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Semiconservative Synthesis of Single-Stranded RNA by Bacteriophage φ6 RNA Polymerase†

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The RNA polymerase in the nucleocapsid of *Pseudomonas phaseolicola* bacteriophage φ6 transcribed large, medium, and small single-stranded RNA from the viral double-stranded RNA genome by a semiconservative (displacement) mechanism. Approximately 23%, 63%, and 65% of the nucleocapsid particles in the assay mixture synthesized at least one round of large, medium, and small single-stranded RNA molecules, respectively. Some of these particles reinitiated synthesis such that an average of 1.5 large, 33 medium, and 24 small single-stranded RNAs were synthesized from each double-stranded RNA.

The genome of φ6, a lipid-containing *Pseudomonas phaseolicola* bacteriophage (19), consists of three unique double-stranded RNA (dsRNA) segments with molecular weights of 2.2×10^6 (small), 3.2×10^6 (medium), and 5.0×10^6 (large) (13, 17). Treatment of the phage with nonionic detergents removes the lipid envelope and leaves a stable icosahedral nucleoprotein capsid (NC) consisting of at least five proteins and the three dsRNA's (15, 16).

The virus also has its own RNA polymerase. Enzyme activity was detected either after a brief heat treatment of the intact phage (18) or with untreated NC (10, 16). The NC RNA polymerase synthesized large quantities of single-stranded RNA (ssRNA) which annealed to denatured medium and small dsRNA's (10); however, large ssRNA synthesis was not detected.

The RNA polymerase could synthesize viral ssRNA's by either a conservative mechanism, whereby the nascent RNA strand is released as ssRNA, or by a semiconservative (displacement) mechanism, whereby the nascent strand replaces one of the two parental strands. In this paper we present direct evidence that the φ6 RNA polymerase synthesizes ssRNA in vitro by a semiconservative mechanism. Furthermore, a small amount of large ssRNA synthesis is detected.

MATERIALS AND METHODS

Materials. [5-^{3}H]CTP (22 Ci/mmol) and [5,6-^{3}H]uracil (40 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass. Phage φ6 NC containing [^{3}H]dsRNA was prepared by growing *P. phaseolicola* on SSM medium (19) at 25°C to a cell concentration of 4×10^8 to 5×10^8 colony-forming units per ml. The culture was then chilled to 0°C, and phage was added at a ratio of 5 PFU/cell. After a 20-min adsorption period, the culture was returned to 25°C and 0.2 μCi of [^{3}H]uracil was added per ml (0 time). An additional 0.6 μCi of [^{3}H]uracil per ml was added at both 15 min and 100 min postinfection. After lysis and release of φ6, phage was purified, and NC was prepared as described previously (10). The NC contained ca. 2.8×10^5 cpm per unit of absorbancy at 260 nm.

RNA polymerase and analysis of products. The standard reaction mixture for the RNA polymerase was as described previously (10), except that assays using [^{3}H]dsRNA NC contained unlabeled nucleoside triphosphates. The reaction products were precipitated with 2.5 volumes of ethanol and taken up in small volumes of buffer E (0.04 M Tris, 0.033 M sodium acetate, 0.001 M EDTA, 0.2% sodium dodecyl sulfate, pH 7.2) (1). The samples were layered on linear log sucrose density gradients (3), equilibrated with SSC buffer (0.15 M NaCl, 0.015 M sodium citrate, pH 7.2), and centrifuged for 16 h at 28,000 rpm at 14°C in a Beckman SW41 rotor. Gradients were scanned photometrically at 254 nm, and 0.25-ml fractions were collected and counted. Alternatively, the reaction products were taken up in buffer E containing 10% Ficoll and electrophoresed for 16 h at 40 V in buffer E on 2% polyacrylamide slab gels containing 0.5% agarose (14). The gels were soaked in water to remove sodium dodecyl sulfate, and then RNA was visualized on the gels with Stains-all (7); radioactive RNAs were detected by fluorography (2, 9). The radioactive RNAs in the gels were quantitated by excising the radioactive zones, eluting the radioactive material, and counting as described previously (18).

RESULTS

Mode of replication. Phage φ6 NC containing [^{3}H]dsRNA was used to determine whether φ6 RNA polymerase synthesizes ssRNA by a conservative or semiconservative mechanism. If

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the reaction is conservative, all of the radioactivity should remain in dsRNA; conversely, if the reaction is semiconservative, at least some of the radioactivity should appear in ssRNA. The products of the polymerase reaction at 30 min were analyzed by sucrose density gradient centrifugation (Fig. 1). The majority of the radioactivity remained with the 3 dsRNA's; however, approximately 19% of the radioactivity (22,300 cpm of the 116,600 cpm present in the NC) appeared in the region of small, medium, and large ssRNA's.

