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In Vitro Translation of the Three Bacteriophage ϕ6 RNAs†

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In vitro translation of the three single-stranded RNAs transcribed in vitro by bacteriophage ϕ6 RNA polymerase revealed that the large RNA codes for phage proteins P1, P2, P4, and P7, the medium RNA codes for P3, P6, and P10, and the small RNA codes for P5, P8, and P9.

ϕ6, a lipid-containing bacteriophage of *Pseudomonas phaseolicola* HB10Y (16), contains 9 to 11 different polypeptides (11, 14) and a double-stranded RNA (dsRNA) genome composed of three unique segments of 2.2×10^6 (small), 3.2×10^6 (medium), and 5.0×10^6 (large) daltons (9, 15). Treatment of the phage with nonionic detergents removes the lipid envelope, leaving a stable nucleocapsid (11, 14) which has RNA polymerase activity (8, 12). The enzyme synthesizes large amounts of small and medium single-stranded RNA (ssRNA) and a small amount of large ssRNA.

The objective of this study was to determine which ϕ6 proteins were formed when each individual ϕ6 ssRNA component was translated in an *Escherichia coli* cell-free protein-synthesizing system.

ϕ6 ssRNA's were synthesized in the standard RNA polymerase reaction and separated by sucrose density gradient centrifugation as described previously (12). The gradients were scanned photometrically at a wavelength of 254 nm and the small, medium, and large ssRNA's were individually collected. The RNAs were precipitated with 2.5 volumes of cold ethanol and then dissolved in 0.5 × SSC buffer (0.075 M NaCl–0.0075 M sodium citrate, pH 7.2). After dialysis against 0.5 × SSC buffer for 6 h at 4°C, the RNAs again were precipitated with ethanol and finally dissolved in water.

A 30,000 × *g* supernatant extract (S30) was prepared from *E. coli* A19 by a method similar to that of Davies and Kaesberg (3). The conditions for in vitro translation of ϕ6 RNA were modified from those described for *Pseudomonas aeruginosa* phage PP7 RNA (2). A 100-μl reaction mixture contained 45 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH adjusted to 7.5 with 1 M KOH), 4 mM Tris, 80 mM NH_4Cl, 1.5 mM dithiothreitol, 2 mM ATP and 0.2 mM GTP (pH adjusted to 7.0), 2.5 mM phosphoenolpyruvate, 7 to 9 mM magnesium acetate (depending on the S30 preparation), 50 μg of leucovorin, 30 μM each of 19 amino acids, 4 μCi of the missing amino acid ([^{3}H]leucine), 2 absorbance units at 260 nm of S30, and 10 μg of ϕ6 ssRNA. The mixture was incubated at 35°C for up to 2 h.

The in vitro translation products were concentrated by precipitation with 10 volumes of 80% acetone at −20°C for 30 min. The precipitates were collected by centrifugation, suspended in buffer (10% [wt/vol] Ficoll, 0.04% [wt/vol] crystal violet, 2% [wt/vol] sodium dodecyl sulfate, 0.06 M Tris [pH 8.8], 0.002 M EDTA, 0.01 M dithiothreitol), and heated at 100°C for 90 s. Equal amounts of radioactivity from different reaction mixtures were placed on a linear 8 to 20% polyacrylamide slab gel (13) and electrophoresed in the buffer system of Laemmli (4) at 24°C at constant voltage for ca. 1,000 V-h. The labeled proteins were visualized by fluorography (1, 5).

For the preparation of ϕ6 proteins labeled with [^{3}H]leucine in vivo, *P. phaseolicola* HB10Y was grown at 25°C in M8 medium (11) to an absorbance of 0.40 at 640 nm. The culture was incubated in the presence of [^{3}H]leucine (1 μCi/ml) for 10 min, then chilled, and infected with ϕ6 (5 PFU/cell). After a 15-min adsorption period, the culture was returned to 25°C, and an additional 1 μCi of [^{3}H]leucine per ml was added 15 and 45 min after infection. After lysis, ϕ6 was purified by sucrose density gradient centrifugation (16). Labeled ϕ6 proteins were prepared for electrophoresis by precipitating the virus with acetone, collecting the precipitate by centrifugation, and resuspending the samples in the same buffer used for the in vitro translation products.

The ssRNA's synthesized by ϕ6 RNA polymerase effectively served as templates for the incorporation of [^{3}H]leucine into hot trichloroacetic acid-insoluble products. Total ssRNA, as well as the individual components (large, medium and small) stimulated incorporation 7- to 16-fold

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over that effected by the *E. coli* S30 extract alone.

