Synthesis of Reovirus Oligo Adenylic Acid In Vivo and In Vitro

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The formation of reovirus double-stranded (ds) RNA and of oligo adenylic acid (oligo A) is inhibited by 5 μ g of actinomycin D per ml added at the time of viral infection. Viral proteins are synthesized and assembled into dsRNA-deficient particles under these conditions. The addition of cycloheximide to infected cells during the mid-logarithmic phase of viral replication terminates protein and dsRNA synthesis, but allows continued oligo A synthesis for about 1 h. The ³H-labeled oligo A formed in the presence of cycloheximide is incorporated into particles whose density in CsCl is identical to that of reovirions. Using the large particulate or virus factory-containing cytoplasmic fraction of infected L-cells, we have established an in vitro system for the synthesis of oligo A. The in vitro product migrates slightly faster in sodium dodecyl sulfate acrylamide gels than marker oligo A. Oligo A synthesis in vitro continues for about 1 h, requires the presence of only one ribonucleoside triphosphate (ATP), is not inhibited by DNase or RNase, but is abruptly terminated by the addition of chymotrypsin to the reaction mixture. Oligo A formed both in vivo and in vitro is released from the factory fraction by chymotrypsin digestion. The enzymes which catalyze the synthesis of oligo A, dsRNA, and single-stranded RNA all exhibit a similar temperature dependence with an optimum of ~45 C. These results indicate that oligo A is formed within the core of the nascent virion after the completion of dsRNA synthesis; they suggest that the oligo A polymerase is an alternative activity of the virion-bound transcriptase and that it is regulated by outer capsomere proteins.

Approximately 20% of the RNA contained within reovirus is a single-stranded oligomer, 6 to 20 nucleotides in length, which is composed principally of adenylic acid (5-7, 19, 23, 29). Each virion contains about 1,200 molecules of these adenine-rich oligoribonucleotides regardless of the temperature at which the virus is grown (7) or the cell type in which it replicates (5). Despite the abundance of these adenylic acid oligomers (oligo A) in reovirus, virtually no information is available concerning the mechanism and intracellular location of its synthesis, its functional role, if any, in the formation of viral single- and double-stranded RNAs, its precise location within the virion, and its importance in the viral replicative cycle. For these reasons, we have examined the synthesis of viral oligo-adenylic acid in vivo and in vitro.

MATERIALS AND METHODS

Cells and virus. L-strain mouse fibroblasts were grown in suspension culture and infected with reovi-

rus type 3 as described previously (18).

Purified ³H-leucine-labeled reovirus was prepared as described (24).

Preparation of the large particulate cytoplasmic fraction (LPF). Infected L-cells were sedimented at 500 $\times g$ at 4 C, washed twice with cold phosphate-buffered saline (PBS) (13), resuspended in ~8 ml of 0.14 M NaCl, 0.01 M Tris (pH 8.4), 0.0015 M Mg acetate, 0.5% NP-40 (Shell Chemical Co.), and disrupted with a dounce homogenizer. The homogenate was centrifuged at 1,000 $\times g$ for 10 min at 4 C, and the supernatant fraction was removed and stored at 4 C. The pellet fraction was resuspended in 3 to 4 ml of homogenization buffer, and was centrifuged as before. The supernatants from both centrifugations were combined and centrifuged at 20,000 $\times g$ at 4 C for 20 min in a Sorvall centrifuge. The resulting pellet, which contains the virus factories, was resuspended in reticulocyte standard buffer (RSB) (32) and used as the source of enzyme for RNA synthesis.

Synthesis of RNA in vitro. In vitro synthesis of single-stranded (ssRNA), double-stranded (dsRNA), and oligo A was carried out as described (1), with the following exceptions. (i) Phosphoenolpyruvate (10 mM), pyruvate kinase (25 μ g/ml), and actinomycin D (10 μ g/ml) were added to all reaction mixtures. (ii) Chymotrypsin (100 μ g/ml) was added to the reaction

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mixture whenever ssRNA synthesis was monitored. (iii) Oligo A synthesis was measured with 0.5 mM ^3H -ATP (S.A. 200 $\mu\text{Ci}/\text{mM}$) as the only nucleotide. Unless otherwise noted, all incubations were for 30 min at 37 C.

Analytical procedures. RNA was extracted with sodium dodecyl sulfate (SDS)-phenol by the method of Tavitian et al. (30) and analyzed as described in the text.

Methylated albumin-silicic acid columns were prepared as previously described (23, 25).

Polyacrylamide gel electrophoresis. (i) Proteins. Viral proteins were analyzed in 10% acrylamide, 0.27% bisacrylamide gels in 0.1% SDS, 6 M urea, 0.1 M NaPO_4 (pH 7.2), 0.02 M NaEDTA as described (24, 28, 36). Samples were prepared as described in the text, solubilized in dissociation buffer (DB) in the presence of 6 M urea at 100 C for 1 min, and run at 5 mA/gel. Gels were cut into 0.9-mm segments, solubilized with 0.25 ml of Soluene (Packard) for each 0.9-mm segment, and assayed for radioactivity as described (24).

(ii) RNA. Oligo A and dsRNA were assayed in 10% acrylamide, 0.27% bisacrylamide gels in 0.1% SDS, 0.1 M NaPO_4 (pH 7.2), 0.02 M NaEDTA as originally described by Bellamy et al. (5). Samples were prepared as described in the text, solubilized in DB (24) at 37 C for 1 h, and run at 6 mA/gel. Gels were cut into 0.9-mm segments (four adjacent segments were combined in each fraction) and assayed for radiolabel as described above for proteins.

