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Intermediates in the Biosynthesis of Double-Stranded Ribonucleic Acids of Bacteriophage ϕ6

(replicative intermediate/replication of double-stranded RNA/*Pseudomonas phaseolicola*)

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ABSTRACT ***Pseudomonas phaseolicola* infected with bacteriophage ϕ6 synthesized all three viral double-stranded RNA segments, three single-stranded RNAs, and three replicative intermediate-like RNAs in the presence of rifampin. The single-stranded RNA intermediates sedimented and electrophoresed along with melted viral double-stranded RNA, annealed with melted viral double-stranded RNA, and were transient in nature. The relative amounts of the single-stranded RNA intermediates varied during the infection cycle and were altered in the presence of chloramphenicol. The replicative intermediate-like RNAs sedimented faster than double-stranded RNA, failed to enter 2.5% polyacrylamide gels, eluted with double-stranded RNA from a CF-11 cellulose column, were precipitated with single-stranded RNA in 2 M LiCl, and yielded three genome-size pieces of double-stranded RNA upon digestion with RNase. These results are consistent with the hypothesis that complementary strands of the ϕ6 double-stranded RNAs are synthesized asynchronously during the infection cycle.**

The genome of ϕ6, a lipid-containing *Pseudomonas phaseolicola* bacteriophage, consists of three unique double-stranded RNA (dsRNA) segments of approximately 2.2, 3.2, and 5.0 $\times$ 10^6 daltons (1, 2). Recent work (Coplin *et al.*, in preparation) indicates that the three segments are synthesized continuously throughout the infection cycle. If infected cells were labeled with [^{3}H]uracil and then "chased" with excess unlabeled uracil, radioactivity continued to accumulate in dsRNA, suggesting that complementary strands of the dsRNAs are synthesized asynchronously, possibly via single-stranded RNA (ssRNA) intermediates. The present paper describes the identification and properties of two classes of RNA intermediates: (*i*) ssRNA transcripts formed from each segment, and (*ii*) the corresponding partially double-stranded branched RNAs which are analogous to the replicative intermediates (RI) of ssRNA bacteriophages.

MATERIALS AND METHODS

Growth and Infection of Bacteria. Phage lysates were prepared by suspending confluent lysis overlays in 5 ml of nutrient broth/yeast extract medium (NBY) (3) and clarifying by centrifugation. Supernatants were stored in 6% sucrose at −80°. *P. phaseolicola* HB10Y, host for phage ϕ6, was maintained on NBY agar (3). All experiments were done in minimal salts/casein (MSC) liquid medium which contains per liter of distilled water: 5.90 g of K_2HPO_4; 2.20 g of KH_2PO_4; 1.32 g of $(NH_4)_2SO_4$; 0.20 g of $MgSO_4 \cdot 7H_2O$; 1.54 mg of $MnSO_4 \cdot H_2O$; 8.8 mg of $ZnSO_4$; 2.42 mg of $FeCl_3 \cdot 6H_2O$; 10.0 g of sucrose; and 1.0 g of Difco casamino acids. The sucrose and casamino acids were autoclaved separately.

To obtain consistent and maximal infection, a culture of the host that had been grown for 12 hr with shaking was subcultured at 26° and allowed to increase from 8 $\times$ 10^7 to 4 $\times$ 10^8 cells per ml. The culture was then chilled to 0° and phage were added at a ratio of 10 plaque-forming units (PFU) per cell. After an adsorption period of 30 to 60 min, infection was initiated by warming the culture to 26°.

