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Base Composition and Hybridization Studies of the Three Double-Stranded RNA Segments of Bacteriophage $\phi 6$[1]

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The three double-stranded ribonucleic acid (dsRNA) segments of the bacteriophage $\phi 6$ were isolated and shown to have similar melting temperatures and base compositions. RNA:RNA hybridization experiments with the isolated segments eliminate the possibility that the two smaller dsRNA segments arise from a cleavage of the large dsRNA segment. The two smaller RNA segments reanneal rapidly even at low temperatures; in contrast, the large dsRNA reannealed only at higher temperatures. Evidence is also presented which suggests that the dsRNAs may contain a short single-stranded RNA tail.

Several plant and animal viruses contain a double-stranded ribonucleic acid (dsRNA) genome. In most cases, these dsRNAs have a guanosine plus cytosine (G + C) content of 38 to 44%, and each virion contains several species of dsRNA with molecular weights ranging from 0.2×10^6 to 2.9×10^6 (11). Previously, we reported the isolation and general characteristics of a morphologically unique lipid-containing bacteriophage, $\phi 6$, of *Pseudomonas phaseolicola* (10). The phage genome was composed of at least three dsRNA segments with estimated molecular weights of 2.2×10^6, 2.8×10^6, and 4.5×10^6 (8). The G + C content of the total dsRNA was 56 to 58%, which is higher than that reported for other dsRNA viruses except for the mycovirus from *Penicillium chrysogenum* (7). The present report describes additional characteristics of the $\phi 6$ dsRNA segments.

MATERIALS AND METHODS

Virus culture, purification, and nucleic acid extraction. The host, strain HB10Y of *P. phaseolicola*, was grown in a semisynthetic medium (SSM) as previously reported (10). The phage was isolated from 7- or 25-liter lysates and was purified by CsCl equilibrium sedimentation (10). The dsRNA was isolated from the purified phage by the single-phase phenol procedure of Diener and Schneider (4) as described previously (8). Phage $\phi 6$ ^{32}P-dsRNA was prepared by adding 50 mCi of $H_3{}^{32}PO_4$ at the start of a 7-liter fermentation in SSM modified by a 10-fold reduction in the phosphate concentration.

Centrifugation procedures. Sedimentation coefficients were estimated in linear-log sucrose density gradients (2) equilibrated with 0.3 M NaCl and 0.03 M sodium citrate, pH 7.0 (2× SSC), and centrifuged in a Spinco SW41 rotor at 37,000 rpm at 14 C for 8 to 12 h. The gradient columns were scanned photometrically at absorbancy at 254 nm (A_{254}) with an ISCO density gradient fractionator (Instrumentation Specialities Co.). Brome mosaic virus RNAs were used as standards for determining sedimentation coefficients.

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed in 2.4 or 3% polyacrylamide containing 0.5% agarose in 0.04 M Tris, 0.02 M sodium acetate, 0.001 M sodium EDTA, 0.2% sodium lauryl sulfate buffer, pH 7.2 (1). The gels (9 cm long, 6 mm diameter) were pre-electrophoresed for 4 h at 5 mA/gel prior to the addition of 0.05- to 0.1-ml samples in 6% sucrose. The samples were electrophoresed at 5 mA/gel for 4 to 8 h (analytical) or for 16 h (preparative) at room temperature. The gels were scanned at A_{260} with a Gilford model 2410 linear transport system (Gilford Instruments), which was coupled to a Beckman DU spectrophotometer. When radioactive profiles were required, the gels were frozen with solid CO_2 and sliced into 1-mm sections. The radioactive material was eluted from the gel slices (12) and the samples were counted as described previously (9).

Strand separation and reannealing of the dsRNA. Several procedures were used to separate the $\phi 6$ dsRNA into single strands. These included heating the dsRNA to 100 C for 90 s in 0.01 M EDTA, pH 7.0, followed by quickly cooling to −8 C. In some experiments samples were heated in 3.7% (vol/vol) formaldehyde and dialyzed overnight against 3.7% formaldehyde in 0.001 M EDTA, pH 6.8, at 40 C (3). In addition, separation of the dsRNA into single strands was attempted in 50% formamide or 85% dimethylsulfoxide (6) by heating at various temperatures and for various periods of time.