Electron microscopy. Cells were fixed, processed, and examined as described (25).

RESULTS

Formation of Oligo A in vivo: (i) Effect of inhibitors of RNA synthesis. To determine whether viral oligo A synthesis is dependent upon the formation of cellular poly A, we used the adenosine analog cordycepin (3 deoxyadenosine), an inhibitor of cellular poly[A] synthesis (12). Cordycepin at a concentration of 50 $\mu\text{g}/\text{ml}$ reduces the incorporation of ^3H -uridine and ^3H -adenosine into cellular RNA by 60 and 66%, respectively (Table 1). Oligo A synthesis is unaffected by this compound, whereas reovirus replication is somewhat reduced (Table 1). Therefore, it seemed unlikely that viral oligo A synthesis and cellular poly[A] synthesis are linked.

Actinomycin D at concentrations above 0.5 $\mu\text{g}/\text{ml}$ inhibits reovirus replication in L-cells (22). To determine whether the actinomycin-mediated inhibition of reovirus replication is linked to inhibition of viral oligo A synthesis by this antibiotic, L-cells were infected with reovirus in the presence of 0.3 μg of actinomycin D per ml, and at various times after infection the actinomycin concentration was increased to 5 $\mu\text{g}/\text{ml}$. The cells were incubated with ^3H -adenosine from 9 to 10 h after infection, and the

distribution of radiolabel in oligo A and viral dsRNA was measured (Table 2). When the actinomycin concentration was increased from 0.3 to 5 $\mu\text{g}/\text{ml}$ 4 h after infection, dsRNA synthesis 9 to 10 h after infection was reduced to 33% of the control value, whereas oligo A synthesis was decreased to 56% of control. In contrast, when the actinomycin concentration was increased 8 h after infection, nearly normal amounts of dsRNA and oligo A were formed.

Polyacrylamide gel electrophoretic analysis of the radiolabeled viral proteins in cells treated with 0.3 and 5 μg of actinomycin per ml revealed no qualitative differences in the viral proteins synthesized (Fig. 1). An electron microscope examination of reovirus infected cells treated with 5 μg of this antibiotic per ml showed small viral factories containing a large proportion of empty particles. (Fig. 2b).

These data provide no support for a selective inhibition of viral oligo A synthesis by actinomycin; rather, they suggest that dsRNA synthesis, and not the formation of oligo A, is primarily affected by high concentrations of actinomycin.

(ii) Effect of inhibition of protein synthesis.

Cycloheximide causes prompt cessation of dsRNA formation in reovirus infected L-cells;

TABLE 1. *Effect of cordycepin (50 $\mu\text{g}/\text{ml}$) on cellular RNA synthesis, oligo A synthesis, and reovirus replication*

Incorporation of ^3H -nucleosides into cellular RNA (% uninhibited control) ^a		Incorporation of ^3H -adenosine into viral oligo A (% uninhibited control) ^b	Viral yield (% uninhibited control) ^c
^3H -uridine	^3H -adenosine		
40	34	98	60

^a Separate cultures of L-cells (10^6 cells per 60-mm petri dish) were incubated for 30 min at 37 C in medium with or without cordycepin (50 $\mu\text{g}/\text{ml}$). ^3H -uridine or ^3H -adenosine (3.3 $\mu\text{Ci}/\text{ml}$) was added to duplicate cultures; the cells were incubated for an additional 3.5 h and assayed for their content of acid-insoluble radioactivity.

^b Cordycepin was added to 5×10^6 cells in suspension culture 11 h after infection with reovirus (~ 15 PFU/cell). 15 min later ^3H -adenosine (1.5 $\mu\text{Ci}/\text{ml}$) was added to the medium and the cells were incubated for an additional 1 h at 37 C. A large particulate cytoplasmic fraction was prepared and assayed by polyacrylamide gel electrophoresis for its content of oligo A.

^c L-cells were infected with reovirus (20 PFU/cell) and incubated in the presence or absence of cordycepin for 14 h. The cultures were assayed for their viral content by plaque assay.

TABLE 2. Effect of actinomycin D (5 μ g/ml) on the synthesis of reovirus dsRNA and oligo A *in vivo*^a

Actinomycin (μ g/ml)	dsRNA (counts/min)	dsRNA (%)	Oligo A (counts/min)	Oligo A (%)	Viral yield (%)
0.3	36,000	100	22,100	100	100
0.3 to 5, 4 h	12,000	33	12,400	56	ND ^b
0.3 to 5, 8 h	34,000	95	20,100	91	ND
5	<500	<1	<250	<1	2

^a Portions of 4×10^7 cells were incubated for 1 h with 0.3 or 5 μ g of actinomycin D per ml. The cells in each sample were then concentrated by centrifugation and infected with reovirus (MOI = 25 PFU/cell) at 4 C for 2 h. The cells were resuspended in warm medium (37 C) containing either 0.3 or 5 μ g of actinomycin per ml and were incubated at 37 C. After 4 and 8 h of infection, the actinomycin concentration of the appropriate sample was increased to 5 μ g/ml. All samples were labeled with ³H-adenosine (2 μ Ci/ml) from 9 to 10 h after infection. An LPF was prepared from each sample and its RNA was extracted and precipitated with ethanol. dsRNA was separated by CF 11 chromatography, re-precipitated with ethanol, and assayed as RNase (8 μ g/ml) resistant acid-insoluble radioactivity as described previously (15). Oligo A was assayed by SDS polyacrylamide gel electrophoresis. Viral yield was measured by plaque assay.