The RNA polymerase products formed from [^{3}H]dsRNA NC at 10, 30, and 60 min were also electrophoresed on a polyacrylamide gel; the gel was stained with Stains-all (Fig. 2A) to visualize total RNA and then processed for fluorography (Fig. 2B) to detect radioactivity. Radioactivity was present in all three dsRNA's, in all three ssRNA's, and in replicative intermediate (RI) RNA. Although the ϕ6 enzyme is a transcriptase and not a replicase, we find it convenient to call dsRNA's, with one or more partially completed ssRNA's, replicative intermediates (RIs) because of their structural similarity to RNA phage RIs. Since radioactive ssRNA's were derived from [^{3}H]dsRNA, the results indicate semiconservative RNA synthesis. Furthermore, since radioactivity appeared in all three ssRNA's, all three RNAs must be synthesized semiconservatively.

Percentage of NC particles synthesizing RNA semiconservatively. To estimate the percentage of NC particles that synthesize at least one strand of ssRNA, the various RNA species were excised from the gel shown in Fig. 2, and the radioactivity was eluted and counted (Table 1). Thirty minutes after the start of the reaction, approximately 19% of the radioactivity was in ssRNA, 71% was in dsRNA, and 10% was in RI RNA.

If one assumes that uracil is present in equal amounts in both strands of each dsRNA, then displacement of one complementary strand of the dsRNA by semiconservative replication would ultimately release 50% of the label into ssRNA if all of the NC particles were active. Therefore, the percentage of the NC particles that exhibit enzyme activity equals [(2 × cpm in ssRNA) + (cpm in RI)]/[cpm in (ssRNA + dsRNA + RI)] × 100. Thus, 30 min after the start of the reaction, a minimum of 48% of the particles synthesized RNA by a semiconservative mechanism [(18,980 + 4,910)/49,900]. Since a similar percentage was obtained at 10 min (42.5%), the majority of the NC particles that are active apparently function immediately.

If the incorporation in the RI region is disregarded (radioactivity into small, medium, and large RI RNAs cannot be resolved), similar calculations allow us to estimate the percentage of NC particles that synthesize small, medium, and large ssRNA's. Thus, at 30 min, 23.4% (5,660 ÷ 24,140), 63.3% (7,580 ÷ 11,980), and 64.6% (5,740

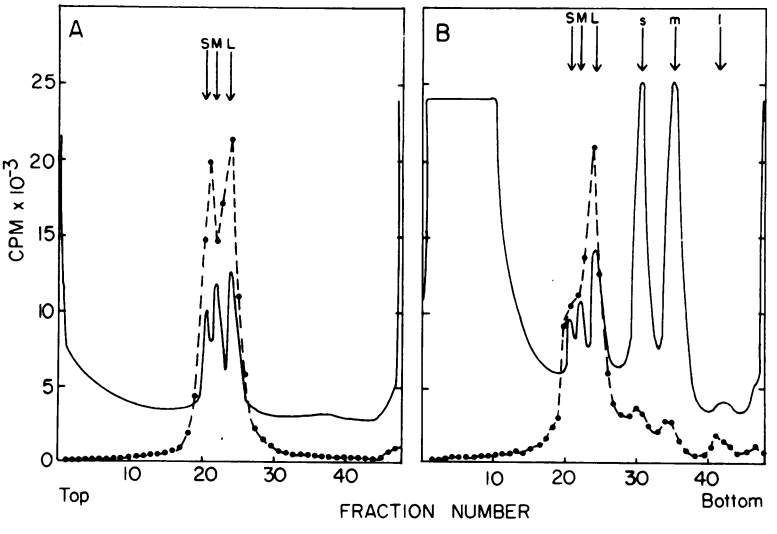

FIG. 1. *Analysis of ϕ6 NC RNA polymerase products by sucrose density gradient centrifugation. [^{3}H]-dsRNA NC served as the source of RNA polymerase, and the reaction was run with unlabeled nucleoside triphosphates. (A) Sample at zero time; (B) sample at 30 min. The symbols S, M, and L, refer to the three dsRNA's (small, medium, and large), and s, m, and l indicate the corresponding ssRNA's. The absorbancy at 254 nm is represented by the solid line, and radioactivity is indicated by the dashed line. The majority of the absorbancy at 254 nm at the top of the gradient in B is due to unincorporated nucleoside triphosphates.*

