Complementary Strands of Bacteriophage ϕ 29 Deoxyribonucleic Acid: Preparative Separation and Transcription Studies

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Bacillus subtilis phage ϕ 29 has a nonpermuted, duplex deoxyribonucleic acid (DNA) with cohesive ends and a molecular weight of 11×10^6 . Denaturation of this DNA yielded two intact polynucleotide chains. Preferential binding of the polyribonucleotide polyuridylic-guanylic acid (poly UG) to the complementary strands of denatured ϕ 29 DNA permitted separation of the strands in neutral CsCl gradients. In analytical CsCl density gradient centrifugation, the separated strands with poly UG appeared as two symmetrical bands, both heavier than the normal denatured DNA band. The strands differed in density by 11 mg/cc. Preparative separation of the ϕ 29 DNA strands resulted in two fractions, heavy (H) and light (L). The H fraction was essentially free from L contamination, whereas L contained up to 25% of H, as determined both by rebanding the separated fractions in CsCl and by electron microscopic examination of self- and mixed-annealed fractions. Pulse-labeled ribonucleic acid (RNA) prepared at intervals after infection was hybridized with the self-annealed DNA strands. Preliminary experiments indicated that both strands of ϕ 29 DNA are transcribed during the development of the virus. Early transcribed ϕ 29-specific RNA hybridizes only with the L strand; at later times, transcription occurs from both the L and H strands.

Phage ϕ 29 is a small, virulent *Bacillus subtilis* phage with an elaborate morphology (1). The deoxyribonucleic acid (DNA) of ϕ 29, characterized by electron microscopy and sedimentation (2), has a length of 5.8 μ m and a molecular weight of 11×10^6 daltons. Thus, ϕ 29 DNA is about one-third the size of lambda DNA and less than half the size of T7 DNA (22). In addition to being extremely small, ϕ 29 DNA is nonpermuted, has cohesive ends, yields two intact polynucleotide chains upon denaturation (2), and has a high infectivity (15). These properties of ϕ 29 DNA have encouraged us to begin analysis of the replication (18) and transcription of the ϕ 29 genome.

Phage ϕ 29 DNA does not exhibit a natural pronounced bias in base distribution between its two complementary strands, as indicated by the unimodal profile of the denatured DNA in both neutral and alkaline CsCl gradient centrifugation (2). This is in contrast to a number of *B. subtilis* phages, for example SP8 (12), SP82

(5), ϕ 14 and ϕ 25 (Reilly, Ph.D. Thesis, Western Reserve Univ., Cleveland, Ohio, 1965), SP50 (3), and SPP1 (17), as well as several *E. coli* phages (4, 8, 11, 21).

In the present study, using techniques evolved by Szybalski and his co-workers (6, 14), we have isolated the complementary strands of ϕ 29 DNA by preferential binding of polyuridylic-guanylic acid (poly UG) to both strands, thereby providing a differential buoyant density shift in the CsCl gradient. The purity and intactness of the isolated strands were studied by self- and mixed-annealing, banding in CsCl, electron microscopy, and transfection. Analysis of the *in vivo* transcription of the ϕ 29 strands demonstrated that both strands are transcribed.

MATERIALS AND METHODS

Bacteria and bacteriophage. *B. amyloliquefaciens* strain H was used for the growth of phage ϕ 29 in liquid culture, and *B. subtilis* strain 12A was the normal plating host. For transfection studies, *B.*

subtilis 168M was employed and a streptomycin-resistant mutant of *B. amyloliquefaciens* strain H was used as the indicator bacterium. Phage $\phi 29$ was originally isolated and described by B. E. Reilly (Ph.D. Thesis, Western Reserve Univ., Cleveland, Ohio, 1965).

Growth and purification of phage $\phi 29$. Phage $\phi 29$ was grown and purified in CsCl as described previously (1).

DNA isolation. DNA was released quantitatively from freshly purified $\phi 29$ in 2 M sodium perchlorate as described previously (1).

Analytical density gradient centrifugation. The buoyant density of denatured $\phi 29$ DNA under different experimental conditions was determined by use of the procedures described by Tewfik (E. M. Tewfik, Ph.D. Thesis, Univ. of Minnesota, Minneapolis, 1967) and Szybalski (25). A saturated stock solution of optically pure CsCl in 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.5) was used to bring the density of the DNA solutions to 1.73 to 1.74 g/cc. Approximately 0.70 ml of the DNA-CsCl solution was injected into a 4° sector 12-mm cell with a Kel-F centerpiece and was centrifuged in an analytical rotor (An-D) with one or two cell assemblies. A Spinco (model E) analytical ultracentrifuge was operated at 44,770 rev/min at 10 C for 20 to 22 hr. The resulting ultraviolet (UV)-absorption photographs were traced with a Beckman model RB-Analytrol film microdensitometer. The buoyant density of the denatured DNA was then calculated by using the position of native $\phi 29$ DNA as a reference (20). The buoyant density of native $\phi 29$ DNA was taken to be 1.696 g/cc.