^b ND, Not done.

FIG. 1. SDS-urea polyacrylamide gel electrophoretic analysis of ¹⁴C-labeled proteins in reovirus infected L-cells incubated in either 0.3 or 5.0 μ g of actinomycin per ml. Monolayer cultures of L-cells (5×10^6 cells per 100-mm petri dish) were treated with actinomycin for 1 h and then infected with reovirus (20 PFU/cell) in the presence of this antibiotic. The cells were incubated at 37 C in actinomycin-containing medium for 8.5 h, at which time 2 μ Ci of ³H-leucine per ml was added to the culture. After a 0.5-h labeling period the cells were harvested, and cytoplasmic extracts were prepared as described by Zweerink et al. (21), precipitated with acetone, and analyzed by SDS-urea polyacrylamide gels. Panel a,

the synthesis of viral ssRNA, however, continues unabated after the addition of this antibiotic (33). We took advantage of these observations to determine whether oligo A synthesis is dependent upon the continued synthesis of proteins or of dsRNA, or both. Infected L-cells were tested for their ability to incorporate ³H-adenosine into oligo A at various times after the addition of cycloheximide to the culture. Although viral replication is inhibited by cycloheximide, oligo A synthesis proceeds normally for about 1 h after the addition of this antibiotic (Fig. 3a), and then stops. As expected, progeny virus and oligo A continue to be formed in the control culture throughout the course of the experiment (Fig. 3b).

To determine whether the oligo A formed in the presence of cycloheximide was incorporated into reovirions, an experiment similar to the one just described was performed, and the radio-labeled virus was purified and centrifuged to equilibrium in CsCl gradients (Fig. 4a,b). During a 1-h labeling period nearly equal amounts of ³H-adenosine were incorporated into particles from the control and cycloheximide-treated cultures (Fig. 4 a,b). These particles exhibited a buoyant density of 1.37 g/cm³. Polyacrylamide gel analyses of the ³H-labeled RNA contained within these particles showed that 97% of the ³H-adenosine incorporated into virions in the presence of cycloheximide was contained in oligo A (Table 3). In contrast, virions formed in the absence of cycloheximide incorporated ³H-

cells treated with 0.3 μ g of actinomycin per ml; total counts per minute incorporated = 385,040. Panel b, cells treated with 5.0 μ g of actinomycin per ml; total counts per minute incorporated = 325,500. Panel c, ³H-leucine-labeled reovirus.

adenosine into both dsRNA and oligo A. The distribution of ^3H -adenosine between dsRNA and oligo A varied, depending upon the length of the labeling period. (Table 3).

Previous studies have established the following. (i) The adenylic acid residues in reovirus RNA are nearly equally divided between dsRNA and oligo A molecules (23). (ii) dsRNA is formed by the synthesis of "minus" strands on preformed "plus" strand templates (21). The "plus" strands which serve as templates for dsRNA accumulate during the beginning of the replicative cycle. Hence, the dsRNA formed late in the replicative cycle (i.e., after the 8th or 9th h of infection) is labeled exclusively in the minus strand (1). (iii) Synthesis of minus strands on plus strand templates occurs within the "core" of the nascent virion (1, 20, 37). From these data and those reported in Fig. 3 and Table 3, it is possible to determine the order of incorporation of dsRNA and oligo A into reovirions.

If oligo A and dsRNA are incorporated into virions concurrently during viral replication, then ^3H -adenosine should label these two classes of molecules in a ratio of 1:2, dsRNA:oligo A (Fig. 5a). However, if the incorporation of dsRNA either precedes or follows the incorporation of oligo A during viral maturation, then the ratio of ^3H -adenosine-labeled dsRNA:oligo A in mature virions should vary with the length of the labeling period. If dsRNA is incorporated into nascent virions before oligo A, the ratio of ^3H -adenosine-labeled dsRNA:oligo A should increase as the length of the labeling period increases (Fig. 5b). Conversely, if dsRNA is incorporated into nascent virions after oligo A, then the ratio of ^3H -adenosine-labeled dsRNA:oligo A should decrease with increased time of labeling (Fig. 5c). The results of the experiment reported in Fig. 3 and Table 3 indicate that dsRNA is incorporated before oligo A, supporting the model described in Fig. 5b. The presence of ^3H -adenosine-labeled oligo A in virions containing unlabeled dsRNA (Fig. 3a, and Table 3, line a) is also in agreement with this model.

Synthesis of oligo adenylic acid in vitro. To examine the synthesis of viral oligo A directly we established a cell-free system by using the large particulate or virus factory-containing fraction of reovirus-infected cells (1, 25). (This fraction has been identified as the site of reovirus ss- and dsRNA synthesis [1, 20, 34, 37]). The large particulate fraction incorporates α - ^{32}P -labeled ATP into oligo A. The product formed under these conditions migrates in polyacrylamide gels together with or slightly faster than oligo A extracted from purified reovirus (Fig. 6).