To determine the rate of reannealing of the dsRNA segments, the samples were heated in 0.01 M EDTA, quickly cooled, and then incubated at various temperatures. Portions were removed at appropriate times,

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quickly adjusted to 2× SSC, and incubated with RNase A (10 μg/ml) at 22 C for 15 min before electrophoresing on polyacrylamide gels. The percentage of reannealing was determined from planimeter measurements of the A_{260} profiles of the dsRNA and compared to equivalent samples which had not been heat treated.

Isolation of the dsRNA segments. After electrophoresis of total ϕ6 dsRNA on 2.4 or 3% polyacrylamide gels for 16 h, the gels were scanned and the three dsRNA regions were excised, frozen at −70 C, and then passed through a fine mesh screen. The dsRNA was eluted from the meshed material by shaking in a small volume of 2× SSC for 2 days at room temperature. After centrifugation to remove the residual polyacrylamide, the RNA was precipitated with 2 volumes of ethanol. The precipitated RNA was dissolved in 2× SSC, centrifuged on sucrose density gradients, and RNA regions were collected and dialyzed extensively against 0.01× SSC containing 0.01 M EDTA, pH 7.0, followed by dialysis against 0.01× SSC, pH 7.0. RNA segments isolated in this manner were used to obtain the melting temperatures (T_m) and base composition of each segment. The ϕ6 dsRNA segments used for the hybridization experiments were isolated by returning the excised dsRNA-containing gels to the gel tubes and by electrophoresing the dsRNA into dialysis sacks. The dsRNAs were dialyzed and concentrated by ethanol precipitation.

Base composition analysis. RNA samples were hydrolyzed by treatment with 0.3 M KOH for 16 h at 37 C. After neutralization with perchloric acid and removal of insoluble potassium perchlorate, the pH was adjusted to 8.8 with NH_4OH. After the addition of 0.05 M ammonium acetate (pH 8.8), alkaline phosphatase was added and the sample was incubated 3 h at 37 C to remove the phosphate residues from the nucleotide mixture. The resulting nucleosides were separated on a Bio-Rad A-26 column by using ammonium formate buffer (0.4 M, pH 4.7). The eluate was monitored at A_{254} and the quantity of each nucleoside was determined from the area of the respective peak. All analyses were performed in triplicate.

Thermal denaturation. Thermal transition studies were performed in a Cary 16 spectrophotometer equipped with an electrically heated 5-position automatic sample changer block. Cuvet temperatures were monitored by a thermistor inserted in a blank cuvet containing buffer. Optical densities and temperatures for each sample were recorded continuously with a heating rate of 0.3 to 0.6 C/min. The sodium ion concentration of all samples was adjusted by dialysis against the appropriate dilution of SSC prior to melting.

RNA:RNA hybridization experiments. Duplicate samples, containing 0.05 A_{260} of each ^{32}P-dsRNA (approximately 70,000 counts per min per A_{260}) segment, were mixed with 0.5 A_{260} of unfractionated, unlabeled ϕ6 dsRNA and adjusted to 0.01 M EDTA, pH 7.0. After a heat treatment at 100 C for 90 s, one set of samples was incubated at 50 C for 60 min to allow reannealing. The samples were then adjusted to 2× SSC and treated with 10 μg of RNase A per ml at 22 C for 15 min prior to electrophoresing for 15 h on 3% polyacrylamide gels. The gels were scanned at A_{260}, sliced, and counted. To prove that strand separation occurred during the original heat treatment, the other set of heat-treated samples was immediately diluted fivefold with ice water, centrifuged on linear-log sucrose density gradients, scanned. and fractions were collected and counted.

ssRNA tail structure. To determine if the ϕ6 dsRNA contained single-stranded regions, ^{32}P-dsRNA (approximately 10^5 counts per min per A_{260}) in 2× SSC was incubated with RNase A (10 μg/ml) and RNase T_1 (4,000 units/ml) at 22 C for 15 min before centrifuging on linear-log sucrose density gradient columns. The amount of radioactivity that sedimented in the top 20% of the gradient was determined after fractionation of the gradient columns. These values were compared with untreated ϕ6 dsRNA samples. In addition, the dsRNA regions in sucrose density gradient columns from RNase-treated samples were dialyzed, precipitated with ethanol, subjected to a second RNase treatment, and centrifuged.