Electron microscopy. DNA molecules were prepared for electron microscopy by a procedure modified from the protein film technique of Kleinschmidt et al. (10). One volume of DNA at 2 μ g/ml in 1 M ammonium acetate was mixed with one-tenth volume of 0.1% (w/v) cytochrome *c*; 0.002 ml of the mixture was spread onto a 5-cm² surface of 0.15 M ammonium acetate. The resulting film was picked up with carbon-coated mica and dehydrated in ethyl alcohol for 30 sec. The procedure of Westmoreland and Szybalski (28) was used in order to demonstrate single-stranded $\phi 29$ DNA in an extended form. Electron micrographs of DNA molecules were taken at a magnification of 10,000 times. Procedures for measuring molecular lengths were described previously (2). All micrographs were taken with a Siemens Elmiskop 1A electron microscope.

Determinations of biological activity. Transfection experiments were carried out with $\phi 29$ DNA as described previously (15).

Preparation and isolation of labeled ribonucleic acid (RNA). Pulse-labeled RNA was made by adding ³H-uridine (0.1 mCi, 22.6 Ci/mmol) to 20 ml of culture for 3 min. The pulse was terminated by rapidly pouring the cells onto frozen TMK buffer (0.01 M Tris-hydrochloride, 0.01 M magnesium acetate, 0.06 M KCl, pH 7.3) followed by centrifugation at 4 C. The cells were washed with iced buffer and suspended in 1 ml of buffer containing lysozyme (200 μ g/ml) and deoxyribonuclease (50 μ g/ml).

Three cycles of freeze-thawing (7) was followed by the addition of sodium lauryl sulfate to 0.1%. After 15 min at room temperature, the lysate was centrifuged at 10,000 $\times g$ for 10 min. Phenol extraction and extensive dialysis of the supernatant were performed as described by Kennell (9). The specific activity of the RNA prepared by this procedure was normally 5×10^3 to 6×10^3 counts per min per μ g of RNA, and greater than 99% of the counts could be solubilized with 0.3 N KOH at 37 C.

DNA-RNA hybridization. Hybridization of RNA with DNA followed the method of Nygaard and Hall (13). Tubes containing 4 to 5 μ g of the self-annealed DNA strands A or B, pulse-labeled RNA (10^5 to 3×10^5 counts/min) and phenol-saturated $2 \times$ standard saline citrate (SSC: 0.15 M NaCl, 0.015 M sodium citrate; final volume, 0.5 ml) were placed at 67 C for 20 hr and then rapidly cooled on ice. The samples were diluted to 10 ml with $2 \times$ SSC and slowly filtered through nitrocellulose filters (25 mm; Schleicher & Schuell B6). The filters were treated with ribonuclease (20 μ g/ml, 1.5 hr at 24 C) which had been preheated in $2 \times$ SSC at 98 C for 10 min and then extensively washed with $2 \times$ SSC (11). The filters were dried at room temperature and radioactivity was determined as described previously (19).

Chemicals and radioisotopes. Polyuridylic acid (poly U), polyguanylic acid (poly G), polyinosinic-guanylic acid (poly IG, 1:1) were obtained from Miles Laboratories, Inc., Kankakee, Ill.; and poly UG (1:1) was obtained from either Miles Labs or Biopolymers, Inc., Chagrin Falls, Ohio. The polymers were dissolved in doubly distilled water and stored at -20 C at a concentration of 1 mg/ml.

Purified CsCl was obtained from Matheson, Coleman & Bell, Norwood, Ohio. Ribonuclease-A (five times crystallized) was obtained from Sigma Chemical Co., St. Louis, Mo. Egg white lysozyme (three times crystallized) and deoxyribonuclease 1 were obtained from Calbiochem, Los Angeles, Calif.

