four ribonucleoside triphosphates is shown in Fig. 1B. As reported previously (40, 41), reovirus precursor particles synthesizing in vitro single- or double-stranded RNAs are heterogeneous in size. The in vitro synthesized RNA that was RNase resistant was believed to reflect the activity of the replicase in particles

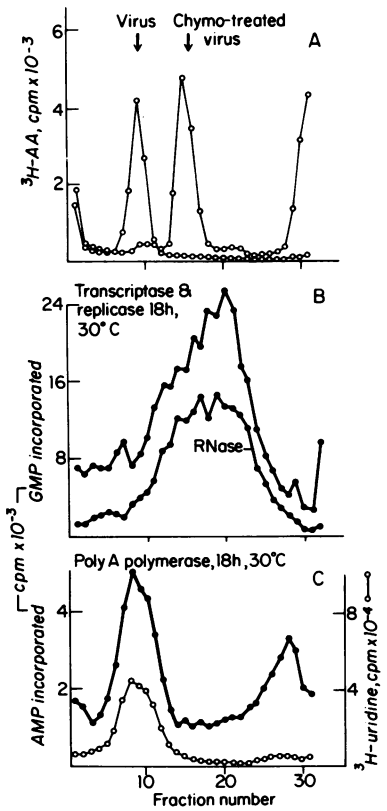

FIG. 1. *Distribution in 20 to 40% glycerol gradients of: (A) purified virus, untreated or treated with chymotrypsin; (B) particles with active transcriptase or replicase; and (C) particles with active poly(A) polymerase. Conditions of centrifugation were in 20 to 40% linear glycerol density gradients in an SW41 rotor at 4°C for 1 h at 30,000 rpm. (A) Distribution in gradient of purified reovirus labeled with ^{3}H-amino acids added after adsorption and maintained at 37°C until harvest at 22 h. The virus was purified as described previously (19). For chymotrypsin treatment, 25 μg of purified virus was treated with 50 μg of alpha-chymotrypsin for 1 h at 39°C in phosphate-buffered saline (8). (B and C) The material analyzed for each enzyme was that which pelleted through the 40% glycerol cushion from cytoplasmic extracts prepared from cells infected with reovirus for 18 h at 30°C. The particles with active replicase and transcriptase were from material containing 1,600 μg of total protein layered on top of the gradient, and those with active poly(A) polymerase were from 2,900 μg of total protein layered on top of the gradient. The assay mixture and conditions of incubation for the replicase and transcriptase are those in the text that describe synthesis in the presence of limited, but highly radioactive, GTP during the first 60 min at 39°C, after which unlabeled GTP and NaCl are added for an additional 30 min at 39°C. The procedure for assay of the poly(A) polymerase is as follows. In a final volume of 0.80 ml were 0.34 ml of each gradient fraction, 80 μmol of Tris-hydrochloride (pH 8.1), 1.9 μmol of manganese chloride, 5.5 μmol of 2-mercaptoethanol, 0.005 μmol of ATP, and 1 μCi of [α-^{32}P]ATP. Incubation was for 1 h at 39°C, after which were added 0.5 μmol of ATP and 130 μmol of NaCl for an additional 30 min at 39°C. The amount of acid-insoluble material in each gradient fraction was determined as described in the text.*

in the particular gradient fraction, and the difference between the total amount of RNA synthesized and that which was RNase resistant reflected the activity of the transcriptase in the same fraction of the gradient. When analyzed at 18 h after infection at 30°C, 60% of the particles synthesizing single-stranded RNAs sedimented between tubes 11 and 25, from 600*S* to 210*S*. Eighty percent of the particles synthesizing double-stranded RNAs were distributed in the same tubes.

The distribution in the gradient of particles with active poly(A) polymerase was different from that of particles with active replicase and transcriptase (Fig. 1C). Synthesis in vitro was in the presence of [α-^{32}P]ATP and Mn^{2+}, which is the preferred divalent cation for the poly(A) polymerase, in contrast to Mg^{2+}, which is used to assay for replicase and transcriptase. Particles with active poly(A) polymerase were distributed in the gradient with the same heterogeneity as most of the in vivo labeled reovirus particles that are completed or nearly completed virus. Of note is the finding that particles with the most transcriptase and replicase activities had little or no poly(A) polymerase activity. When a similar analysis was done on cells infected for 24 h at 30°C, the particles in tubes 5 through 12 synthesized twice the amount of product that was synthesized in the 18-h sample because there were more virions, complete and immature, present at 24 h than at 18 h. There was acid-insoluble material labeled with ^{32}P from [^{32}P]ATP at the top of the gradient shown in Fig. 1C. However, the amount of ^{32}P in this material did not increase from 18 to 24 h, and it was not studied further.

