Origin of Reovirus Oligo(A)

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Reovirus contains about 1,200 molecules per virion of oligo(A) of chain length 10 to 15 nucleotides in addition to the 10 double-stranded genome segments. Virions purified from infected BHK, HeLa, or L cells had similar amounts of oligo(A) of the same composition, indicating that it is a virus-specific product. Although conversion of virions to cores by chymotryptic digestion resulted in an almost complete loss of oligo(A) and a marked decrease in infectivity, the infectivity could be partially restored by adsorbing cores to cells in the presence of Kaopectate. Core-infected cells yielded virions that contained a normal complement of oligo(A). The results indicate that oligo(A) is not essential for infectivity or required as a primer/template for its own synthesis.

In addition to 10 double-stranded (ds) RNA genome segments, reovirus contains two classes of single-stranded (ss) RNA (3, 25, 32). One class comprises 2,500 oligomers per virion of chain length two to nine nucleotides and includes pyrimidine-rich oligomers terminated at their 5' end with (p)ppG. A second class of ss RNA consists of 1,200 molecules per virion of oligo(A) of chain length 10 to 15 nucleotides with 5'-terminal pppA, ppA, or pA. Treatment of purified virus with chymotrypsin degrades the outer structural protein and results in the loss of both classes of oligomers and a decrease in specific infectivity (15). A constant amount of oligo(A) is present in virions grown at 31 to 40 C, whereas a reduced amount of pyrimidine-rich oligomers is obtained in virions grown at 31 or 34 C (18). These results suggest that oligo(A) may be required for infectivity and led us to examine the origin and possible role of oligo(A) in the replicative cycle of reovirus.

MATERIALS AND METHODS

Virus and cells. Reovirus type 3 (Dearing strain) was propagated in suspension cultures of L (mouse), HeLa S3 (human), and BHK 21 (hamster) cells, the latter generously provided by D. T. Rubin, Rutgers Medical School. Virus was purified by fluorocarbon extraction and isopycnic sedimentation in CsCl as described previously (2, 29).

Purification of cores. Purified reovirus (500 μ g/ml) was digested with chymotrypsin (200 μ g/ml) in 0.05 M Tris buffer (pH 8.0) for 1.5 h at 41 C. The resulting cores were purified either by sedimentation (27,000 rpm, 45 min, 5 C, SW50 rotor) onto a 1.5-ml cushion of CsCl solution (in 0.01 M Tris buffer [pH 8.0]; $\rho = 1.50$ g/ml) or through a 0.5-ml layer of CsCl

($\rho = 1.40$ g/ml) onto a 1.5-ml denser cushion of CsCl ($\rho = 1.50$ g/ml). The core band was removed from the side of the tube with a syringe to reduce contamination by material above and below the band. The cores had a buoyant density of $\rho = 1.44$ to 1.46 g/ml in CsCl (35,000 rpm, 18 h, 5 C, SW50 rotor) and consisted of polypeptides λ_1 , λ_2 , and σ_2 when solubilized and analyzed by electrophoresis in 7.5% polyacrylamide gels in 0.1% sodium dodecyl sulfate as described previously (29). [³H]adenosine-labeled cores were prepared from purified virions grown in the presence of [³H]adenosine (5 μ Ci/ml), and ³H-labeled RNA was extracted with phenol:chloroform (1:1) (27). The presence of oligo(A) was assayed by binding to poly(U)-impregnated filters (31) and gel filtration in Sephadex G-100. Both methods, used as described previously (32, 34), differentiate between ds RNA and oligo(A).