Rifampin Treatment, Pulse Labeling, and Extraction of RNA. Rifampin was routinely added to bacterial cultures to give a final concentration of 200 μg/ml 10 min before labeling. RNA was labeled with 3 μCi/ml of [*5*-^{3}H]uracil (20.4 Ci/mmol) or [*5,6*-^{3}H]uracil (40.7 Ci/mmol). Labeling was stopped by pouring cells over crushed frozen MSC medium, and the cells were harvested by centrifugation at 8000 $\times$ *g* for 5 min. For extraction of dsRNA and RI, the cells were resuspended in 1.0 ml of STE buffer [50 mM Tris, 0.1 M NaCl, 5 mM EDTA (pH 8.0)] containing 0.1 ml of 25% sodium dodecyl sarcosine and 400 μg of washed bentonite. The suspension was placed at 65° for 5 min and then deproteinized with an equal volume of phenol-cresol (100 g of phenol, 11 ml of STE buffer, 14 ml of *m*-cresol, 100 mg of 8-hydroxyquinoline) at 65° for 5 min. The aqueous phase was removed and the sodium concentration adjusted to 0.3 M with 5 M sodium acetate, pH 5.5. RNA was precipitated with 3 volumes of 95% ethanol at −20°. ssRNA was extracted in the same manner except that it was deproteinized with equal volumes of phenol-cresol and chloroform at room temperature to prevent aggregation.

Nucleic acids were purified by chromatography on CF-11 cellulose fiber columns (4). ssRNA eluted in 15% ethanol, while dsRNA and RI were eluted in buffer without alcohol. RI was separated from dsRNA by overnight precipitation at 4° with 2 M LiCl in STE buffer.

Centrifugation and Electrophoresis. RNA samples were dissolved in 0.5 ml of 0.10 M NaCl, 10 mM Tris (pH 8.9) and centrifuged for 10.5 hr at 37,000 rpm at 4° in an SW 41 rotor on linear-logarithmic sucrose density gradients (5) equilibrated with the same buffer. The gradients were scanned photometrically at $A_{254\,nm}$ with an ISCO density gradient fractionator, 0.25 ml fractions were collected, and the radioactivity was

Abbreviations: dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; RI, replicative intermediate; NBY, nutrient broth/yeast extract medium; MSC, minimal salts/casein medium; STE buffer, 0.1 M NaCl, 50 mM Tris, 5 mM EDTA (pH 8.0).

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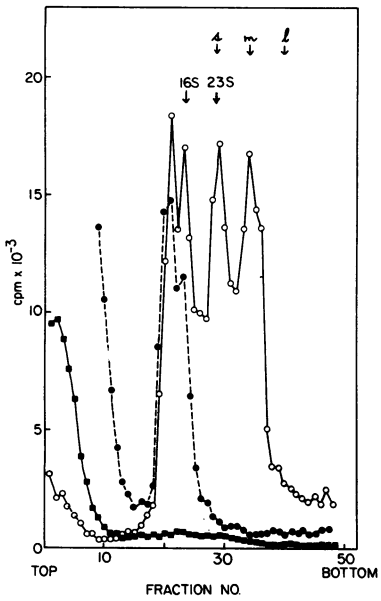

FIG. 1. Rate zonal sedimentation of [^{3}H]RNA extracted from (*i*) uninfected cells pulsed 10 min after the addition of rifampin (■), (*ii*) ϕ6-infected cells treated with rifampin at 40 min after infection and pulsed from 50 to 53 min after infection (○), and (*iii*) cells pulsed as in *ii* but sample pretreated with 2.5 μg of RNase A in 0.3 M NaCl, 50 mM Tris (pH 8.5) (●). Positions of bacterial rRNAs and ssRNA species (large, *l;* medium, *m;* and small, *s*) obtained by melting ϕ6 [^{32}P]dsRNA are indicated.

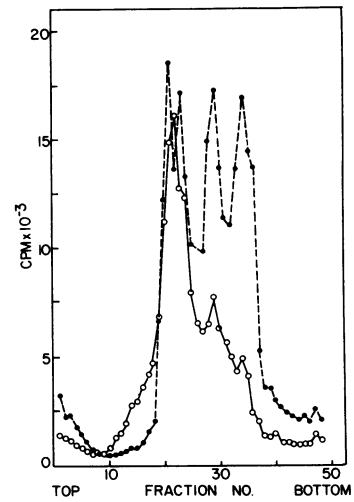

FIG. 2. Rate zonal sedimentation in linear-logarithmic sucrose gradients of RNA from infected cells treated with rifampin and pulse-labeled with [^{3}H]uracil as in Fig. 1, method *ii*. RNA extracted immediately after pulse (●). Cells washed and incubated with excess unlabeled uracil for 10 min before RNA extraction (○).

determined. Alternatively, if the samples were to be treated with RNase, they were dissolved in 0.3 M NaCl, 50 mM Tris, pH 8.5 (high salt buffer) and centrifuged on gradients equilibrated with the same buffer.