By using ϕ6 ssRNA as a template, nine ϕ6 proteins (P1 and P3 through P10) were readily detected within 60 min of the start of the translation reaction (Fig. 1B). Protein P2 was observed only if the reaction proceeded an additional 60 min (data not shown). Although there was some cross-contamination among the three ϕ6 ssRNA segments, slots C, D, and E in Fig. 1 indicate which proteins are coded for by each segment. Thus, the large ssRNA segment codes for P1, P2, P4, and P7, the medium ssRNA segment codes for P3, P6, and P10, and the small ssRNA codes for P5, P8, and P9. In addition, the small ssRNA appears to code for a protein that migrates slightly slower than P9.

The large ssRNA appears to be translated more efficiently than the other two ssRNA's.

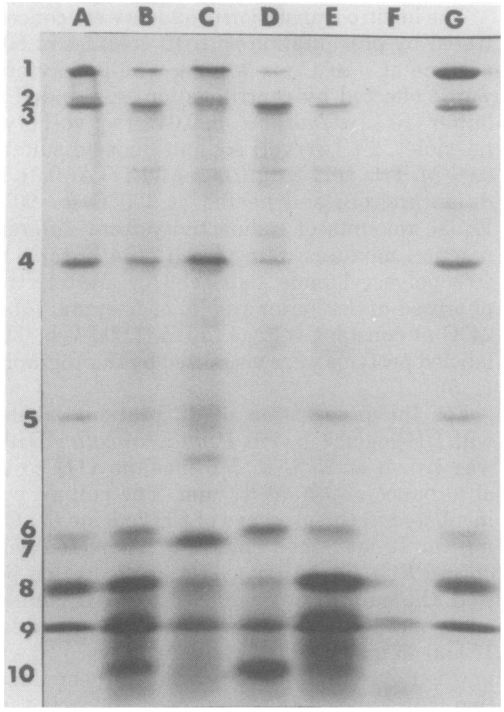

FIG. 1. *Polyacrylamide gel electrophoresis of ϕ6 proteins synthesized in an E. coli cell-free protein synthesizing system. The proteins were translated from ϕ6 total ssRNA (B), ϕ6 large ssRNA (C), ϕ6 medium ssRNA (D), ϕ6 small ssRNA (E), or no ϕ6 RNA (F). A and G contained ϕ6 proteins labeled in vivo. Each slot (with the exception of F, in which an equivalent volume was taken) contained 75,000 cpm (from [^{3}H]leucine). X-ray film was exposed to the gel for 4 days. The positions of ϕ6 proteins P1 to P10 are indicated on the left. P10 appears as a faint band in A and G if the gel is exposed to X-ray film for a longer period of time (10 days).*

Large ssRNA is transcribed in small quantities in the RNA polymerase reaction and comprises less than 5% of the total ssRNA synthesized (12). Nonetheless, proteins P1, P4, and P7 (Fig. 1, slot B) were produced in amounts similar to those of the other proteins when unfractionated ssRNA's were used as mRNA. Incorporation with a mixture of 15 ^{3}H-amino acids, instead of [^{3}H]leucine, gave similar results.

Our results support the genetic data of Mindich and his colleagues (6, 7), who used temperature-sensitive and nonsense mutants to show that P1, P2, and P7 map together (linkage set A), P3, P6, and P10 map together (linkage set B), and P5, P8, and P9 map together (linkage set C). We provide direct evidence that the genes for set A are located on the large RNA, those for set B are on the medium RNA, and those for set C are on the small RNA. Furthermore, our results predict that P4 will map in set A.

Mindich and his colleagues (7, 10, 11) have reported that ϕ6 RNA codes for two additional proteins: P11, a possible precursor of P5, with a molecular mass of 25,000 and P12 with a molecular mass of 20,000. We were unable to detect these proteins with the in vitro translation system, despite varying the experimental conditions employed in Fig. 1. Neither labeling the translation products with 15 ^{3}H-labeled amino acids (instead of ^{3}H-labeled leucine) nor electrophoresing them on a 15% or linear 15 to 25% polyacrylamide gel as used by Mindich and his colleagues (6, 11) resulted in the appearance of either protein. Prolonged exposure (3 weeks) of the gels to X-ray film also failed to reveal them. Synthesis of P11 and P12 may require some component of the *Pseudomonas* host cell. This possibility is supported by the finding of Davies and Benike (2) that, under appropriate conditions, *Pseudomonas* ribosomes synthesize three different proteins using pseudomonad phage PP7 RNA as a template, whereas *E. coli* ribosomes synthesize only one protein.

In summary, we have demonstrated that ϕ6 large RNA codes for P1, P2, P4, and P7; ϕ6 medium RNA codes for P3, P6, and P10; and ϕ6 small RNA codes for P5, P8, and P9. These results confirm genetic studies with ϕ6 and also show that the ϕ6 RNA polymerase produces plus strands (mRNA's).

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