Infection of cells with cores or virions. Cores, purified by centrifugation through two CsCl layers as described above, and purified virions were dialyzed against 0.01 M Tris buffer (pH 8.0) for 18 h at 5 C. The protein content of particle preparations was measured by the method of Lowry et al. (22), and infectivity was assayed as described previously (29). Cores or virions (added multiplicity of infection = 2 PFU/cell) were adsorbed at a cell density of 10^6 to 2×10^6 /ml in Eagle medium at 37 C in the presence of 0.25% Kaopectate (Upjohn Co.). After 30 min, the suspended cells were diluted by addition of 20 volumes of Eagle medium containing 5% fetal calf serum and incubated at 34 C. In those experiments involving radioactive precursors, 0.4 μ g of actinomycin per ml was also added at this time. For growth of ³²P-labeled virus, phosphate-free medium was used. At 2 h after infection, 95% of the cells were viable as measured by trypan blue exclusion and registered as infectious centers. [³H]adenosine (5 μ Ci/ml) or [³²P]phosphoric acid (10 μ Ci/ml) was added at 2 h after infection. The same concentrations of isotope were added again at 24 and 48 h, and the virus was extracted and purified at 72 h after infection.

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DEAE-cellulose chromatography. Oligo(A) was separated from ds RNA by gel filtration and chromatographed on a DEAE-cellulose column in the presence of 7 M urea as described previously (32).

Base composition analysis. Base composition of oligomers was determined by paper electrophoresis of 0.3 N KOH digests (37 C, 18 h) as described previously (32).

Chemicals. [^3H]adenosine (specific activity, 5 Ci/mmol) was purchased from Schwarz-Mann, Orangeburg, N.Y. [^{32}P]phosphoric acid (carrier-free) was from New England Nuclear Corp., Boston, Mass. Kaolin was purchased from Fisher Scientific Co., Springfield, N.J.

RESULTS

Specificity of reovirus A-rich RNA. A-rich RNA has been detected in reovirus type 3-infected mouse L cells but not in growing, uninfected cells (3). Type 3 virus purified from L, BHK, or HeLa cells contains A-rich RNA, as do types 1 and 2 from L cells (30) and avian reovirus from chick kidney cells (17). If the A-rich RNA is a virus-specific product, its size and base composition should be similar in virions purified from different host cells. To test this, BHK, HeLa, and L cells were infected at 37 C with reovirus and grown in the presence of [^{32}P]phosphoric acid. Viral RNA was extracted from purified progeny virus, and the A-rich RNA was separated from the ds RNA by Sephadex G-100 chromatography. All three ^{32}P -labeled preparations contained 60% of the radioactivity in ds genome RNA and 40% in the low-molecular-weight A-rich RNA. The low-molecular-weight RNAs were further analyzed by DEAE-cellulose chromatography in the presence of 7 M urea (Fig. 1). The elution profiles were very similar for RNA from virus grown in all three cell types, and consisted of a mixture of single-stranded oligomers rich in pyrimidines (peaks I through VI) and oligo(A) (peak VII) as reported previously for virus grown in L cells (32). The relative proportion of radioactivity in peaks I through VII was similar for all three virus RNA preparations (Table 1). In addition, the base compositions of the separated oligomers isolated from virus grown in BHK cells (data not shown) were also found to be similar to the values reported previously for the corresponding oligomers isolated from virus grown in L cells (32). These data suggest that the oligomers, including oligo(A), are virus-specific products.

Infectivity of oligo(A)-deficient particles. Treatments that result in the loss of oligo(A) from reovirus also decrease infectivity (15). To determine whether oligo(A) is necessary for infectivity, we compared the infectivity of virions with cores that are deficient in oligo(A).

FIG. 1. DEAE-cellulose chromatography of oligonucleotides from reovirus grown in three cell types. ^{32}P -labeled reovirus was purified from L, BHK, or HeLa cells, and RNA was extracted with phenol-chloroform. ss RNA was separated from ds RNA by gel filtration and applied to a DEAE-cellulose column (1 by 16 cm) in 0.5 ml of 0.05 M Tris-hydrochloride (pH 8.0), 0.05 M NaCl, 7 M urea. The sample was eluted at a flow rate of 15 ml/h with a linear NaCl gradient (0.05 to 0.5 M, 120 ml each) in 0.05 M Tris-hydrochloride (pH 8.0) and 7 M urea. Fractions of 2 ml were collected, and samples (1 ml) were assayed for radioactivity as previously described (32). Salt concentrations were determined by conductivity measurements.