Response to homopolymers of particles in reovirus-infected cells. In our study of the RNA-synthesizing capacity of the particles of different sizes present in the extracts from reovirus-infected cells, we wanted to define whether there were any components that could synthesize RNA under the direction of exogenously added homopolymers as template, poly(A), polyribouridylate [poly(U)], polyriboguanylate [poly(G)], and poly(C). It was known that the gradient fractions containing completed virus or virus near completion could syn-

thesize poly(A) in the absence of added template RNA (Fig. 1C). In the absence of added template, there was no enzymatic activity present in material in any of the gradient fractions shown in Fig. 1B or C, which incorporated UMP, CMP, or GMP from the respective triphosphate into an RNA that was acid insoluble.

When homopolymer was added to the reaction mixture and synthesis was allowed to proceed in the presence of the respective complementary nucleoside triphosphate, there was a response to synthesize RNA with only one, poly(C). With poly(G) and poly(A), no RNA was synthesized by material in any fraction. With poly(U) as template, the pattern of synthesis was identical to that shown in Fig. 1C, which demonstrated that no RNA was synthesized in addition to the poly(A) synthesized in the absence of added template. Figure 2 shows the capacity of material in each gradient fraction from the cytoplasm of reovirus-infected cells to incorporate GMP into RNA in response to added poly(C). There was material in almost every gradient fraction that responded to added poly(C). The distribution of particles in the gradient with poly(C)-dependent polymerase was thus heterogeneous, with most sedimenting from 300*S* to 550*S*. The material in the peak fraction, tube 15, had a sedimentation value of 460*S*. Substitution of GTP in each reaction mixture by GDP resulted in no synthesis of RNA. No other nucleoside triphosphate was incorporated in response to poly(C), and the synthesis was unaffected by the presence of UTP and CTP in addition to GTP in the reaction mixture. In particular, there was no synthesis of labeled RNA when [α-^{32}P]CTP and unlabeled GTP were used. In the presence of the four nucleoside triphosphates, virus-specific RNAs were synthesized (Fig. 1B). Under suitable conditions, 2′-*O*-methyl-poly(C) [poly(rC_m)] can be used as template by the reverse transcriptase from tumor viruses (11). Figure 2 shows that there was no synthesis in vitro of RNA by the enzyme in the particles from reovirus-infected cells when poly(rC_m) was used as template. This confirms the specificity of the response to poly(C). In addition, if poly(C) and poly(rC_m) were both present in the same reaction mixture, they did not compete for the binding site of the poly(C)-dependent polymerase as the amount of product synthesized in response to poly(C) was identical whether or not poly(rC_m) was present.

New protein synthesis was required for the appearance at 30°C of the particles responding to poly(C), as none appeared during infection when cycloheximide was present at 20 μg/ml

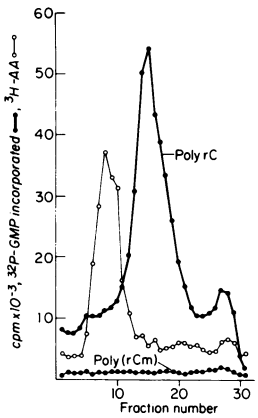

FIG. 2. *Distribution in 20 to 40% glycerol gradients of particles synthesizing RNA in vitro in response to poly(C) or poly(rC_m). The conditions of centrifugation and analysis were those described in the legend to Fig. 1, except that the material analyzed was from reovirus-infected cells labeled with ^{3}H-amino acids after adsorption until harvest at 24 h. The particles that responded to poly(C) were from material containing 2,400 μg of protein layered on each gradient. The conditions for assay of the poly(C)-dependent polymerase are detailed in the text. Incubation was identical to that described in the legend to Fig. 1, with both unlabeled GTP and NaCl added at 60 min for the final 30 min of incubation.*