RESULTS

Strand separation of the dsRNA. One unusual feature of ϕ6 dsRNA noted previously was the disproportionate quantities of the three single-stranded (ss) RNA species which appeared after heating the dsRNA to 100 C for 90 s in 0.01 M EDTA, pH 7.0, quickly cooling to −8 C, and electrophoresing on polyacrylamide gels (8; Fig. 1). Almost all of the large dsRNA segment existed as ssRNA, whereas only a small amount of the two smaller dsRNA segments electrophoresed as ssRNA species after this treatment. Variations in the length of the heat treatment and decreased concentrations of EDTA did not alter this pattern except that prolonged heating increased the amount of polydisperse low molecular weight RNA. Attempts to separate the dsRNAs into ssRNAs in 50% formamide or 85% dimethyl sulfoxide followed by electrophoresis on polyacrylamide gels also resulted in profiles similar to those shown in Fig. 1. Two possible explanations for this phenomenon are (i) the large dsRNA segment contained a lower G + C content than the two smaller segments and thus was the only segment that separated into single strands or (ii) all three segments were separated into single strands but the two smaller segments reannealed much faster than the large segment. The following experiments indicate that (ii) is probably the explanation for this phenomenon. (i) The sharp 40% increase in the hyperchromicity of the ϕ6 dsRNA described previously (8) suggested that all three segments had separated into single strands at the same temperature. (ii) If the dsRNA was exposed to 100 C for 90 s in 0.01 M EDTA, and immediately incubated with RNase A in 2× SSC, approximately 90% of the RNA was degraded (0 time point in Fig. 2).

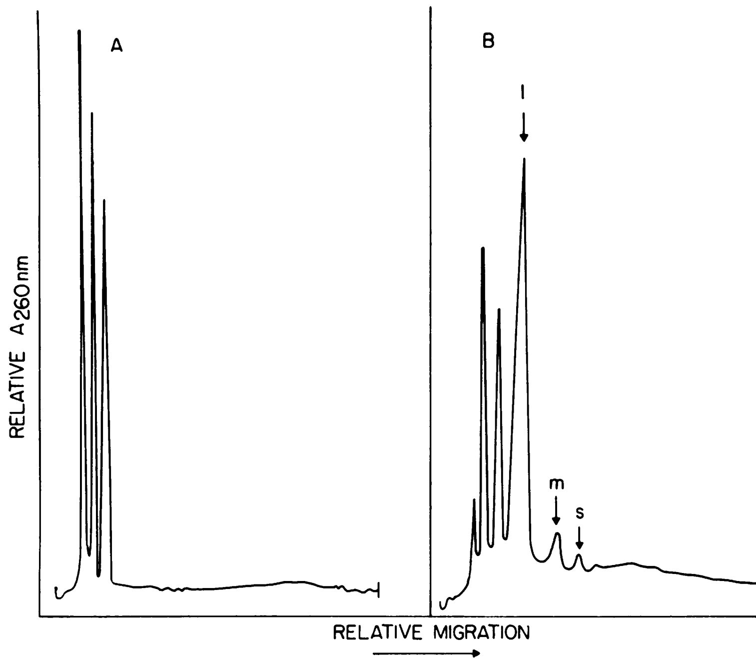

FIG. 1. *Electrophoresis of φ6 RNA on 2.4% polyacrylamide gels for 4 h at 5 mA/gel. (A) Untreated φ6 RNA showing the three dsRNA segments. (B) Phage φ6RNA which was heated for 90 s at 100 C in 0.01 M EDTA, pH 7.0, and quickly cooled to −8 C prior to electrophoresis. The arrows indicate the three ssRNAs arising from the three dsRNAs.*

Thus all three dsRNAs must have separated into single strands with the heat treatment. (iii) If the melted RNAs were incubated at 24 C, the small dsRNA segment reannealed rapidly, followed by the medium size dsRNA; essentially no reannealing occurred with the large dsRNA at 24 C (Fig. 2). In contrast, all three dsRNAs reannealed rapidly at 50 C (Fig. 2). Separate experiments established that the optimal temperature for reannealing in 0.01 M EDTA was between 50 and 60 C. (iv) If the dsRNA was heated to 100 C for 90 s in 0.01 M EDTA, cooled rapidly, and immediately diluted fivefold with ice water before centrifuging on sucrose density gradients, three fast-sedimenting species of ssRNA were observed which had S values of 24.7, 29.4, and 33.0 (Fig. 3). The A_{254} peaks for the three ssRNA species were in about the ratio expected if all three dsRNA segments had separated into single strands. (v) Determinations of the T_m values and the base compositions of the three isolated dsRNA segments were nearly identical (Table 2).