Gel electrophoresis was performed in 2.5% polyacrylamide (4% crosslinking) containing 0.5% agarose (6). Viral ssRNAs were separated by electrophoresis at 6 mA per gel for 4–5 hr, whereas dsRNAs were usually electrophoresed for 8–9 hr.

RNA·RNA Hybridization. Samples containing radioactive viral ssRNA, 0.2–0.4 A_{260} unit of virion dsRNA, and 1 μmol of EDTA (pH 7.2) in a total volume of 0.1 ml were heated for 90 sec at 100° and then incubated at 50° for 2 hr to allow annealing. After incubation, 0.1 ml of 0.6 M NaCl and 60 mM sodium citrate containing 10 μg/ml of RNase A and 5 μg/ml of RNase T1 were added and the samples incubated at 34° for 45 min. The reaction was terminated with 0.2 ml of 10% trichloroacetic acid and the samples were stored at 0° for 2 hr. Trichloroacetic acid precipitates were collected on Whatman GF/A glass fiber filters, washed, dried, and counted.

Materials. [^{3}H]Uracil was obtained from New England Nuclear, rifampin from Calbiochem, chloramphenicol from Sigma, RNases A and T1 from Worthington Biochemicals, and electrophoresis reagents from Eastman Kodak. Antiserum to ϕ6 was prepared as described (7).

RESULTS

Effect of Rifampin. In order to study viral RNA synthesis, it was necessary to inhibit host RNA synthesis because only about 50% of the bacteria were infected under optimum conditions. Host RNA synthesis was completely inhibited within 7 min by rifampin, at 200 μg/ml, but viral RNA synthesis continued at a gradually diminishing rate for at least 40 min. Formation of infectious particles was prevented if rifampin was added within 30 min after infection. Rifampin added 40 min or later after infection allowed formation of a reduced number of infectious particles; however, lysis was delayed. Under normal conditions, in MSC medium, phage release occurs between 60 and 80 min after infection.

Viral RNA Synthesis in Infected Cells. Infected cells were treated with rifampin at 40 min after infection and then labeled with [^{3}H]uracil from 50 to 53 min after infection. RNA was immediately extracted and analyzed by density gradient centrifugation. Peaks of radioactive material which sedimented at about 15 S, 17 S, 24 S, and 29 S were obtained from infected cells but not from uninfected cells labeled under similar conditions (Fig. 1). The peaks at 17 S and 15 S contain genome-sized dsRNA; the medium (15.5 S) and small (14.5 S) segments are not resolved under these conditions. If the sample was pretreated with 2.5 μg/ml of RNase A in high salt buffer before centrifugation, material in the 24 and 29S peaks was degraded, but not that in the peaks at 15 and 17 S, indicating that the former is single-stranded RNA (Fig. 1). [^{32}P]ssRNA derived from melted virion dsRNA sedimented with the material of the 24 and 29S peaks. Under these conditions, very little if any ^{3}H-labeled material sedimented with the melted large [^{32}P]dsRNA segment. Results similar to those reported in Fig. 1 were obtained when the experiment was repeated with [^{3}H]adenine.

A "pulse and chase" experiment was done to determine whether the ssRNAs were stable or transient in nature. Infected cells were treated with rifampin at 40 min after infection and labeled with [^{3}H]uracil from 50 to 53 min after infection. After the pulse the cells were washed and resuspended in fresh medium containing rifampin and 0.5 mg/ml of unlabeled uracil. Incubation was continued for additional 10 and 20 min periods, after which RNA was extracted and centrifuged on sucrose density gradients. The radioactivity profiles of the control and of samples chased for 10 min are shown in Fig. 2. The profile of the sample chased for 20 min (data not shown) is similar to that in Fig. 2. It is apparent that radioactivity in the ssRNAs is transient and can be chased out. Fractions from a similar gradient were treated