from the beginning of infection. There were also no particles in uninfected cells that synthesized RNA in vitro in response to the addition of poly(C). Purified complete reovirus cores derived after treatment of purified virus with chymotrypsin in low salt (3, 13, 17, 31) or subviral particles obtained after chymotrypsin treatment in phosphate-buffered saline (8, 22) did not synthesize RNA in response to poly(C) in the presence of [α-^{32}P]GTP. Moreover, subviral particles derived from inoculum virus in cells infected in the presence of cycloheximide (7, 20, 35) were active in the synthesis of reovirus single-stranded RNAs in the presence of the four nucleoside triphosphates, but they were inactive in response to poly(C) in the presence of only GTP. These results indicate that the particles responding to poly(C) shown in Fig. 2 were not virus or any of the well-characterized particles derived from virus.

Effect of Mn^{2+}, Mg^{2+}, and the primer GpG on the response to poly(C). Optimal synthesis occurred over a broad range of concentrations of Mn^{2+} from 3 to 6 mM. Synthesis in the presence of 2.5 mM Mn^{2+}, which was used for all experiments presented in this paper, was 10 to 15% lower than what was synthesized optimally. Figure 3 shows that the amount of product

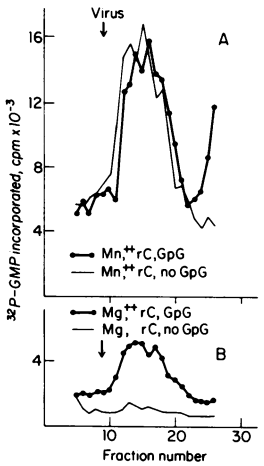

FIG. 3. *Synthesis of RNA in response to poly(C) by particles in the 20 to 40% glycerol gradient in the presence of 2.5 mM Mn^{2+} (A) or 8 mM Mg^{2+} (B) and the effect of GpG added as primer during synthesis. Conditions of centrifugation were in 20 to 40% linear glycerol density gradients as described in the legend to Fig. 1. The material analyzed was from cells infected with reovirus and labeled with ^3H-amino acids for 2.5 h before harvest at 24 h. Layered on top of each gradient was material containing 560 μg of protein. The enzymatic activity was tested in particles distributed in tubes 5 through 26. Conditions of assay and incubation were those described in the legend to Fig. 1. Where indicated, 50 μg of GpG was present in each gradient tube during RNA synthesis. To correct for the variation in the amount of material layered on each gradient, the counts per minute in [^{32}P]RNA synthesized in vitro were adjusted so that each gradient contained the same amount of radioactivity in ^3H.*

synthesized was at least four times higher when synthesis proceeded in the presence of 2.5 mM Mn^{2+} than in the presence of 8 mM Mg^{2+} and 50 μg of GpG. Addition of the primer GpG to the reaction mixture in the presence of 2.5 mM Mn^{2+} did not significantly alter the amount of product synthesized in vitro (Fig. 3A). In contrast, response to poly(C) was minimal in the presence of 8 mM Mg^{2+} when no primer was present in the reaction mixture. Addition of 50 μg of GpG markedly stimulated the capacity of the particles to synthesize RNA (Fig. 3B). A primer was thus necessary for the synthesis of product in the presence of Mg^{2+}. More than 50 μg of GpG did not increase further the amount of product synthesized in the presence of 8 mM Mg^{2+}. Primers longer than GpG have not been tested, and it was not determined whether the primer was incorporated into the product. However, the size of the product synthesized in the presence of Mn^{2+} with or without added GpG and in the presence of Mg^{2+} and GpG was identical (see below).

Kinetics of synthesis of product by particles responding to poly(C). Figure 4 shows the kinetics of synthesis of RNA by particles sedimenting from 300S to 550S. The amount of product was obtained by summing the total RNA synthesized at each time point by particles in gradient tubes 12 through 20 (gradient similar to that shown in Fig. 2). Synthesis occurred at a maximal rate during the first 10 min and at a slightly reduced rate from 10 to 40 min. After that, the rate of synthesis increased more slowly up to 4 h.