The specificity of the oligo A synthesizing enzyme for adenylic acid was examined by adding each of the other three radiolabeled ribosomal nucleoside triphosphates (rNTP's) to the reaction mixture. ^3H -CTP or ^3H -GTP are not incorporated into a polynucleotide similar in size to oligo A (Fig. 7). A small amount of ^3H -UTP is incorporated into a polynucleotide which co-electrophoreses with oligo A (Fig. 7).

To gain further information about the structure and base composition of these in vitro synthesized oligonucleotides, oligo A was synthesized by using ^3H - or α - ^{32}P -labeled ATP. The oligonucleotide products of these reactions were purified, and then hydrolyzed with alkali; the resulting radiolabeled nucleotides were analyzed by paper electrophoresis and compared with the alkaline hydrolysis products of in vivo synthesized ^3H -adenosine-labeled oligo A (Table 4). Eighty percent of the radiolabel in the in vivo synthesized oligo A co-electrophoresed with AMP and 10% co-electrophoresed with adenosine. Five percent of the label migrated together with, or slightly ahead of, ADP and ATP, and was identified as pAp and ppAp (Table 4). In contrast, in two separate experiments, 52 and 71% of the radiolabel in the in vitro synthesized oligo nucleotides co-electrophoresed with adenosine, 40 and 20% of the radiolabel was recovered as AMP, and less than 10% was identified as pAp, ppAp, and pppAp. A similar analysis of in vitro synthesized α - ^{32}P -ATP-labeled oligo A showed that 67% of the radiolabel was incorporated into AMP, 32.5% into UMP, and 0.5% into ppAp (Fig. 8 a and b).

To be certain that the radiolabeled compound identified at pH 3.5 as UMP was a pyrimidine, and not a phosphorylated purine, the nucleotides were hydrolyzed in acid prior to electrophoretic analysis. Under these circumstances no radiolabel co-migrated with AMP, whereas the radiolabel in UMP was conserved (data not shown).

Several important conclusions may be drawn from these data. (i) The oligo A polymerase incorporates ATP into an adenylic acid-rich polymer in vitro. (ii) A minor proportion (less than 10%) of these in vitro synthesized oligomers are formed ab initio since they contain pAp, ppAp, or pppAp at their 5' termini. (iii) A moderate proportion (between 20 and 40%), of the ^3H -ATP incorporated in vitro resides in internal linkages, since 20 to 40% of the nucleotides in an alkaline hydrolysate are recovered as ^3H -AMP. (iv) A major proportion (52 to 71%) of the radiolabeled ATP is incorporated into the 3' termini of preformed oligonucleotides and reflects chain termination. (v) One third of the

FIG. 3. Effect of cycloheximide on the synthesis of reovirus oligo A. L-cells (2×10^6) were infected with reovirus (25 PFU/cell) and incubated in medium containing $0.3 \mu\text{g}$ of actinomycin per ml for 9 h. The cells were then divided into two portions. Cycloheximide ($20 \mu\text{g}/\text{ml}$) was added to one portion and 15 min later ^3H -adenosine ($3 \mu\text{Ci}/\text{ml}$) was added to both samples. At 1, 2, and 4 h after addition of cycloheximide the cells were harvested from cycloheximide-treated (a) and control (b) cultures, and were assayed for their content of oligo A as described in Materials and Methods. BB, Bromophenol blue dye marker. At suitable intervals (9, 12.25 and 13.25 h after infection), samples were also taken from the control and cycloheximide-treated cultures and monitored for their content of infectious virus (insert c).

α - ^{32}P -ATP is incorporated into an oligonucleotide containing the sequence UpA but lacking the sequences CpA or GpA.

Previous work (1) established that the large particle fraction catalyzed synthesis of dsRNA proceeds for a limited time (10 to 15 min) and is inhibited by adding RNase to the reaction mixture at the beginning of the incubation. We took advantage of these observations to determine whether the *in vitro* synthesis of oligo A is linked to the formation of dsRNA, and whether it is dependent upon ssRNA templates.

Synthesis of oligo A, in contrast to the synthesis of dsRNA, proceeds *in vitro* for over an hour, suggesting that these two biosynthetic events are not closely linked (Fig. 9). That oligo A synthesis is indeed independent of dsRNA synthesis and of the presence of ssRNA templates was confirmed by measuring the amount of oligo A formed after addition of nucleases to the reaction mixture at the beginning of the incubation. RNase, which inhibits dsRNA formation by destroying template plus strands (1), had no effect upon the amount or size of the oligo A formed. Similar results were obtained when DNase was added to the reaction mixture.

Effect of chymotrypsin. The enzymes which

FIG. 4. Effect of cycloheximide on the incorporation of oligo A into reovirus. L-cells (4×10^6) were infected with reovirus ($\text{MOI} = 5$ PFU/cell) and incubated in medium containing $0.3 \mu\text{g}$ of actinomycin per ml for 11 h. The cells were then divided into three portions at a concentration of 3.3×10^6 cells/ml. Cycloheximide ($20 \mu\text{g}/\text{ml}$) was added to one portion, and 10 min later ^3H -adenosine ($10 \mu\text{Ci}/\text{ml}$) was added to all three samples. The cycloheximide-treated portion and one portion of the control cells were harvested after a 1-h labeling period. The cells in the second control culture were collected after a 2-h labeling period. Virus contained in each sample was then purified and centrifuged to equilibrium in CsCl gradients as described (24). The gradients were fractionated and the radiolabel in each fraction was measured.