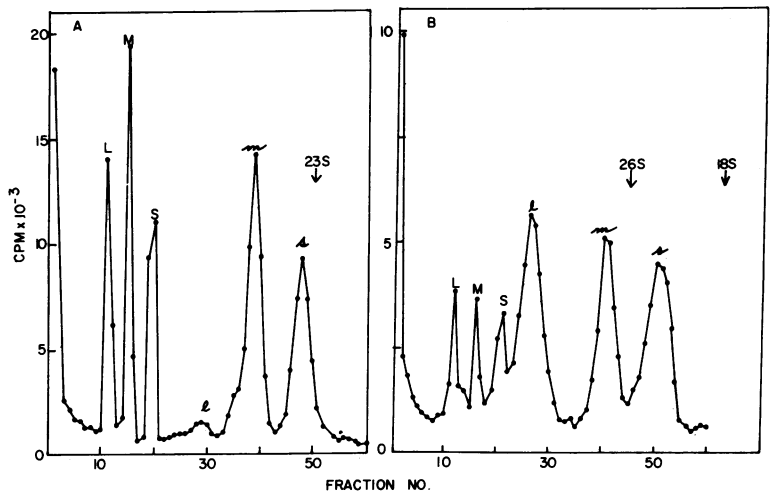

FIG. 3. Polyacrylamide gel electrophoretic analysis of viral RNA extracted from (A) cells treated with rifampin at 40 min after infection and pulsed with [^{3}H]uracil from 50 to 53 min after infection and (B) cells as in (A) but treated with chloramphenicol at 30 min after infection. *Arrows* show positions of *P. phaseolicola* 23S rRNA and *Rhizopus stolonifer* 18 and 26S rRNA markers. L, M, and S refer to the large, medium, and small dsRNA segments; *l*, *m*, and *s* are the corresponding ssRNAs. The direction of migration is from left to right.

with RNase A in high salt, and nearly all the radioactivity remaining in the 24 and 29S regions after the chase period was resistant to degradation. This material is believed to be RI molecules.

Effect of Chloramphenicol on Viral RNA Synthesis. Chloramphenicol inhibits protein synthesis in *P. phaseolicola* and prevents the formation of infectious ϕ6 particles if added within 30 min after infection (Coplin *et al.*, unpublished data). Infected cells were treated with 100 μg/ml of chloramphenicol at 30 min after infection and 200 μg/ml of rifampin at 40 min after infection. The cells were pulsed with [^{3}H]uracil from 50 to 53 min after infection; RNA was extracted and analyzed by polyacrylamide gel electrophoresis (Fig. 3A and B). Addition of chloramphenicol produced a 2- to 3-fold reduction in dsRNA synthesis and altered the pattern of viral ssRNA synthesis. The large ssRNA segment is barely detectable in the control culture (Fig. 3A); however, after chloramphenicol treatment the large ssRNA is labeled to the same extent as the medium and small ssRNAs (Fig. 3B).

Determination of the Molecular Weights of Viral ssRNA. The molecular weights of the ϕ6 ssRNAs from infected bacteria were estimated to be 1.09 $\pm$ 0.02, 1.51 $\pm$ 0.07, and 2.23 $\pm$ 0.06 $\times$ 10^6 by electrophoresis of these RNAs with ^{14}C-labeled *Rhizopus stolonifer* rRNAs. These values agree with melted virion RNA molecular weights of 1.12, 1.41, and 2.26 $\times$ 10^6 obtained by gel electrophoresis under the same conditions (2).

Hybridization of ^{3}H-Labeled ssRNAs to Virion RNA. Further evidence that the three ssRNAs found in infected cells were viral products was obtained by annealing experiments with dsRNA obtained from purified virus. RNA was extracted from infected cells treated with rifampin and chloramphenicol at 40 min after infection and pulsed from 50 to 55 min after infection with [^{3}H]uracil. The [^{3}H]RNA was fractionated by chromatography on CF-11 columns to remove dsRNA and RI-like RNA from the ssRNA. Although the ssRNA preparation contained primarily rRNA, gel electrophoresis revealed that 16S rRNA and presumably 23S rRNA were not labeled and the material was free of dsRNA. The isolated ssRNAs were mixed with 0.36 A_{260} unit of virion dsRNA, melted, allowed to reanneal, treated with RNases in high salt, and assayed for trichloroacetic acid-precipitable radioactivity (*Materials and Methods*). Table 1 shows that such a sample containing 4400 cpm was only 17% ribonuclease-resistant. However, when annealed with ϕ6 dsRNA, an additional 78% of the radioactivity became RNase-resistant. In contrast, host nucleic acids did not hybridize with the viral RNA. The hybrids thus formed electrophoresed on polyacrylamide gels with virion dsRNA, and the distribution of radioactivity among the three dsRNA hybrids was the same as that among the ssRNAs from which they were constructed, indicating that all three species hybridized with virion dsRNA to the same extent.