Kinetics of appearance during infection of particles responding to poly(C). To quantitate the activities of replicase, transcriptase, and poly(C) polymerase, equal amounts of cells were harvested at different times after infection, and cytoplasmic extracts were prepared and fractionated in 20 to 40% glycerol gradients as before. The respective enzymatic activities of material in each fraction were determined. As shown for the 18- and 24-h samples in Fig. 5, the total activity of replicase and transcriptase decreased 50 to 75% from 18 to 24 h. In contrast, the activity of particles responding to poly(C)

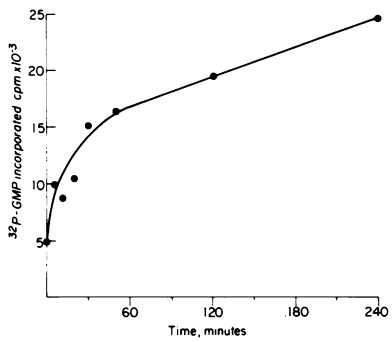

FIG. 4. *Time course of GMP incorporation by particles sedimenting from 300S to 550S that were present in gradient fractions 12 through 20. Conditions of centrifugation were in 20 to 40% linear glycerol density gradients as described in the legend to Fig. 1. The material analyzed was from cells infected with reovirus and labeled throughout infection with ^3H-amino acids before harvest at 24 h. Material containing 1,600 μg of protein was committed to each of seven gradients, and one gradient was used for each time point analyzed. The conditions for assay of poly(C)-responding particles are those described in the text, except that GTP was present in each tube at 0.2 μmol and a specific activity of 62,270 cpm/nmol from the beginning of incubation at 39°C and no NaCl was present. The [^{32}P]GMP incorporated was adjusted so that each gradient contained the same amount of particles labeled with ^3H.*

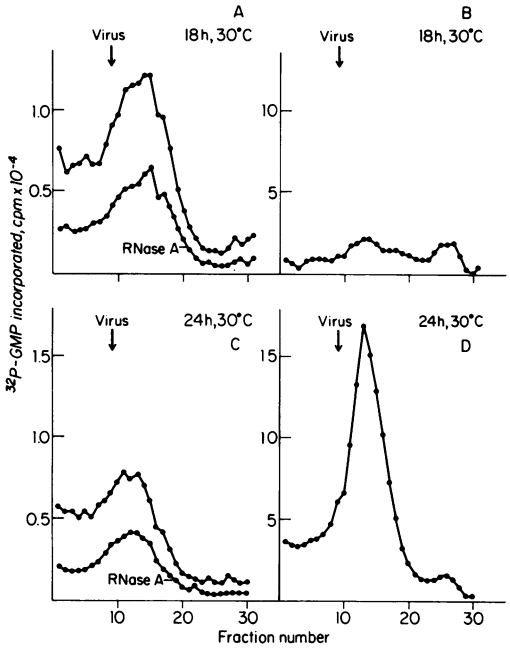

FIG. 5. *Distribution of particles with active replicase, transcriptase, and poly(C) polymerase in 20 to 40% glycerol gradients in material from cytoplasmic extracts of cells infected with reovirus and harvested at 18 and 24 h after infection at 30°C. The conditions of centrifugation and analysis were those described in the legends to Fig. 1 and 2. The particles with active replicase, transcriptase, and poly(C) polymerase were from material containing 1,400 μg of protein layered on top of each gradient. [³H]uridine was present in the growth medium from the end of adsorption until harvest.*

was small at 18 h, and there was a marked increase between 18 and 24 h.

Figure 6 shows similar data for the harvest at each time point and the growth curve of reovirus at 30°C in L cells. We concentrated on the period from 15 h after infection at 30°C until maximal amounts of virus were produced as the changes in poly(C) response occurred during that time period. For this kinetic analysis, we compared the amount of product synthesized at each time point by particles sedimenting from 300S to 550S. The activities of transcriptase and replicase were maximal at 15 and 18 h after infection. Thereafter, there was a marked decrease until base line levels were reached at 31 h, the time of onset of exponential growth of virus. Simultaneous with the onset of the decrease in activities of transcriptase and replicase, there appeared particles responding to poly(C). The exponential increase in poly(C)-responding particles began at 23 h, and maximal activity was present by 31 h, the beginning of exponential increase in virus.