To insure that the cores to be assayed for infectivity were depleted of oligo(A), RNA from [^3H]adenosine-labeled virions and cores was analyzed by gel filtration (Fig. 2). RNA extracted from virus included 43% of the radioactivity as ds RNA and 57% as the A-rich oligonucleotide fraction. In contrast, cores prepared by treatment of virions with chymotrypsin contained 99% of the [^3H]adenosine in ds RNA (Fig. 2). The radioactivity in the remaining 1% (fractions 10 through 20) bound to poly(U)-impregnated filters as described previously for reovirus oligo(A) (34). These results are consistent with the presence in cores of less than 0.5%

TABLE 1. Analysis of radioactivity in reovirus oligonucleotides by DEAE-cellulose chromatography

Peak	L cells (%)	HeLa (%)	BHK (%)
I	5.6	5.4	13.3
II	14.2	16.7	17.5
III	10.1	11.6	10.4
IV	6.3	6.0	6.7
V	4.2	6.6	3.7
VI	6.1	8.1	5.5
VII	50.0	45.5	42.9

FIG. 2. Gel filtration of RNA from virions and cores. [³H]adenosine-labeled reovirus (500 µg/ml) grown at 34 C was incubated with chymotrypsin (200 µg/ml) in 0.05 M Tris, pH 8.0. After 90 min at 41 C, the chymotrypsin reaction mixture of 3.5 ml was layered onto a 1.5-ml cushion of CsCl in 0.01 M Tris, pH 8.0 ($\rho = 1.5$ g/ml) and centrifuged as described in Materials and Methods. Cores were isolated from the side of the tube and dialyzed against 0.01 M Tris, pH 8.0. RNA was extracted from the purified cores or virions with phenol-chloroform, and the resulting ds and ss RNAs were applied at 0 C to columns (1.5 by 15 cm) of Sephadex G-100 equilibrated with 0.02 M Tris, 0.001 M EDTA, pH 8.0. Samples of 1 ml were collected and counted in Aquasol (New England Nuclear Corp.). (A) Virion RNA; (B) core RNA.

of the amount of oligo(A) found in intact virions.

The specific infectivity of virus decreased 10⁵-fold after treatment with chymotrypsin to produce cores (Table 2). However, infectivity can be partially recovered by the addition of certain "facilitators" (Tables 2 and 3). Kaolin (0.25%) has been reported to partially restore the infectivity of heat-inactivated reovirus (11). In our experience, this concentration of kaolin enhanced core infectivity somewhat, but higher

reactivation levels were achieved with Kaopectate, a commercial preparation of kaolin and pectin. A 0.25% solution of Kaopectate enhanced core infectivity as much as 400-fold, and the effect was specific since Kaopectate had only a small effect on virus infectivity (Table 2). Similarly, intermediate subviral particles (SVPI) prepared from virions by partial chymotryptic digestion are infectious (29), and their infectivity was not enhanced by Kaopectate. The reactivation of core infectivity was directly proportional to the concentration of facilitator used at concentrations below 0.5% (Table 3). At higher concentrations, the colloid quickly settled out of solution, and stimulation of infectivity was no longer a direct function of concentration. In the experiments shown in Table 2 the facilitator was incubated with cores for 1 min at room temperature and diluted before adsorption to cells. However, the degree of enhancement was the same when facilitator was present during the adsorption period. Neither DEAE-dextran nor a level of exogenous oligo(A) comparable to, or greater than, that in virions enhanced infectivity (Table 2). These results indicate that cores deficient in oligo(A) are infectious under appropriate conditions and suggest that oligo(A) is not necessary for infectivity.