Exposure of the dsRNA to 100 C for 90 s in the presence of 0.01 M EDTA containing 3.7% formaldehyde, followed by overnight dialysis against 3.7% formaldehyde at 40 C, resulted in the appearance of three ssRNA species which sedimented at 17.8, 20.1, and 23.5S (Fig. 3C). By using these S values and the formula $S = 0.083\ M^{0.38}$ for converting sedimentation coefficients of formaldehyde-treated ssRNA to molecular weights (3), one obtains values of 1.36×10^6, 1.88×10^6, and 2.84×10^6 for the three ssRNA components. These values give molecular weights of 2.72×10^6, 3.76×10^6, and 5.68×10^6 for the three dsRNAs and a total molecular weight of 12.16×10^6 for the phage genome (Table 1). These values are approximately 15 to 30% larger than those calculated directly from the sedimentation coefficients of the dsRNA using the formula $M = 2.24S^{5.16}$ (ref. 5) or those derived previously from polyacrylamide gel electrophoresis and by electron microscopy (8; Table 1).

Characteristics of the isolated dsRNA segments. The three dsRNA segments of φ6, isolated after separation by polyacrylamide gel electrophoresis, were free of cross-contamination (Fig. 4). The T_m values and the base compositions were virtually identical for each of the three separated segments (Table 2).

Since the sum of the molecular weights of the two smaller dsRNA segments are approximately equal to the molecular weight of the large dsRNA segment, it is possible that the

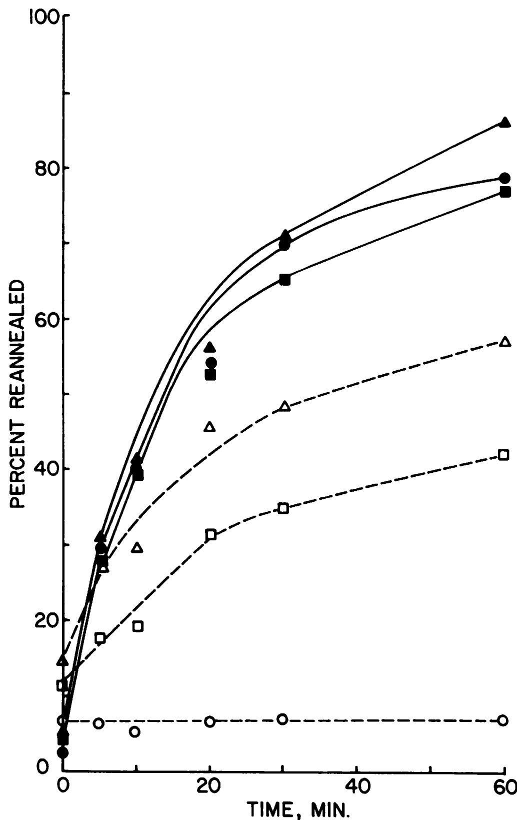

FIG. 2. *The rate of reannealing of the three ϕ6 dsRNA segments at 24 and 50 C. The protocol for the experiment is described in Materials and Methods. The dashed lines and open symbols were obtained at 24 C and the solid lines and solid symbols at 50 C. The symbols ●, ■, and ▲ represent the large, medium, and small dsRNA segments, respectively.*

smaller segments arise from a specific cleavage of the large dsRNA segment. If such a cleavage occurred we would have expected more than the six RNA peaks observed in Fig. 1 because large ssRNA would have reannealed with small and medium ssRNA; such results have never been observed. Additional evidence that the two smaller dsRNA segments were not derived from the large dsRNA segment is presented in Fig. 5. Isolated ϕ6 ^{32}P-dsRNA segments allowed to reanneal with a 10-fold excess of unlabeled, unfractionated ϕ6 dsRNA revealed that each segment reannealed only with strands of the same size (Fig. 5A, C, and E). Figures 5B, D, and F show that strand separation of both the radioactive and unlabeled ϕ6 dsRNA actually occurred during the original heat treatment; thus all three unlabeled strands had the opportunity to reanneal with each ^{32}P-RNA segment. These data eliminate the possibility that the two smaller dsRNA segments arise from a cleavage of the large dsRNA segment.