Nature of the product of the poly(C)-responding particles. Thus far, the amount of RNA synthesized in vitro was taken as the amount of radioactive label incorporated into an acid-insoluble product from [α-^{32}P]GTP. That this was a true estimate was verified by direct analysis of the product. When the product synthesized in vitro was collected by ethanol precipitation from the whole assay mixture, followed by phenol extraction and subsequent chromatography through Sephadex G-50, from 1 to 3% of the ethanol-precipitated material labeled with radioactivity eluted in the void volume during the chromatography. This 1 to 3% was, however, equal to 35 to 100% of the amount of radioactive RNA indicated by precipitation with trichloroacetic acid (e.g., that shown in Fig. 2 and 3). It is this RNA in the void volume from chromatography that was analyzed (see below) for size and nature. The radioactive material that was retarded during the chromatography consisted of equal amounts of GTP and GDP that were precipitated by ethanol along with the particles and the in vitro

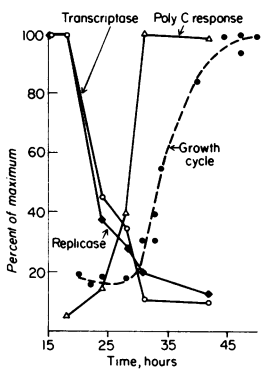

FIG. 6. *Kinetics of appearance during infection at 30°C of particles with active replicase, transcriptase, and poly(C) polymerase and the growth cycle of reovirus in L cells at 30°C. The conditions of centrifugation, analysis, and in vitro RNA synthesis were those described in the legends to Fig. 1 and 2. Equal numbers of infected cells were harvested at each time point for the analysis. After adsorption, the cells were washed three times with growth medium to remove unadsorbed inoculum virus before further incubation at 30°C. The particles with active replicase, transcriptase, and poly(C) polymerase were from material containing 850 μg of protein layered on top of each gradient. At each time point, 3 to 5 ml of infected cell suspension was removed and frozen and thawed three times. Nuclei and cell debris were removed by low-speed centrifugation, and the amount of virus in the supernatant fluid was determined by plaque assay (19).*

synthesized product. It is presumed that the GDP resulted from the activity of the phosphohydrolase known to be present in reovirus particles and putatively present in the precursor particles in the gradient fractions.

We determined the size of the product, eluting in the void volume during chromatography, by sucrose density centrifugation, using the double-stranded reovirus RNA segments as markers (2, 33). Most of the product sedimented between 10.5 and 12S when the poly(C) used in the assay mixture had an S value of 8 to 13 (Fig. 7A). The size of the product was identical