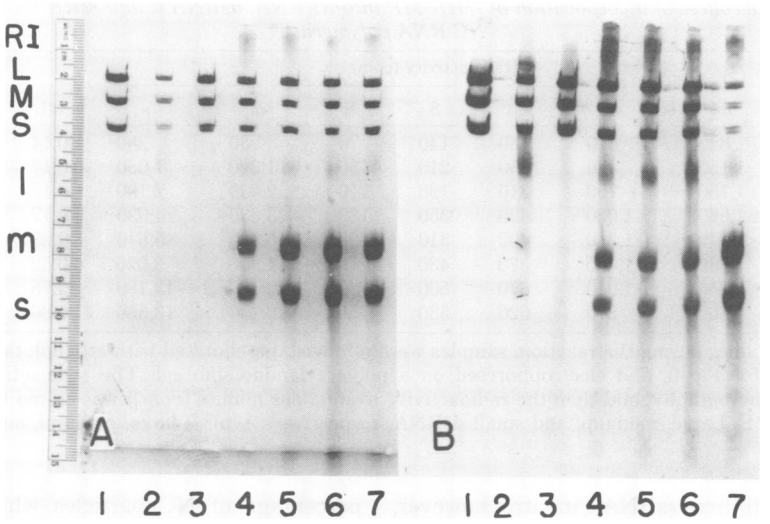

FIG. 2. *Analysis of φ6 NC RNA polymerase products by polyacrylamide gel electrophoresis. Slot 1 contained [^{14}C]dsRNA, and slot 2 contained [^{14}C]dsRNA that had been heated to 100°C for 2 min and quickly cooled. Slots 3 to 6 contained products from the [^{3}H]dsRNA NC reaction at 0 min (slot 3), 10 min (slot 4), 30 min (slot 5), and 60 min (slot 6). Slot 7 contained a 30-min reaction product using unlabeled NC as an enzyme source and assayed with [^{3}H]CTP. (A) Gel stained with Stains-all; (B) fluorogram of the same gel. Symbols are defined in the legend to Fig. 1.*

TABLE 1. *Distribution of radioactivity into RI RNA, dsRNA's, and ssRNA's from [^{3}H]dsRNA NC at various times after initiating the RNA polymerase reaction*[a]

RNA[b]	Time							
	0 min		10 min		30 min		60 min	
	cpm	%	cpm	%	cpm	%	cpm	%
RI RNA			5,980	11.7	4,910	9.8	3,450	7.0
L dsRNA	26,740	46.6	22,140	43.4	21,310	42.7	20,470	41.8
M dsRNA	17,930	31.3	8,520	16.7	8,190	16.4	8,730	17.8
S dsRNA	12,670	22.1	6,530	12.8	6,010	12.1	6,980	14.2
Total dsRNA			37,190	72.8	35,510	71.2	36,180	73.8
l ssRNA			1,670	3.3	2,830	5.7	2,940	6.0
m ssRNA			3,680	7.2	3,790	7.6	3,440	7.0
s ssRNA			2,500	4.9	2,870	5.8	2,990	6.1
Total ssRNA			7,850	15.4	9,490	19.0	9,370	19.1
Total	57,340	100	51,020	100	49,900	100	49,010	100

[a] Radioactivity (counts per minute [cpm]) was eluted from the gel shown in Fig. 2 and counted.
[b] L, l, Large; M, m, medium; S, s, small.

+ 8,880) of the NC particles have synthesized at least one strand of large, medium, and small ssRNA, respectively.

Number of ssRNA's synthesized from the dsRNA's. To estimate the average number of ssRNA's synthesized from each dsRNA, we measured [^{3}H]CTP incorporation directed by unlabeled NC into each of the three dsRNA's, the three ssRNA's, and RI RNA (Table 2). The ratio of radioactivity in the ssRNA to that in the corresponding dsRNA for each segment approximates the number of ssRNA's synthesized from that dsRNA with the qualification that the first strand of ssRNA displaced is unlabeled. Therefore, 30 min after the start of the reaction, about 1.5, 33, and 24 strands of large, medium, and small ssRNA's, respectively, are synthesized per dsRNA (Table 2). These values are qualitatively consistent with the optical density profiles of the reaction products on sucrose gradients (Fig. 1B), correcting for the fraction of active particles. Thus, the NC RNA polymerase reinitiates the