Purity of core particles. The differential stimulation of virus and core infectivity by Kaopectate described in Table 2 indicates that cores possess inherent infectivity and are not contaminated by a fraction of chymotrypsin-resistant virus. However, after chymotrypsin treatment, cores tended to aggregate and could trap virions. Such aggregates could copurify with cores, adsorb to cells in the presence of Kaopectate, and contribute oligo(A). To demonstrate that cores were free of such aggregates, we examined the sedimentation rate of Kaopectate-enhanced infectious particles. Cores purified by pelleting onto a layer of CsCl ($\rho = 1.5$ g/ml) were mixed with purified virus in the proportion 1,000 cores per virion on the basis of protein concentration. The mixture was analyzed by centrifugation in a 10 to 50% sucrose gradient. Virus was sedimented as a marker in a separate gradient under the same conditions. Fractions were collected and assayed for infectivity in the presence and absence of 0.25% Kaopectate (Fig. 3). In the absence of the facilitator, infectivity coincided with the position of virus as measured for the marker virus by absorbance at 260 nm and polypeptide composition in sodium dodecyl sulfate-polyacrylamide gels (data not shown). The infectivity due to cores was obscured by the background of virus infectivity. However, in the presence of Kaopec-

TABLE 2. *Specific infectivity of cores, virus, and SVPi*

Facilitator	Core		Virus		SVPi	
	Specific infectivity ^a	Enhancement ^b	Specific infectivity ^a	Enhancement ^b	Specific infectivity ^a	Enhancement ^b
None	5×10^{4a}		2×10^{10b}		4×10^9	
Kaolin, 0.25%	2×10^6	40	8×10^{10}	4		
Kaopectate, 0.25%	2×10^7	400	1×10^{11}	5	4×10^9	1
Reovirus oligo(A) ^c						
30 μ g	4×10^4	1				
200 μ g	4×10^4	1				

^a Values expressed as PFU per milligram of virus protein, using equivalent numbers of virus, SVPi, or cores. SVPi and cores had 75 and 30%, respectively, of the virus protein content.

^b Infectivity ratio: +facilitator/-facilitator.

^c Reovirions contained approximately 30 μ g of oligo(A) per mg of virus.

TABLE 3. *Effect of facilitator concentration on production of infectious centers*

Facilitator	Core		Virus	
	Infectious centers	Enhancement ^a	Infectious centers	Enhancement ^a
DEAE-dextran (μ g/ml) ^b				
None	10^4		2.3×10^5	
100	1.2×10^4	1	1.4×10^5	1
300	10^4	1	3.0×10^4	<1
400	10^4	1	3.0×10^4	<1
Kaopectate (%) ^c				
None	10^4		7.0×10^4	
0.05	2.7×10^5	27	7.0×10^4	1
0.125	5.3×10^5	53	5.0×10^4	1
0.25	1.3×10^6	130	7.5×10^4	1
0.5	2.0×10^6	200	1.5×10^5	2

^a Infectivity ratio: +facilitator/-facilitator.

^b Cells (2×10^6 /ml) were incubated with DEAE-dextran for 1 min at 37 C and then mixed with virus at a multiplicity of infection (MOI) = 0.5 or cores at MOI = 0.01. After 30 min at 37 C, 0.3 ml of an appropriate dilution was plated on a monolayer of L cells. Values are expressed as infectious centers per 2×10^6 cells.

^c Cells (10^6 to 2×10^6 /ml) were incubated with Kaopectate for 1 min at room temperature and then mixed with virus at MOI = 0.2 or cores at MOI = 0.01. After 30 min at 37 C, 0.3 ml of an appropriate dilution was plated on a monolayer of L cells. Values are expressed as infectious centers per 2×10^6 cells.

tate, a broad band of enhanced infectivity was detected sedimenting more slowly than the virus marker. Stimulation of the slowly sedimenting band was 20-fold greater than that of banded or pelleted virus. Thus, the enhanced infectivity sedimented more slowly than virus, as expected for the smaller core particles.

To establish that the enhanced infectivity was not in SVPi, which would also sediment more slowly than virus, the cores ($\rho = 1.45$

g/ml) in a volume of 3.0 ml were further purified by sedimentation through a 0.5-ml CsCl layer ($\rho = 1.40$ g/ml) onto a 1.5-ml CsCl cushion ($\rho = 1.50$ g/ml). The material in the 0.5-ml layer ($\rho = 1.40$ g/ml), which would be expected to contain SVPi ($\rho = 1.40$ g/ml), was enhanced only sixfold by 0.25% Kaopectate (Table 4). In contrast, infectivity in the CsCl cushion ($\rho = 1.50$ g/ml) was enhanced about 300-fold, indicating that particles prepared under these conditions consisted almost entirely, if not completely, of cores.