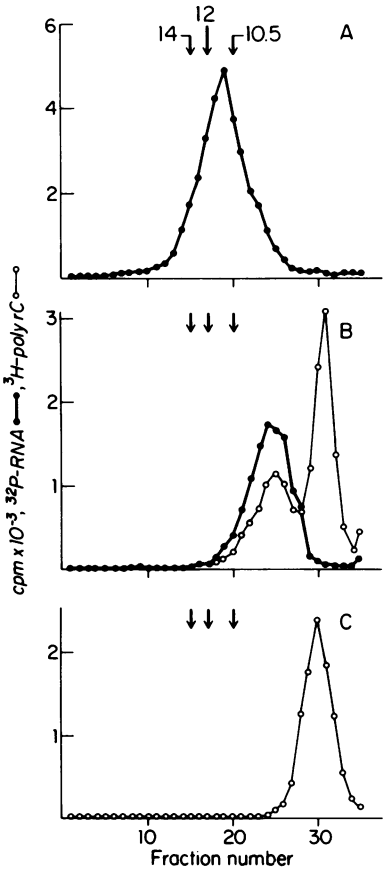

FIG. 7. *Size in sucrose density gradients of product synthesized in vitro, using poly(C) of two different sizes as template. The cells were harvested at 24 h after infection at 30°C. The particles with active poly(C) polymerase were from material containing 1,000 μg of protein layered on two 20 to 40% glycerol gradients. The particles used for synthesis were those that were distributed in tubes 12 through 20, from 300S to 550S. Conditions for synthesis were those described in the legend to Fig. 4. GTP was added to each gradient fraction at 0.2 μmol, and it had a specific activity of 52,600 cpm/nmol. Poly(C) (S_{20} of 8 to 13) was used as template for the product shown in (A), and [^{3}H]poly(C) (S_{20} of 4.9) was used as template for the synthesis of the product shown in (B). Synthesis was for 2.25 h at 39°C. After synthesis, the material in tubes 12 through 20 was pooled and brought to 0.3 M in sodium acetate and 0.0001 M EDTA, and 2 volumes of ethanol were added for precipitation at −28°C. The procedure for the preparation of product for determination of size as outlined in the text includes extraction with phenol and chromatography through Sephadex G-50. (C) Sedimentation of [^{3}H]-poly(C) before it was used as template for the synthesis of the product shown in (B). Before centrifugation, all samples were resuspended in 0.001 M EDTA, and sodium dodecyl sulfate, Brij 58, and EDTA were added to final concentrations of 1.95%, 0.5% and 0.005 M, respectively. Centrifugation in 15 to 30% sucrose density gradients was in an SW27 rotor at 18°C for 22.25 h at 26,000 rpm. Equal fractions of 1 ml were collected from below, and 100 μg of bovine plasma albumin and 2 ml of 10% trichloroacetic acid were added. Acid-insoluble material was collected on membrane filters (Millipore Corp.), and the radioactivity was determined in a Packard scintillation spectrometer.*

if 50 μg of GpG was present during the synthesis in 2.5 mM Mn^{2+} or if synthesis proceeded in 8 mM Mg^{2+} and 50 μg of GpG. When [^{3}H]poly(C) (S value of 4.9) was used as template for synthesis, the product was smaller. It sedimented between 6 and 8S (Fig. 7B). Figure 7C shows the sedimentation profile of the [^{3}H]poly(C) before synthesis. The profile for the [^{3}H]poly(C) shown in Fig. 7B was different in that much of the template poly(C) after synthesis sedimented with the product labeled with [^{32}P]GMP. These results indicated that the size of the product depended on the size of the template poly(C) used during RNA synthesis and that the poly(C) was a component of the product. The results suggested that the product was poly(C)·poly(G) in a double-stranded configuration.

To confirm that the product was double-stranded poly(C)·poly(G), we determined its sensitivity to RNase T1 and RNase A before and after heating the product in 0.001 M EDTA at 100 or 130°C. Sensitivity of double-stranded RNAs to nuclease is markedly influenced by the ionic environment (2) in which hydrolysis occurs in that undenatured reovirus RNAs in 0.001 M EDTA were hydrolyzed to completion by RNase A. In contrast, when suspended in 0.3 M NaCl and 0.001 M EDTA, they were completely resistant to hydrolysis by the same concentration of RNase A. Table 1 shows that, when not heated, the poly(G) strand of the product in 0.001 M EDTA was completely resistant to hydrolysis by RNase T1. In the same ionic environment, it was, however, almost

TABLE 1. *Effect of nucleases on poly(G) strand of product*[a]

Enzyme	0.3 M NaCl	Nuclease resistant (% of total)		
		Unheated	Heated to 100°C	Heated to 130°C
None	–	100	100	100
RNase T1	–	100	100	3
RNase T1 + A	–	5	11	1
	+	100	75	17

[a] The products in 0.001 M EDTA that were analyzed were those shown in Fig. 7A and B. One portion was left unheated, and two other equal portions were placed into sealed vials that were heated for 10 min at 100 or 130°C in a mineral oil bath. After heating, the samples were plunged into an ice-water mixture. The conditions for nuclease treatment are outlined in the text. After each treatment, the samples were layered on sucrose density gradients and centrifuged as stated in the legend to Fig. 7. The amount of radioactivity in acid-insoluble material at the position of untreated product (shown in Fig. 7A and B) that had remained nuclease resistant was determined. The figure of 100% represents 10,000 cpm in [^{32}P]GMP incorporated into the in vitro synthesized RNA.

completely hydrolyzed by the combined activities of RNase T1 and RNase A. In separate experiments, we found that when [^{3}H]poly(C) was used as template, the labeled poly(C) strand in the product was also resistant, as expected, to hydrolysis by RNase T1, but it was completely hydrolyzed by RNase A in 0.001 M EDTA. These data would suggest that the poly(G) strand of the product was hydrolyzed by RNase T1 only when the poly(C) strand of the product was simultaneously hydrolyzed by RNase A.