TABLE 2. *Time course of incorporation of [^{3}H]CMP into RI RNA, dsRNA's, and ssRNA's by unlabeled φ6 NC RNA polymerase*[a]

Time (min)	Radioactivity (cpm)							Ratio		
	RI	L	M	S	l	m	s	l/L	m/M	s/S
3	820	510	160	140	70	230	280	0.14	1.4	2.1
5	1,150	470	250	210	80	1,320	1,080	0.17	2.8	5.2
7	1,150	750	310	190	110	2,330	2,100	0.15	7.5	11.2
10	1,580	1,030	390	350	180	3,330	4,090	0.17	8.5	11.8
15	1,940	1,350	430	310	300	6,580	5,640	0.23	15.4	18.0
30	1,280	1,480	370	430	770	11,950	9,920	0.52	32.2	22.9
60	950	1,040	670	500	780	22,420	13,180	0.75	33.3	26.3
90	660	870	620	530	750	20,190	13,560	0.86	32.3	25.5

[a] At various times during the reaction, samples were removed, precipitated with ethanol, taken up in buffer E containing 10% Ficoll, and electrophoresed on a polyacrylamide slab gel. The radioactive regions were visualized by fluorography, and then the radioactivity (counts per minute [cpm]) was eluted from the gel and counted. L, M, S, Large, medium, and small dsRNA, respectively; l, m, s, large, medium, and small ssRNA, respectively.

synthesis of all three ssRNA's in vitro; however, reinitiation of large ssRNA synthesis is limited.

DISCUSSION

Since the φ6 NC RNA polymerase reaction displaces radioactivity from prelabeled NC dsRNA into all three ssRNA products, the RNA polymerase synthesizes all three ssRNA's semiconservatively. This is consistent with previous indications of semiconservative φ6 ssRNA synthesis in vitro (10) and in vivo (5, 6, 12). Thus φ6 RNA polymerase differs from the dsRNA reovirus transcriptase which synthesizes ssRNA's conservatively (for review, see reference 8). The enzyme, however, is similar to the RNA polymerase in the dsRNA virus-like particles of *Penicillium stoloniferum* (4) and *Aspergillus foetidus* (11). Semiconservative replication in the virus-like particles from *P. stoloniferum* was demonstrated by showing incorporation of 5-bromouridine monophosphate into one strand of the dsRNA genome. The resultant dsRNA had a "hybrid" buoyant density in Cs_2SO_4 gradients. Unfortunately, 5-bromouridine triphosphate severely inhibits the activity of φ6 RNA polymerase (Van Etten, Burbank, and Cuppels, unpublished data).

The φ6 RNA polymerase synthesizes large ssRNA as well as medium and small ssRNA's. However, the latter ssRNA's are synthesized in much greater amounts, mimicking in vivo φ6 RNA synthesis (5). The in vitro regulation of φ6 ssRNA synthesis then is at least qualitatively sufficient to explain in vivo regulation.

In vitro synthesis with NC RNA polymerase clearly indicates that at least some NC particles reinitiate transcription and synthesize many copies of medium and small ssRNA's. In contrast, the polymerase reinitiates large ssRNA synthesis only to a limited extent. Likewise, the percentage of NC particles which synthesize large ssRNA (ca. 23%) is much less than those synthesizing medium and small ssRNA (ca. 64% each). However, it is obvious that in vitro some NC particles must be synthesizing more than one type of ssRNA, i.e., at least medium and small segments.

The ssRNA's synthesized by the NC RNA polymerase are plus strands, since they can direct the synthesis of identifiable polypeptide products in an *Escherichia coli* cell-free protein-synthesizing system (Cuppels and Van Etten, unpublished data). Thus, the φ6 NC RNA polymerase functions as a transcriptase. It might be imagined that the NC RNA polymerase synthesizes both plus and minus strands but in unequal proportions and that the strands anneal to yield dsRNA plus ssRNA, or that the NC particles also use the newly synthesized plus strands as templates to synthesize complementary minus strands to form dsRNA. These possibilities appear unlikely since we have never detected increased amounts of dsRNA as a result of the RNA polymerase reaction (e.g., compare Fig. 1A with 1B). However, limited synthesis of both strands cannot be ruled out.

In summary, φ6 RNA polymerase transcribes all three φ6 dsRNA's in vitro by way of a semiconservative mechanism.

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