FIG. 2. Electron microscopy of reovirus factories in L-cells incubated in either 0.3 or $5.0 \mu\text{g}$ of actinomycin per ml. a, L-cells were treated with $0.3 \mu\text{g}$ of actinomycin per ml for 1 h prior to infection with reovirus (250 PFU/cell). The infected cells were incubated at 37°C in medium containing $0.3 \mu\text{g}$ of actinomycin per ml for 12.5 h, harvested, and processed for electron microscopy. b, Reovirus-infected L-cells were treated as described in Fig. 3a except that $5 \mu\text{g}$ of actinomycin per ml was used throughout. Note the large proportion of particles lacking electron-dense dsRNA cores, and the "empty" particles containing small electron-opaque structures at their centers. These centrally located electron-opaque structures may represent the ssRNA's which serve as templates for dsRNA formation.

TABLE 3. Distribution of ^3H -adenosine between dsRNA and oligo A^a

Time of ^3H -adenosine labeling (h)	Cycloheximide	Total counts/min applied to gel	Counts/min recovered on gel	Counts/min in oligo A	Recovered counts/min in oligo A (%)	Counts/min in dsRNA	Recovered counts/min in dsRNA (%)	Ratio counts/min dsRNA/oligo A
1	+	64,330	57,932	56,406	97.4	1,516	2.6	0.027
1	-	72,530	66,328	57,886	87.3	8,442	12.7	0.15
2	-	121,500	103,647	83,181	80.3	20,446	19.7	0.24

^a The peak fractions (8 to 11) of each of the CsCl gradients described in Fig. 5 were combined and dialyzed overnight against 0.15 M NaCl. The RNA was extracted with SDS-phenol and precipitated with alcohol. The precipitated RNA was dissolved in 500 μl of dissociation buffer and 100- μl portions of each sample were analyzed on 10% acrylamide gels. Under these conditions all of the dsRNA remains near the origin of the gel, whereas oligo A migrates ahead of the bromophenol blue dye marker. The amount of radiolabel in each species of RNA was assayed as described in Materials and Methods.

FIG. 5. Models of the radiolabeling pattern of dsRNA and oligo A. Symbols: —, radiolabeled molecules; ----, unlabeled molecules.

FIG. 6. Size comparison of *in vivo* and *in vitro* synthesized oligo A by acrylamide gel electrophoresis. An LPF from 6×10^8 L-cells infected for 14 h with reovirus (MOI = 20 PFU/cell) was suspended in 1.5 ml of RSB. A 0.2-ml amount of this material was incubated in a 1-ml RNA reaction mixture containing

catalyze the synthesis of reovirus ss- and dsRNA's are resistant to chymotrypsin digestion (1, 3, 27). To determine whether the enzyme(s) which catalyzes oligo A synthesis is also resistant to proteolysis by chymotrypsin, a

FIG. 7. Nucleotide specificity of the oligo A polymerase. An LPF prepared from 8×10^8 L-cells infected for 13 h with reovirus (MOI = 70 PFU/cell) was suspended in 2 ml of RSB. Four 0.4-ml portions of this material were incubated separately for 30 min (total volume of each reaction mixture = 1.6 ml) with the usual concentrations of salts and buffers containing 0.5 mM of only one nucleoside triphosphate (either ^3H -ATP, ^3H -GTP, ^3H -CTP, or ^3H -UTP [200 $\mu\text{Ci}/\mu\text{mol}$]). The reaction mixtures were then centrifuged at 20,000 $\times g$ for 30 min at 4 C, and the RNA in the resulting pellets was extracted with SDS-phenol, precipitated with ethanol, dissolved in DB, and analyzed on 10% SDS-acrylamide gels.

200 μCi of $\alpha\text{-}^{32}\text{P}$ -ATP (specific activity 200 $\mu\text{Ci}/\mu\text{M}$). After 30 min, ^3H -adenosine-labeled RNA extracted from reovirus was added. The mixture was precipitated with 5% trichloroacetic acid, and the precipitate was washed with ethanol, dissolved in DB, and analyzed on 10% SDS gels.

TABLE 4. Electrophoretic analysis of the nucleotide composition of reovirus oligoribonucleotides labeled *in vivo* and *in vitro*

Product analyzed	Label	Adenosine		AMP		pAp		ppAp		pppAp	
		Counts/min	%	Counts/min	%	Counts/min	%	Counts/min	%	Counts/min	%
In vivo synthesized oligo A	³ H-adenosine ^a	397	12	2,540	76	220	6.5	190	5.5		
In vitro synthesized oligo A	³ H-ATP ^b	2,320	71	635	20	140	4	170	5		
	³ H-ATP ^c	1,590	52	1,245	40					220	7

^a Reovirus-infected cells (5×10^6 ; MOI = 10 PFU/cell) in medium containing 0.3 μ g of actinomycin D were labeled with ³H-adenosine from 11.5 to 14.5 h after infection. The RNA contained in the LPF of these cells was extracted, alcohol-precipitated, resuspended in 0.01 M NaH₂PO₄ (pH 7.2), and analyzed by electrophoresis in SDS-polyacrylamide gels. The gel fractions containing ³H-oligo A were incubated for 12 h at 37 C in 0.3 N KOH. The alkaline digest was neutralized with 70% perchloric acid and centrifuged, and the clear supernatant was spotted on Whatman 3 MM paper and electrophoresed at 220 V for 20 h in 0.02 M Na citrate buffer (pH 4.5). The paper was cut into 1-cm segments and the radiolabeled nucleotides were eluted with water and counted in Aquasol. Identity of labeled nucleotides was established by comparison with known standards.