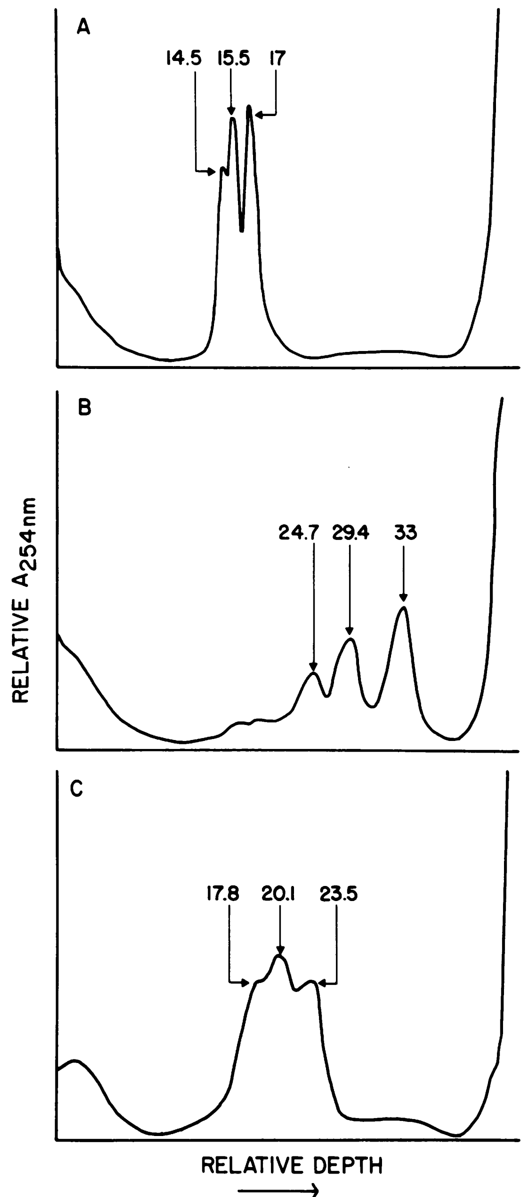

FIG. 3. *Rate zonal sedimentation of ϕ6 dsRNA in linear-log sucrose density gradients. (A) Untreated dsRNA; (B) dsRNA heated to 100 C for 90 s in 0.01 M EDTA and quickly diluted fivefold with ice water; (C) dsRNA heated as in (B) but in the presence of 3.7% (vol/vol) formaldehyde and then dialyzed for 16 h at 40 C in the presence of 3.7% formaldehyde. The sedimentation coefficients are indicated in the figures.*

TABLE 1. *A comparison of the molecular weights of phage φ6 dsRNAs as determined by four procedures*

Segment	Molecular weight × 10^6				
	Electron microscopy[a]	Polyacrylamide gel electrophoresis[a, b]	Formaldehyde-treated ssRNA[c]	Calculated from the *S* value of the dsRNA[d]	Avg
Small	2.0	2.24	2.72	2.20	2.29
Medium	2.87	2.82	3.76	3.11	3.14
Large	4.78	4.52	5.68	5.00	5.00
Total	9.65	9.58	12.16	10.31	10.4

[a] Value reported in reference 8.
[b] Determined from melted single-stranded φ6 RNA (8).
[c] Determined from sedimentation coefficients of single-stranded φ6 RNA obtained after formaldehyde treatment.
[d] Determined directly from sedimentation coefficients of 14.5, 15.5, and 17.0 of the dsRNA (8) by using the formula of $M = 2.24\ S^{5.16}$ (ref. 5).

TABLE 2. *The T_m's of the three phage φ6 dsRNA segments at several salt concentrations and the nucleoside composition of these segments*

Segment	T_m values (C) sodium salt concn			Base composition[a]					
	0.0017	0.0099	0.0463	U	G	A	C	A/U	G/C
Small	70.1	81.7	93.0	23.0	28.7	20.9	27.4	0.91	1.05
Medium	70.3	82.9	94.0	21.6	30.0	20.2	28.3	0.94	1.06
Large	70.2	82.2	94.0	22.9	29.2	20.4	27.6	0.89	1.06
Total	70.0	83.0	94.2	22.1	28.9	20.4	28.6	0.92	1.01

[a] Standard deviation was ± 0.36 calculated on the assumption that the variance for each base determination was identical.