Temporal Synthesis of Viral ssRNA after Infection. To determine the temporal pattern of viral ssRNA synthesis, the culture was synchronized by addition of antiserum at 5 min after infection to remove unadsorbed phage. Aliquots were removed from an infected culture at 10 min intervals and

TABLE 1. *Annealing of ^{3}H-labeled ssRNA from uninfected cells or rifampin-treated infected cells with unlabeled virion dsRNA**

Source of [^{3}H]RNA	Added ϕ6 dsRNA	Treated with RNase	Acid-precipitable cpm	Percent RNase-resistant
ssRNA from	−	−	4400	—
infected cells	−	+	735	16.7
	+	+	4190	95.1
ssRNA from un-	−	−	5210	—
infected cells	−	+	762	14.6
	+	+	845	16.2

* Aliquots of [^{3}H]ssRNA were added to 0.36 A_{260nm} of virion dsRNA. The RNAs were melted, allowed to anneal, treated with RNase, precipitated with trichloroacetic acid, and filtered. Radioactivity was determined. (See *Materials and Methods.*) ssRNA was extracted from infected cells pulsed from 50 to 55 min after infection with [^{3}H]uracil in the presence of rifampin and chloramphenicol and was fractionated by column chromatography on CF-11 cellulose. Nucleic acids were extracted from untreated healthy cells pulsed for 5 min with [^{3}H]uracil. Each treatment is the average of three determinations.

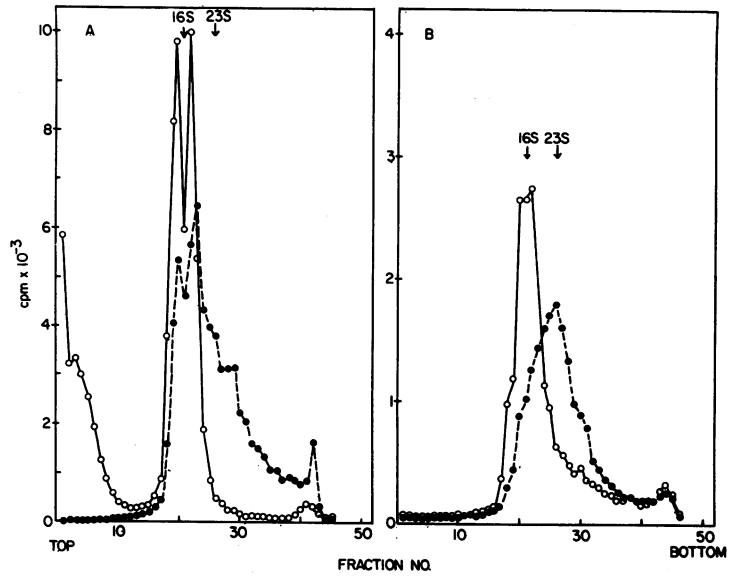

FIG. 4. Rate zonal sedimentation of pulse-labeled dsRNA purified by cellulose fiber CF-11 chromatography. A. dsRNA fraction untreated (●) and pretreated with RNase A in 0.3 M NaCl, 50 mM Tris (pH 8.5) (○). B. Precipitate (●) and supernatant (○) from 2 M LiCl fractionation.

pulsed with [^{3}H]uracil for 3 min. Electrophoresis of the RNA revealed that the large ssRNA species was always labeled to a much less extent than the medium and small species (Table 2). The percentage of label in the large species decreased from 10 to 12% at 10–20 min after infection to about 4% at 60 min after infection, while small ssRNA decreased from 64% to 47%. Over the same period the proportion of medium ssRNA increased from about 25% to 50%.