^b An LPF was prepared from 5×10^6 L-cells 14.5 h after infection (MOI = 15 PFU/cell). The LPF was resuspended in 1 ml of RSB and incubated at 45 C for 30 min in a 5-ml reaction mixture containing 0.5 mCi of ³H-ATP and the usual concentrations of other components. The reaction was terminated by centrifugation at 12,000 rpm at 4 C in a Sorvall SS34 rotor, and the RNA in the pellet fraction was extracted and precipitated. Oligo A was separated by gel electrophoresis and its content of radiolabeled nucleotides was analyzed as described above.

^c An LPF was prepared from 2×10^6 L-cells 13 h after infection (MOI = 25 PFU/cell). The LPF was resuspended in 8 ml of RSB and incubated at 37 C in a 20-ml reaction mixture containing 2.5 mCi of ³H-ATP. The reaction was terminated and RNA was extracted as described in *b* above. RNA was chromatographed on a methylated albumin silicic acid column (60 by 6 mm) equilibrated with 0.1 M NaCl, 0.02 M NaPO₄ (pH 7.1). The RNA was eluted with 0.8 M NaCl in the same buffer, dialyzed versus RSB, and precipitated with alcohol, and its content of labeled nucleotides was analyzed as described above.

FIG. 8. Electrophoretic analysis of an alkaline hydrolysate of oligonucleotides synthesized *in vitro* with α -³²P-ATP. An LPF was prepared from 2×10^6 L-cells infected for 13.5 h (MOI = 3 PFU/cell). The LPF was resuspended in 2 ml of RSB and incubated for oligo A synthesis in a 10-ml reaction mixture containing 100 μ Ci of α -³²P-ATP per ml at 41 C for 30 min. RNA was extracted and processed, and the nucleotides were assayed as described in Table 4a. Electrophoresis in 0.02 M Na citrate, pH 4.5; b, electrophoresis in 0.02 M Na citrate, pH 3.5.

FIG. 9. Time course of oligo A synthesis *in vitro*. Three 0.2-ml samples of the LPF described in Fig. 7 were incubated for oligo A synthesis in separate 1-ml reaction mixtures for 10, 30, or 60 min, and centrifuged at $20,000 \times g$ for 30 min at 4 C. The pellet fractions were resuspended in 1 ml of RSB, precipitated with 5% trichloroacetic acid, washed with ethanol, dissolved in DB, and analyzed on 10% SDS-acrylamide gels. The radiolabel under each oligo A peak was combined, corrected for background, and plotted versus time.

large particle fraction was incubated with chymotrypsin and then assayed for its capacity to catalyze the incorporation of ^3H -ATP into oligo A. Chymotrypsin abolished oligo A synthesis (Fig. 10) but, as expected, enhanced the overall incorporation of ^3H -ATP into acid-insoluble products when all four ribonucleoside triphosphates were present, thus the enzyme(s) responsible for oligo A synthesis differs in its chymotrypsin sensitivity from the enzyme(s) responsible for the formation of ss- or dsRNA's.

Chymotryptic digestion of intact reovirus removes the outer layers of viral capsomeres and releases the particle-bound oligo A (3, 14, 29). We took advantage of these observations to identify the location of the oligo A synthesized *in vitro* within the virus factory.

A large particle fraction was incubated with ^3H -ATP and then divided into two samples. One sample was digested with chymotrypsin. The samples were then centrifuged and the supernatant and pellet fractions were assayed for their content of oligo A. The ^3H -labeled oligo A in the sample that had not been exposed to chymotrypsin remained in the sedimentable fraction (Fig. 11a). However, the ^3H -oligo A in the sample incubated with chymotrypsin was released into the supernatant fraction (Fig. 11b). The capacity of chymotrypsin to release *in vitro* synthesized oligo A from the large particle fraction was compared with the ability of this

FIG. 10. Effect of chymotrypsin on oligo A synthesis *in vitro*. An LPF was prepared from 8×10^8 cells infected for 14 h with reovirus (MOI = 20 PFU/cell) and resuspended in 2 ml of RSB. Two 0.5-ml portions were incubated for oligo A synthesis in separate 3.0 ml reaction mixtures. Chymotrypsin (100 $\mu\text{g}/\text{ml}$) was added to one mixture at the start of the reaction. The reaction was terminated by addition of SDS. RNA was extracted with phenol, precipitated with ethanol, and analyzed on 10% SDS acrylamide gels.