³H-uridine (22.6 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Patterns of interaction of denatured $\phi 29$ DNA with synthetic polyribonucleotides. Perchlorate-extracted $\phi 29$ DNA, at a concentration of 20 μ g/ml in SSC, was heated at 100 C for 5 min in the presence or absence of an equal amount of one of the synthetic polyribonucleotides poly U, poly G, poly UG, or poly IG. It was then rapidly chilled in an ice-water bath, and the density was adjusted to 1.73 to 1.74 g/cc with a saturated CsCl stock solution. After the addition of 1 μ g of native $\phi 29$ DNA as a density marker, the samples were analyzed by centrifugation in a model E ultracentrifuge.

Microdensitometer tracings of the resulting UV-absorption photographs are shown in Fig. 1. Under these experimental conditions, denatured $\phi 29$ DNA bound poly U and poly IG only

FIG. 1. Microdensitometric tracings of UV-absorption photographs of $\phi 29$ denatured DNA (dN) alone (top tracing), and in the presence of the synthetic polyribonucleotides poly U, poly G, poly UG, and poly IG, after CsCl analytical ultracentrifugation according to the procedure described in the text. Native $\phi 29$ DNA (NN) was added as a density marker.

slightly. Poly G was bound to a greater extent, but the binding to both strands of DNA seemed to be equal. Poly UG, however, showed preferential binding to one of the complementary strands, as indicated by the bimodal banding profile of the denatured $\phi 29$ DNA-poly UG complex. Thus, it appeared that poly UG could be used to separate the complementary strands of $\phi 29$ DNA. Attempts were made to increase the separation of the strands by varying the poly UG to DNA ratio. Figure 2 presents the results of this study. It is apparent that a poly UG to DNA ratio of 1.0 gave optimal separation; increasing

FIG. 2. Effect of varying the poly UG to DNA ratio on the density increase of $\phi 29$ DNA heavy (H) and light (L) strands.

the amount of synthetic polymer caused no additional density difference between the two strands.

To carry out additional studies with the complementary strands of $\phi 29$ DNA, it was necessary to prepare large quantities of each strand in a relatively pure form. However, the degree of separation was not sufficient under our initial experimental conditions to allow preparative isolation of the strands. In addition, the commercial source of poly UG was found to have a pronounced effect on the degree of separation of the strands. Only two preparations [Biopolymers lot M-1 and alkali-treated (23) Miles Lot 1] proved adequate, and these were used in all of the studies presented in this paper. The following section describes the modifications in procedure and experimental conditions required for maximal separation of the $\phi 29$ DNA strands.

Preparative separation of the complementary strands. Prior to denaturation, freshly released DNA was dialyzed overnight against 0.001 M ethylenediaminetetraacetic acid (EDTA), pH 8.5 to 9.0, at 4 C. Freshly dialyzed $\phi 29$ DNA (600 μ g) was mixed with an equal quantity of poly UG, in a final volume of 6.0 ml of 0.001 M EDTA, heated at 100 C for 2 min, chilled, and brought to a density of 1.73 to 1.74 g/cc with a saturated CsCl stock solution.

A fraction of the mixture was diluted fourfold with a CsCl solution of the same density, and 1 μ g of native $\phi 29$ DNA was added as a density marker. This mixture was centrifuged in an analytical centrifuge as described previously. The results of this run are shown in Fig. 3. The $\phi 29$ DNA forms two nearly symmetrical bands. The strand with the higher affinity for the poly UG will be designated H (heavy), since it bands lower in the CsCl gradient than the L (light) strand,

FIG. 3. Microdensitometric tracing of a UV-absorption photograph of an analytical density gradient centrifugation run, showing the poly UG-effected separation of the complementary strands of ϕ 29 DNA under the optimal conditions described in the text. Native DNA (NN) was added as a density marker. The position of a peak representing the denatured (dN) ϕ 29 DNA is indicated by the dashed line.

which has less affinity for the poly UG. The calculated buoyant densities of these bands, with the native DNA band used as a reference, were 1.745 g/cc for the L band and 1.756 g/cc for the H band; both were more dense than denatured ϕ 29 DNA in the absence of poly UG (1.713 g/cc).

The remainder of the undiluted reaction mixture (approximately 30 ml) was equally divided into six polyallomer tubes ($\frac{5}{8}$ by 3 inches; 1.6 by 7.6 cm); the tubes were filled and balanced with mineral oil and were centrifuged in fixed-angle rotor 65 of a Spinco model L2 centrifuge at 5 C and 43,000 rev/min for 22 hr. At the end of the run, the polyallomer tubes were pierced through the bottom, and their contents were passed at a constant flow rate through the micro flow-through cell of a Zeiss spectrophotometer; the optical density at 260 nm was continuously recorded, and 4-drop fractions were collected. The results of a typical preparative run are illustrated in Fig. 4. The main L and H peak fractions shown in Fig. 4 as shaded areas (three or four fractions of 4 drops each per peak) were pooled. The identical fractions from the six centrifuge tubes were combined and will be referred to in the following discussion as the L and H fractions. All fractions were stored at 4 C. It was noted that the problem of renaturation encountered with

ϕ 29 DNA because of its small size was greatly diminished through the use of a solvent of low ionic strength (0.001 M EDTA), and by use of low temperatures during centrifugation (5 to 10 C).