Evidence to suggest a ssRNA region in the dsRNA. Previously, we reported that phage φ6 contained a RNA polymerase which mediated the incorporation of ribonucleoside triphosphates into dsRNA and we suggested that the enzyme might be completing one of the complementary strands of the dsRNA (9). If this suggestion was correct, φ6 dsRNA might contain a small ssRNA "tail" region. Phage φ6 ^{32}P-dsRNA was incubated with RNase A and RNase T_1 in 2× SSC prior to layering on linear log sucrose density gradients. As noted in Table 3, 1 to 2.5% of the RNA layered on the gradient was degraded so that it sedimented in the top 20% of the gradient. Less than 0.1% of the radioactivity in the nonenzyme-treated control was observed in this region of the gradient. If the dsRNA region from sucrose density gradients of RNase-treated samples was collected, reprecipitated with ethanol, RNase treated a second time, and centrifuged on sucrose gradient columns, less than 0.2% of the radioactivity layered on the gradient was degraded to slowly sedimenting material. No obvious differences in the sucrose gradient A_{254} profiles of any of the samples were observed. Therefore, these data are consistent with the concept that φ6 dsRNA contains a short ssRNA "tail" region.

DISCUSSION

The results provide additional information about the three segments of the phage φ6 dsRNA genome. Previously, we were unable to explain the disproportionate quantities of ssRNA that occurred after heating the dsRNA to 100 C prior to electrophoresing the RNA on polyacrylamide gels (8). From the experiments presented, it is clear that the two smaller RNA segments reanneal much more rapidly at low temperatures than the large RNA segment. This disparity in the rate of reannealing occurs even though all three RNA segments have similar base compositions. The rapid reannealing of the two smaller dsRNA segments also occurred after treatment with 85% dimethyl sulfoxide and 50% formamide since A_{260} profiles of polyacrylamide gels of dsRNA denatured with these agents were similar to those reported in Fig. 1. However, three ssRNA segments were observed if the samples were diluted fivefold and subjected to sucrose density gradient centrifugation.