Oligo(A) in progeny virions from core-infected cells. Cores purified by the above procedure were used to determine whether oligo(A) is necessary as a template or primer for its own synthesis. L cells were infected with purified cores or virus at a multiplicity of infection of 2 in 0.25% Kaopectate and labeled with [³H]adenosine in the presence of actinomycin D. After incubation for 72 h at 34 C, cells infected with cores or virus produced comparable virus yields, about 100 PFU/cell. The progeny virions were purified, and RNA was extracted with phenol-chloroform and analyzed. RNA from both preparations consisted of the high-molecular-weight ds RNA and the low-molecular-weight ss fraction which was included in G-100 Sephadex (Fig. 4). In a similar experiment, the low-molecular-weight RNA from virions purified from core-infected cells and labeled with both [³H]adenosine and [³²P]phosphoric acid was fractionated further by DEAE-cellulose chromatography (Fig. 5). As described previously for virions grown at 31 to 34 C (18), the pyrimidine-rich oligomers (peaks I through VI) represented a smaller proportion of the ss RNA than is present in virions grown at 37 C. The oligo(A) which eluted at 0.3 M NaCl (peak VII) comprised 70% of the ³²P-labeled oligomers. Low-molecular-weight ss RNA from virions purified from virus-infected cells also consisted of 70% oligo(A) which eluted at 0.3 M

FIG. 3. Sedimentation analysis of reovirus and cores. Reovirus cores (1 mg) purified as described in the legend to Fig. 2 were mixed with 1 μ g of purified virus, layered onto a 10 to 50% sucrose gradient in 0.1 M Tris, pH 8.3, 0.001 M EDTA and centrifuged at 25,000 rpm for 45 min at 15 C in an SW27 rotor. Fractions (1 ml) were collected from the top of the gradient. Every other fraction was assayed for infectivity in the presence or absence of 0.25% Kaopectate. The infectivity in the peak fraction (no. 16) was 5.5×10^5 PFU. The pelleted material resuspended in 0.1 ml of the same buffer had a titer of 2×10^5 PFU, which was increased 3.5-fold after addition of 0.25% Kaopectate. The arrow indicates the position of virions assayed by absorbance at 260 nm in a separate gradient under identical conditions (fractions 14 through 17 had detectable absorbance). Percentage of total infectivity without kapectate (●); infectivity + kapectate/—kapectate (—).

NaCl (data not shown). These results indicate that cells infected with cores that contain less than 0.5% of the normal amount of oligo(A) yield infectious virus with a full complement of oligo(A).

DISCUSSION

Synthesis of oligonucleotides rich in adenylic acid has been detected in Ehrlich ascites and calf thymus cells (8) and in L cells after infection with reovirus (4). In contrast to eu-

karyotic cell mRNAs and several viral mRNAs which possess poly(A) at their 3' termini (1, 7, 9, 10, 12-14, 16, 19-21, 23, 26, 28, 36, 37), oligo(A) is not covalently bound to reovirus mRNA at early or late stages of infection (34). However, ss RNA in reovirions purified from three different cell types, L, HeLa, and BHK cells, consisted of oligo(A) and pyrimidine-rich oligomers in constant amounts, indicating that these oligonucleotides are virus-specific products.

Although oligo(A) is a virus-specific product, it does not appear to be necessary for infectivity. Cores, produced by chymotryptic digestion of virus, contain less than 0.5% of the amount of oligo(A) found in intact reovirus. The specific infectivity of virus decreases 10^5 -fold as a result of this treatment, but infectivity can be partially restored under appropriate conditions. Kaopectate (0.25%) enhanced core infectivity as much as 400-fold. Although cores direct a productive infection when cells are infected in the presence of 0.25% Kaopectate, the facilitator

TABLE 4. Infectivity of cores isolated by sedimentation onto CsCl layers

CsCl (g/ml)	PFU	PFU + 0.25% Kaopectate	Enhancement ^a
1.4	5.0×10^3	2.9×10^4	6
1.5	1.1×10^4	3.2×10^6	291

^a Infectivity ratio: +Kaopectate/—Kaopectate.