The secondary structure of the product also remained essentially intact when it was heated to 100°C because the poly(G) strand was still completely resistant in 0.001 M EDTA to hydrolysis by RNase T1. The poly(G) strand was only completely hydrolyzed in 0.001 M EDTA by RNase T1 alone when the product had been heated to 130°C. This indicated that, at 130°C, the two strands of the product had separated and that the melting temperature of the product was thus between 100 and 130°C. That the poly(G) strand of the product was hydrolyzed even in 0.3 M NaCl by RNase A and RNase T1 only if it had been denatured at 130°C confirms that the strands had separated (Table 1).

When the product containing 100,000 cpm in [^{32}P]GMP was hydrolyzed with alkali or RNase T2 and its base composition was determined, GMP was the only labeled mononucleotide. In particular, CMP was not labeled. This would indicate that there were no CpG linkages in the product, which would have been expected if the poly(G) synthesis had begun by end addition to the poly(C) template. This confirms that the poly(G) strand of the in vitro synthesized product was a strand separate from the template.

DISCUSSION

We have found that particles that synthesize RNA in response to poly(C) accumulate at 30°C in the cytoplasm of reovirus-infected cells. Most of the particles sedimented from 300S to 550S. The particles responding to poly(C) were distributed in the same size range and with the same heterogeneity as those present at earlier times during infection with replicase and transcriptase active in the absence of added template. The template poly(C) is single stranded, and the product synthesized in vitro by the particles was poly(C)·poly(G), a double-stranded RNA. As yet, we do not know whether we are detecting the activity of the replicase, transcriptase, or some other enzyme. We presume that the enzyme responding to poly(C) is the replicase because the templates for both enzymes are single-stranded RNAs and the products of both are double-stranded RNAs. In contrast, the templates for the transcriptase are the genome double-stranded RNAs, and the products synthesized in vitro are reovirus single-stranded RNAs of one polarity.

The particles responding to poly(C) were virus specific as no particles were present in uninfected cells and no particles were found when infection proceeded in the presence of cycloheximide. In addition, the response to poly(C) was specific as the particles did not respond to any of the other homopolymers tested, including poly(rC_m). The response to only poly(C) suggested that there may be a poly(C) sequence in one of the strands of the double-stranded reovirus RNAs, most likely in the strands with the same polarity as mRNA's that are the templates for double-stranded RNA synthesis. Alternatively, only poly(C) among the homopolymers can assume in solution the same configuration as the sequence in reovirus single-stranded RNAs to which the replicase binds and begins the replication of the template into the double-stranded reovirus RNAs.

If there were a poly(C) segment present in either strand of reovirus double-stranded RNAs, it would be resistant to hydrolysis by RNase T1. In preliminary experiments, we determined the size of the T1-resistant fragments obtained from denatured reovirus double-stranded RNAs uniformly labeled with ^{32}P added after adsorption and present throughout infection. The size of the T1-resistant fragments was obtained by electrophoresis in 15% polyacrylamide gels in the presence of 8 M urea (29). We found that the maximal size of the

resistant fragments was 20 to 24 nucleotides, the smallest size that we could detect under our experimental conditions. We concluded that if an oligocytidylic acid sequence was present in either strand of reovirus double-stranded RNAs, this sequence was smaller than 20 to 24 nucleotides.

An exponential increase in particles responding to poly(C) occurred 7 h before the onset of exponential growth of virus, and the maximal amount of responding particles had accumulated by the onset of exponential growth of virus. Coincident with the appearance of poly(C)-responding particles, there occurred a marked decrease in numbers of precursor particles, with replicase and transcriptase active in the absence of added template. This suggests to us that there may be a limited and defined number of reovirus single-stranded RNAs committed to be templates for double-stranded RNA synthesis and that, from 18 to 31 h, excess precursor particles continue to be assembled for which a deficient amount of templates is available. It is these putative partially or completely empty precursor particles that we presume are responding to added poly(C).

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