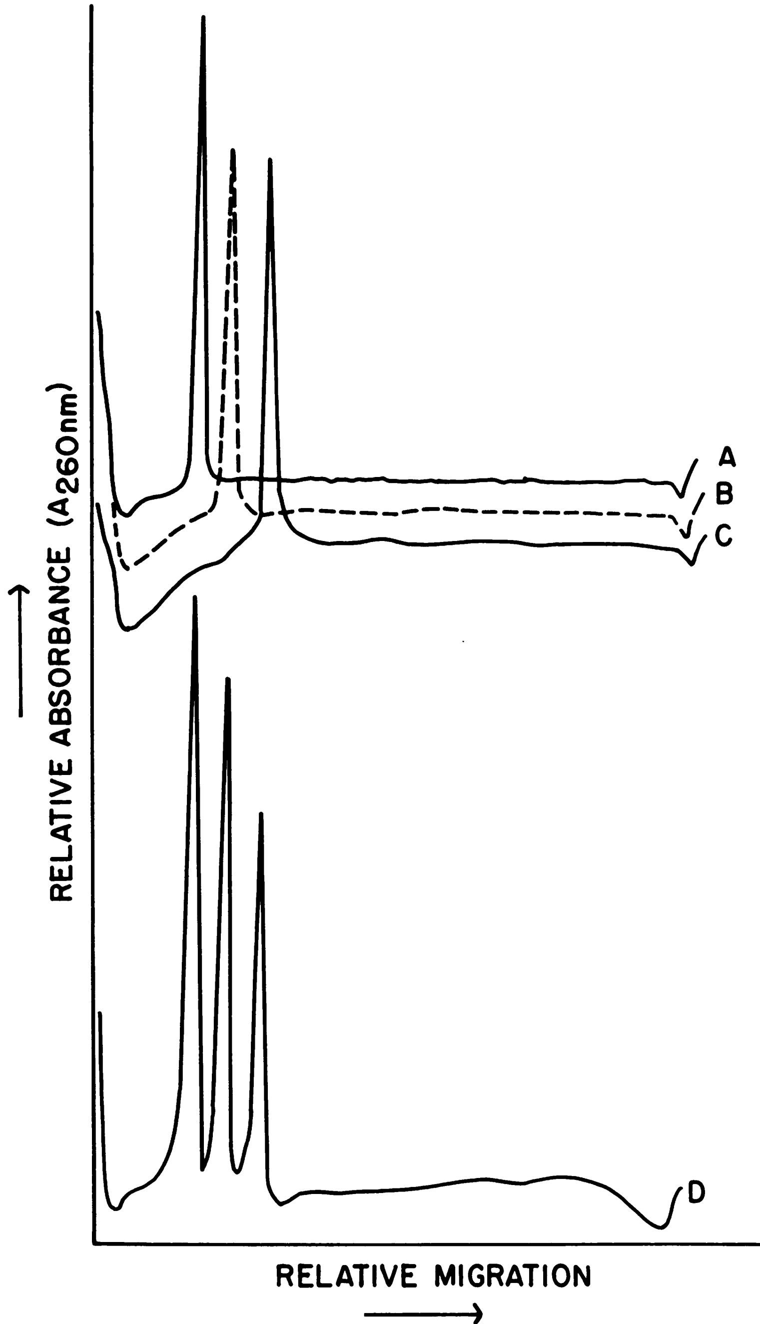

FIG. 4. *Electrophoresis of isolated φ6 dsRNA segments on 2.4% polyacrylamide gels for 6 h at 5 mA/gel (A, B, and C) and compared to an unfractionated sample of φ6 dsRNA electrophoresed at the same time (D). Note that the three isolated components (large, A; medium, B; and small, C) are essentially free of cross-contamination.*