FIG. 11. Effect of chymotrypsin on the location of *in vitro* synthesized oligo A. An LPF was prepared as described in Fig. 11 and incubated for oligo A synthesis in a 12-ml reaction mixture. The reaction mixture was then centrifuged at $20,000 \times g$ for 30 min at 4 C, and the pellet was resuspended in 2 ml of RSB and divided into two portions. Chymotrypsin (100 $\mu\text{g}/\text{ml}$) was added to one portion and both samples were incubated for 15 min at 37 C. The samples were again centrifuged at $20,000 \times g$ for 30 min at 4 C and each sample was divided into pellet (a) and supernatant (b) fractions. The RNA in these fractions was extracted, precipitated with ethanol, and analyzed on 10% SDS acrylamide gels.

enzyme to release oligo A from a similar fraction labeled *in vivo* with ^3H -adenosine. The *in vivo* synthesized oligo A was completely released from the large particulate fraction by chymotrypsin digestion (data not shown) regardless of whether the chymotrypsin digestion was carried out in the presence of RSB (low salt) or the oligo A reaction mixture (high salt). The effect of chymotrypsin on the large particulate fraction of reovirus-infected cells was monitored directly by polyacrylamide gel electrophoretic analysis of the protein content of this fraction before and after chymotrypsin digestion. The principal viral polypeptides remaining in the large particle fraction after chymotrypsin digestion were λ_1 , λ_2 , μ_1 , δ (24), and σ_2 . Thus, the effect of chymotrypsin on the large particle fraction resembles the effect of this enzyme upon the intact virion. In both cases the core polypeptides are retained and the outer capsid proteins are removed.

To further confirm the presence of oligo A within virion-like particles an LPF was incubated with ^3H -ATP for oligo A synthesis. The particles were concentrated by centrifugation and resuspended in TL buffer (26), and then layered over 0.3 ml of 0.25 M sucrose which itself overlays a continuous CsCl gradient. The gradient was centrifuged at 4 C for 3 h at 37,000 rpm in an SW 50.1 rotor. A major proportion of the radiolabel remained at the sucrose CsCl interface, but a small proportion sedimented

into the CsCl gradient and was recovered at a density of 1.36 to 1.38 g/cm³. When the radiolabeled particles in these fractions were again centrifuged to equilibrium in CsCl, a sharp peak of radiolabel was recovered at a density of 1.365 g/cm³.

Temperature optimum of oligo A synthesis.

The particle-bound ssRNA transcriptase exhibits the unusual temperature optimum of 45 to 50 C (15). We took advantage of this finding to explore the relationship between the enzyme(s) catalyzing the synthesis of ss- and dsRNA and of oligo A. All three biosynthetic activities show increased activity at 45 C (Fig. 12 a,b,c), and comparison of the temperature dependence of these three activities shows that they increase in parallel to one another.

DISCUSSION

Actinomycin at a concentration of 5 µg/ml strongly inhibits the formation of reovirus dsRNA and of oligo A (Table 2). All known species of reovirus polypeptides are formed in the presence of high concentrations of this antibiotic (Fig. 1), and these proteins have been shown to form particles, some of which may contain ssRNA precursors of dsRNA (Fig. 2b). These data, when viewed together with the fact that synthesis of dsRNA precedes the formation of oligo A, indicate that the presence of particle-bound dsRNA is required for oligo A formation. The absence of oligo A in "top component" (28) is in agreement with this hypothesis.

Inhibition of protein synthesis with cycloheximide causes rapid termination of ongoing dsRNA synthesis (33), but allows oligo A synthesis to continue for about an hour (Fig. 9). Thus, although oligo A is not formed in the absence of dsRNA, oligo A synthesis is not dependent upon the concurrent synthesis of dsRNA. That the presence of dsRNA and not its synthesis is required for oligo A formation is supported by our findings that oligo A synthesis in vitro continues for about an hour (Fig. 9) and in the presence of ATP alone, whereas the formation of dsRNA in vitro terminates in 10 to 15 min (1) and requires the simultaneous presence of all four ribonucleoside triphosphates.

Oligo A synthesized in vitro migrates in SDS-polyacrylamide gels slightly faster than the in vivo product (Fig. 6). We assume that the increased electrophoretic mobility of the in vitro synthesized oligo A reflects a slightly shorter chain length than the in vivo oligomer. This conclusion is supported by the data in Table 4 which show that 52 to 71% of the ³H-ATP incorporated into oligo A in vitro was recovered,

after alkaline digestion, in ³H-adenosine.

Thus, although a small proportion of oligo A molecules are initiated in vitro, the major proportion of the ³H-ATP incorporated is at the 3' end of the molecule and reflects termination of chain growth. The data in Table 4 also indicate that several adenine nucleotides (pAp, ppAp, and ppAp) are recovered from the 5' end of the in vivo and the in vitro products; Bellamy and Hole (7) and Stolz and Banerjee (29) have reported that pAp is the 5' nucleotide in in vivo synthesized oligo A. We cannot explain these differences. However, a similar variation in the 5' guanosine nucleotides recovered from in vitro synthesized ssRNA has been reported (4, 17, 19) and has been attributed to the presence of nucleotide phosphohydrolases within the virion (4, 10, 16, 19).

The incorporation of ³H-UMP into a product which co-electrophoreses with oligo A, and the finding that about one-third of the α-³²P-ATP incorporated is recovered in UMP after alkaline hydrolysis of the oligonucleotides indicates that the in vitro synthesized oligo A is contaminated with a uridylic acid-containing oligomer.

These findings raise the possibility that some of the in vitro synthesized oligonucleotides represent breakdown products of newly formed ssRNA's. Two observations make this possibility highly unlikely. (i) ssRNA synthesis requires the simultaneous presence of all four rNTP's; the oligonucleotides we have described are synthesized in the presence of ATP alone. (ii) Addition of ribonuclease to the reaction mixture has no effect on the amount or size of oligonucleotide formed. Further work will be required to characterize the uridylic acid-containing oligomer which serves, in vitro, as an acceptor for adenine ribonucleotides. One candidate for such an oligomer is the trinucleotide (pGpCpU) described by Bellamy et al. (7). However, it is clear that the major proportion (67%) of the α-³²P-ATP incorporated in vitro has as its nearest neighbor an adenine nucleotide and reflects the elongation or the termination of adenylic acid oligomers, or both.