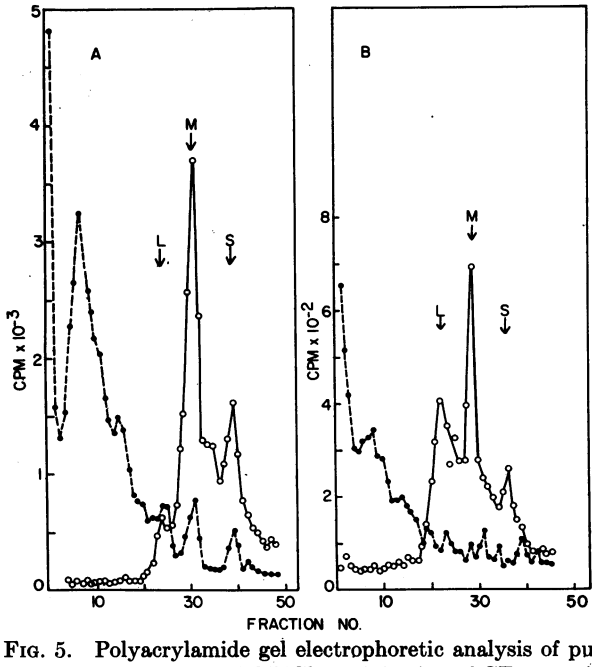

FIG. 5. Polyacrylamide gel electrophoretic analysis of pulse-labeled RI obtained by 2 M LiCl precipitation of CF-11 purified dsRNA. Cells pulsed in presence of rifampin from 50 to 51 min after infection. A. Untreated cells. B. Chloramphenicol-treated cells at 30 min after infection. Untreated RNA (●), RNase-treated before electrophoresis (○). *Arrows* indicate position of virion dsRNA markers. Direction of migration is from left to right.

Characteristics of RI-Like RNA. An RNA fraction that had the properties expected for RI RNA was separated from a dsRNA fraction, purified on CF-11 cellulose by precipitation in 2 M LiCl overnight at 0°. Sucrose density gradient radioactivity profiles of the dsRNA fraction purified on CF-11 cellulose, before and after RNase digestion in high salt, are shown in Fig. 4A and the LiCl-precipitable and -soluble fractions in Fig. 4B. The dsRNA fraction contained a broad, faster sedimenting shoulder that was not present after RNase digestion or in dsRNA extracted from purified virions (Fig. 4A). This fast sedimenting RNA precipitated in 2 M LiCl and subsequently sedimented as a heterodisperse peak ranging from about 15 to 30 S (Fig. 4B).

The majority of the LiCl-precipitable counts barely entered 2.5% polyacrylamide gels (Fig. 5) and migrated much more slowly than the large, medium, and small dsRNA segments; however, ribonuclease digestion in high salt, while releasing

TABLE 2. *Temporal synthesis of φ6 ssRNA intermediates after infection**

Time of pulse (min)	cpm†			Percentage		
	Large	Medium	Small	Large	Medium	Small
10–13	874	2140	5260	10.6	25.9	63.5
20–23	1200	2940	6000	11.8	29.0	59.2
30–33	3050	12900	15700	9.7	40.7	49.6
40–43	5880	39000	38900	7.0	46.6	46.4
50–53	7150	75900	65400	4.8	51.1	44.1
60–63	7160	88400	83900	4.0	49.3	46.8

* At intervals, 2.5 ml samples were removed from a synchronously infected culture, treated with rifampin for 10 min, and then pulsed for 3 min with [^{3}H]uracil (*Materials and Methods*). The isolated RNA was electrophoresed on polyacrylamide gels, and the radioactivity in the large, medium, and small ssRNA intermediates was determined.

† Average value of the three separate experiments.

about half of the radioactivity, yielded labeled dsRNA which was electrophoretically identical to virion dsRNA. The dsRNA obtained in this manner was labeled primarily in the medium and small segments (Fig. 5A). Prior treatment of the cells with chloramphenicol greatly increased the relative proportion of label in the large segment (Fig. 5B). CF-11 derived RI, collected from the fast sedimenting shoulder from a sucrose gradient (Fig. 4A), exhibited similar properties.