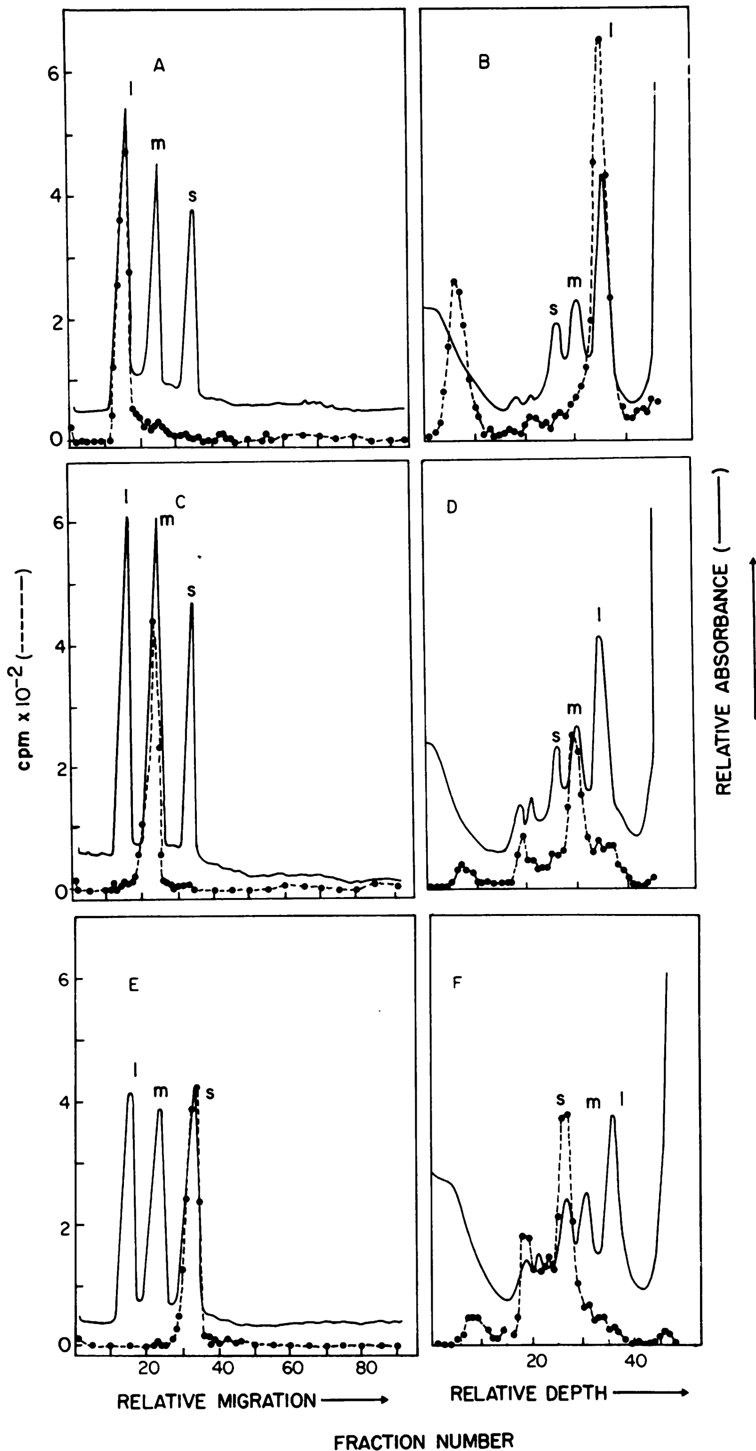

FIG. 5. *Ability of the isolated $\phi 6$ ^{32}P-dsRNA segments to reanneal with a 10-fold excess of unfractionated, unlabeled $\phi 6$ dsRNA (A, C, E). B, D, and F indicate that both the labeled and unlabeled dsRNAs had separated into single strands during the heat treatment. The protocol of the experiment is described in Materials and Methods. The solid line represents the A_{260} on gels (A, C, and E) and A_{254} on sucrose density gradient columns (B, D, and F); the dashed line represents the counts per minute. The experiments shown in A and B were conducted with the large ^{32}P-dsRNA segment; C and D were conducted with the medium ^{32}P-dsRNA segment; and E and F were conducted with the small ^{32}P-dsRNA segment.*

TABLE 3. *The sensitivity of $\phi6$ ^{32}P-dsRNA to RNase in high salt buffer*

Treatment[a]	Counts/min layered per gradient	Counts/min in top 2.5 ml of the gradient	Counts/min in top 2.5 ml of the gradient (%)
Expt 1[b]			
None	96,400	92	0.095
+RNases A and T_1	96,400	2,363	2.45
None	193,000	118	0.061
+RNases A and T_1	193,000	4,231	2.19
+RNases A and T_1[c]	74,740	133	0.18
Expt 2[b]			
None	19,131	0	0
+RNases A and T_1	17,974	202	1.12
+RNases A and T_1[c]	50,645	61	0.12

[a] ^{32}P-RNA was incubated with RNase's A (10 µg/ml) and T_1 (4,000 units/ml) for 15 min at 22 C in 2× SSC prior to layering on linear log sucrose density gradients. The gradients were centrifuged in an SW41 rotor for 13 h at 6 C at 37,000 rpm and fractionated into 24 fractions (0.5 ml per fraction). The counts released into the top five fractions (2.5 ml) were taken to indicate RNase released counts. $\phi6$ dsRNA sediments in fractions 9 through 16.

[b] Values determined from ^{32}P-dsRNA isolated from two different fermentations.

[c] $\phi6$-dsRNA which was collected from the RNase's A and T_1 treatment, precipitated with ethanol, and then subjected to the RNase treatment a second time as described in footnote *a*.

Since the base compositions of the three dsRNA segments are similar and the sum of the molecular weights of the two smaller dsRNA segments is near that of the large dsRNA, it was possible that the two smaller segments were derived from a specific cleavage of the large dsRNA segment. Alternatively, the large dsRNA segment might be formed by the joining of the two smaller dsRNA segments. However, the absence of hybridization between the various RNA segments eliminates this possibility.

Previously, we reported that phage $\phi6$ contained an RNA polymerase which mediated the incorporation of ribonucleoside triphosphates into all three dsRNA components, and we suggested that the enzyme was completing one of the complementary strands of the dsRNA (9). The observation that 1 to 2.5% of $\phi6$ dsRNA is RNase sensitive in high salt (2× SSC) supports this concept. However, the experiments do not provide conclusive evidence that $\phi6$ dsRNA actually contains a short ssRNA "tail" region. For example, it is possible that a "contaminating" ssRNA sediments in a sucrose density gradient in the same region as $\phi6$ dsRNA and that this "contaminant" RNA is the source of the RNase-sensitive material. Another possibility is that a small percentage of the $\phi6$ virions contain incomplete dsRNA molecules and that the majority of the phage genomes are completely double stranded. Experimental attempts to resolve these three possibilities have been inconclusive.

The average sum of the molecular weights of the three dsRNA segments as determined by four different procedures is 10.4×10^6 (Table 1). Previously, we reported that the phage was composed of about 13% RNA (10). Because of the segmented properties of the virus genome it is possible that the virion might contain more than one copy of identical dsRNA segments. However, a ^{60}Co γ-ray inactivation experiment indicated that this does not occur (J. Sands, personal communication). If the phage contains one species of each of the dsRNA segments per virion, this would indicate that the molecular weight of the $\phi6$ particle is about 80×10^6. This value is consistent with the 60- to 70-nm size of the phage observed with the electron microscope (10). Although it is possible that each of the three dsRNA segments consists of distinct populations of dsRNAs, it would seem to be unlikely since repeated attempts to separate the dsRNA into additional dsRNA segments by gel electrophoresis on 3 to 7% polyacrylamide have been unsuccessful.

In conclusion, the phage genome is composed of three unique species of dsRNA whose base compositions are similar.

ACKNOWLEDGMENTS

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