Release of both the in vivo and the in vitro formed oligo A from the large particulate cytoplasmic fraction by chymotrypsin digestion is similar to the release of oligo A from reovirions by this enzyme (14). Moreover, the viral polypeptides which remain in this fraction after chymotrypsin digestion are the same ones conserved after digestion of reovirions. These facts lead us to suggest that oligo A, like dsRNA (1, 20, 37), is synthesized within the maturing virion. The presence of in vitro synthesized oligo A within particles of density 1.365 g/cm³ in

FIG. 12. Effect of temperature on the synthesis of ssRNA, dsRNA, and oligo A. An LPF was prepared from 5×10^6 cells infected 13 h previously with reovirus (20 PFU/cell). The fraction was resuspended in 2 ml of RSB and used as the source of enzyme for RNA synthesis. Incubations were conducted at 31, 37, and 45 C. a, Samples (0.2 ml) of enzyme (equivalent to 5×10^6 cells) were incubated for dsRNA synthesis in 0.5-ml reaction mixtures for 5 min at the indicated temperatures. The reactions were stopped by addition of 1 ml of cold RSB, and the dsRNA was extracted and precipitated. ds RNA was measured as ribonuclease-resistant ($4 \mu\text{g/ml}$) acid-insoluble radioactivity in 0.3 M NaCl as described (1). b, Samples (15 μl) of enzyme (equivalent to 5×10^6 cells) were incubated for ssRNA synthesis in 0.3-ml reaction mixtures at the

CsCl, and the exclusion of oligo A from the soluble cytoplasm of reovirus infected cells (5), support this hypothesis.

Brief incubation of intact reovirus at elevated temperatures activates the RNA transcriptase within the virion, presumably by removing or altering the outer layer of capsids (9). However, the enhanced ssRNA synthesis at elevated temperatures reported here did not result from alterations in permeability of virions and nascent particles since the LPF was incubated with chymotrypsin prior to the measurement of ssRNA synthesis. As noted earlier, the effect of chymotrypsin digestion on the viral polypeptides within the LPF closely resembles the effect of this enzyme on purified virions. Similarly, chymotrypsin treatment of the LPF had no effect on the rate of dsRNA synthesis at 31, 37, or 45 C. Since chymotrypsin treatment of the LPF abolishes oligo A synthesis, similar control experiments regarding the effect of temperature on the availability of nucleotides for oligo A formation cannot be performed. Our finding that the ssRNA transcriptase, dsRNA replicase, and oligo A polymerase all have the same unusual temperature optimum (45 C)

FIG. 13. Model of reovirus maturation.

temperatures indicated. At appropriate times 40 μl of the reaction mixture were analyzed for acid-insoluble radioactivity as described (24). c, Samples (0.4 ml) of enzyme (equivalent to $\sim 10^6$ cells) were incubated for oligo A synthesis in 1-ml reaction mixtures for 30 min at the indicated temperatures. The reaction mixtures were then centrifuged at $20,000 \times g$ for 30 min at 4 C, and the oligo A contained in the pellet fractions was extracted and analyzed as described in Fig. 11.

suggests that they are alternative activities of the same enzyme(s). However, the inhibition of oligo A synthesis by chymotrypsin does not appear to be consistent with this hypothesis since chymotrypsin stimulates the ssRNA transcriptase (3, 27) and has no inhibitory effect on the dsRNA synthetase (1). One explanation of this apparent paradox is that activities of the oligo A polymerase and the ssRNA transcriptase (2) are regulated inversely by outer capsid proteins.

A model for the assembly of reovirions which is consistent with these data is presented in Fig. 13. dsRNA is synthesized within the core of the nascent virion by the formation of minus strands on plus strand templates. As dsRNA strands are completed, the dsRNA replicase functions as an ssRNA transcriptase and catalyzes the formation of ssRNA plus strands. Finally, the ssRNA transcriptase is converted to an oligo A polymerase and oligo A synthesis commences within the maturing virion. Although the initiation signal for the formation of oligo A has not been identified, we assume that the assembly or modification of outer capsid proteins exerts a regulatory effect upon the particle-bound ssRNA transcriptase, altering its conformation and converting it to an oligo A polymerase. A similar proposal regarding the mechanism of oligo A formation has been made by Nichols et al. (19).

Unfortunately, these studies have yielded no clue to the function of reovirus oligo A. Reovirions uncoated in vivo (11), or under specific conditions in vitro (14), retain oligo A yet they exhibit the same RNA transcriptase activity as particles lacking these adenylic acid oligomers (14). Neither the plus strands which function as templates for dsRNA synthesis nor the plus strands which serve as mRNA's contain oligo A (31), and we have been unable to identify a virion-bound activity which joins oligo A to the ssRNA products of the RNA transcriptase (S. Silverstein and G. Acs, unpublished observation). Moreover, reovirus "top component" (which contains no RNA) is indistinguishable in polypeptide composition from virions containing dsRNA and oligo A (28). Thus, whatever its function, oligo A is not required for the activity of the particle-bound RNA transcriptase, for the formation of functional mRNA or of plus strand precursors of dsRNA, or for the assembly of viral proteins into virions.

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