DISCUSSION

Double-stranded RNA viruses such as reovirus replicate via ssRNA intermediates which serve as templates for the synthesis of their complementary strands (8). These ssRNA intermediates in reovirus are termed plus strands because they can also function as mRNA. Evidence that the ssRNAs described in this paper are viral RNAs and are probable intermediates in the replication of ϕ6 dsRNA includes: (*i*) they are found only in infected cells and are labeled in the presence of rifampin, (*ii*) they are transient in nature and are no longer labeled after a chase period, (*iii*) they have electrophoretic and sedimentation properties that are identical to those of melted virion dsRNA, and (*iv*) they hybridize specifically with all three segments of virion dsRNA. These ssRNAs may function as mRNAs since all three species can be isolated from a polysomal fraction obtained from infected cells (Coplin *et al.*, unpublished data). Thus, we have named these ssRNAs plus strands. However, unlike the plus strands of reovirus, those of ϕ6 probably contain several cistrons because the phage contains at least 8 to 10 structural polypeptides (Van Etten *et al.*, unpublished data). Phage ϕ6 plus strands, therefore, would represent polycistronic rather than monocistronic messengers.

The plus strands are not synthesized in constant proportions throughout the infection cycle. The vast majority of the ssRNA intermediates synthesized late in infection consist of medium and small RNA transcripts. Possibly these two ssRNA species code for most of the capsid and other proteins required in large amounts. In contrast, the early gene products, such as regulatory and noncapsid proteins, may be coded for by the large RNA species.

It is interesting that chloramphenicol reduces dsRNA synthesis and alters the pattern of ssRNA synthesis so that an increased amount of large plus strands accumulates. Cycloheximide similarly inhibits dsRNA synthesis in reovirus-infected cells and allows the synthesis of plus strands to continue according to the transcription pattern at the time of its addition (9, 10). In the case of ϕ6, the basis for the effect of chloramphenicol is not understood. However, dsRNA synthesis might be reduced because of the inability to synthesize proteins required for dsRNA (minus strand) synthesis, thereby allowing plus strands to accumulate. Alternatively, the antibiotic may block formation of regulatory proteins whose function is to repress synthesis of the large plus strands.

The properties of ϕ6 RI-like RNA agree with those reported by Franklin (4) for the RI of phage R17: it (*i*) sediments faster than dsRNA, (*ii*) does not enter 2.5% gels, (*iii*) elutes with dsRNA from a CF-11 cellulose column, (*iv*) precipitates in high salt, and (*v*) yields genome-sized pieces of dsRNA upon digestion with ribonuclease. These data are consistent with a structure composed of a full-length dsRNA molecule and one or more partially completed ssRNA transcripts. Although subsequent studies on the RI of ssRNA phages have shown that these structures actually exist *in vivo* as single-stranded complexes that anneal upon deproteinization (11), we do not believe this is the case with ϕ6. Since ϕ6 is double-stranded within the virion, it is unlikely that it would be entirely single-stranded *in vivo* since this would require a direct conversion of dsRNA to ssRNA.

Reovirus transcribes plus strands in a conservative manner (12). If an RI exists for reovirus, one might not expect a rapid labeling of the double-stranded portion of the molecule on the basis of a conservative model. Thus, the observation that ϕ6 RI-like RNA is rapidly labeled in both single-stranded and double-stranded portions of the molecule suggests that transcription of reovirus and ϕ6 may differ, i.e., ϕ6 may replicate semiconservatively.

It is also significant that the distribution of labeled uracil among the three plus strands and among the three dsRNA segments obtained from RI by ribonuclease digestion is very similar. Furthermore, treatment with chloramphenicol increases the relative synthesis of both large plus strand and large RI. This suggests a precursor–product relationship between RIs and plus strands.

Our current model is that phage ϕ6 replicates in a manner analogous to that of reovirus, in that complementary strands are synthesized at different times. Plus strands are transcribed from dsRNA via a RI-like structure. These plus strands probably first serve as polycistronic mRNA and then as templates for synthesis of their complementary strands to form dsRNA. Unlike reovirus, however, the rapid labeling of the double-stranded regions of RI suggests that ϕ6 transcription may not be a conservative process.

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