FIG. 4. Gel filtration of RNA from progeny virions purified from virus- or core-infected cells. [3 H]adenosine-labeled virus was purified from cells infected with virus or cores. RNA was extracted with phenol-chloroform and analyzed on Sephadex G-100 columns as described in the legend to Fig. 2. (A) Virion RNA from virus-infected cells; (B) virion RNA from core-infected cells.

FIG. 5. DEAE-cellulose chromatography of oligonucleotides from progeny virions from core-infected cells. Core-infected cells were incubated at 34 C in the presence of [^3H]adenosine and [^{32}P]phosphoric acid. The labeled progeny virions were purified, and the extracted RNA was separated into its ss and ds components by gel filtration. The ss RNA was applied to DEAE-cellulose and eluted as described in the legend to Fig. 1. The elution position of the pyrimidine-rich oligomers (peaks I through VI) and oligo(A) (peak VII) are indicated. ^{32}P , $\circ$; [^3H]adenosine, $\bullet$.

increased the specific infectivity of cores to only 0.2 to 0.5% of virus specific infectivity. Perhaps full infectivity was not restored because saturating concentrations of facilitator could not be attained. Alternatively, failure of the facilitators tested to restore infectivity completely may indicate that a specific association between oligo(A) and cores is required to attain the specific infectivity of virions. The mechanism by which this facilitator reactivates infectivity is unknown, but it may stimulate nonspecific uptake by cells. Cells infected with cores in the presence of Kaopectate produce particles with characteristics identical to those of reovirus. These particles band at a density of 1.37 g/ml in CsCl and contain ds RNA and oligo(A). Thus, cores which are devoid of oligo(A) direct synthesis of virus particles containing a normal complement of the oligomer; i.e., oligo(A) is not required as a template or primer for its own synthesis in infected cells. The decrease in infectivity after chymotrypsin treatment is probably not due to depletion of oligo(A) but is more likely due to loss of the outer shell polypeptides, which account for 70% of the virion protein and may constitute, together or in part, a receptor site for cell attachment.

Both classes of reovirus oligonucleotides may arise by a transcription process since they have polyphosphorylated 5' termini (25, 32). It has been suggested that the (p)ppG-terminated oligomers arise as a result of abortive transcription by the virion-associated transcriptase because their base sequences resemble the 5'-terminal sequence of at least one species of mRNA (5). Synthesis of (p)ppG-terminated oligomers appears to be temperature dependent; the

amount of these oligonucleotides is reduced in virions grown at 31 to 34 C as compared with 37 C (18). In contrast, a constant amount of oligo(A) is present in virions grown at various temperatures, suggesting that a different mechanism is involved in the synthesis of oligo(A). A poly(A)-synthesizing activity has recently been demonstrated in reovirus, and it may be responsible for synthesis of oligo(A) (33). This enzyme activity is present in virus and SVPi, but not in cores. The enzyme does not utilize exogenous ribopolymers as primers for oligo(A) synthesis, consistent with the observation that oligo(A) is not required as a primer in core-infected cells. If oligo(A) is synthesized and encapsidated during a late step in virus assembly, progeny virus from core-infected cells would be expected to contain a normal complement of oligomers, as observed.

It should be noted that early in the infectious cycle, parental virus is converted to subviral particles which lose most of the outer shell proteins but retain the A-rich RNA (6). Active transcription of 4 of the 10 genome segments occurs during the early period of infection (24, 35) suggesting that oligo(A) or the outer shell proteins may be involved in regulation of reovirus mRNA transcription. It will be of interest to study the pattern of early mRNA synthesis in cells infected with cores.

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