Annealing of H and L fractions. If fractions H and L actually represent the separated ϕ 29 DNA strands, it should be possible, under the appropriate conditions, to anneal the strands and form native duplex DNA. Equal amounts of the H and L fractions were gently mixed, preheated at 100 C for 2 min, and annealed at 65 C for 2 hr at a concentration of 20 μ g of DNA/ml in 6 M CsCl. On analytical centrifugation of this sample, all of the DNA appeared as a single band at a position corresponding very closely to the density of native ϕ 29 DNA (1.696 g/cc). In fact, when native ϕ 29 DNA was added after annealing of the two strands and the sample was analyzed as above, only one distinct band could be detected. Thus, the H and L fractions represent the complementary strands of ϕ 29 DNA.

Analysis of the purity and integrity of the separated strands. Before additional studies with the ϕ 29 DNA strands could be carried out, it was

FIG. 4. Continuous absorbance (260 nm) recording of the poly UG-effected preparative separation of the ϕ 29 DNA complementary strands under conditions described in the text. The two shaded areas indicate the individual fractions which were combined as the two major fractions H and L.

FIG. 5. Two superimposed microdensitometric tracings of UV-absorption photographs, each representing one of the two separated complementary strands H and L of $\phi 29$ DNA rebanded in CsCl density gradient centrifugation. The native $\phi 29$ DNA peak was used as a common reference.

necessary to evaluate the relative purity of each of the preparations. When a sample of fraction H was banded in an analytical CsCl gradient, a single symmetrical band was formed with a buoyant density of 1.756 g/cc. In contrast, when a sample of fraction L was similarly banded, two bands were formed, a major band with a buoyant density of 1.745 g/cc and a minor band which had a density similar to that of the H fraction (Fig. 5). The minor band is illustrated as a shoulder on the L peak. Depending on the fractions pooled, the amount of DNA in the minor band can be as high as 25% of the total DNA banded. This finding indicated that, whereas the H fraction was essentially free from L strands, the L fraction was contaminated with H strands. The absence of additional peaks in the above analysis implied that no significant fragmentation of the separated strands occurred during our experiment.

Another test of the purity of each strand was the observation of self- and mixed-annealed H and L strands by electron microscopy. Samples of the L and H fractions were dialyzed overnight against $0.01 \times$ SSC to remove the CsCl which interfered with electron microscopy, and to initiate dissociation of the poly UG-DNA complexes. Partial dissociation was evidenced by a lowering of the densities of both strands to values closer to the density of denatured DNA in the absence of poly UG. The strands were then self- and mixed-annealed in $2 \times$ SSC at a concentration of $50 \mu\text{g}$ of DNA/ml. Heating at 100°C for 2 min was carried out prior to heating at 65°C for 2 hr. Figures 6 and 7 demonstrate the types

of molecules observed in each of the above preparations. Table 1 shows the relative distribution of the types of molecules observed in the self-annealed H and L fractions. Fraction H is essentially pure because mainly single-stranded molecules were observed. Such molecules can be seen in Fig. 6a as puddles of uniform size which are typical of collapsed single-stranded DNA prepared in the absence of formaldehyde or formamide. As expected, fraction L presented a more complicated set of data (Table 1). Although the bulk of the molecules were single-stranded, there were a considerable number of completely duplex DNA molecules and some partially annealed molecules. The latter are illustrated in Fig. 6b as a molecule consisting of a partial duplex terminating in a puddle, and in Fig. 6c as a molecule with alternating double- and single-stranded sections.

The mixed-annealed H plus L preparation (Fig. 7) contained mainly intact duplex molecules (Fig. 7a) of the same length as native $\phi 29$ DNA (Fig. 8). In addition, a few clusters of several molecules with partial duplex formations were observed (Fig. 7b).

The biological activity of the dialyzed self- and mixed-annealed preparations described above was assayed by use of transfection. Preliminary results of such analyses are presented in Table 2. The number of infective centers per microgram of DNA in the mixed-annealed preparation was approximately 500-fold lower than that of the untreated native DNA. This indicates that only a small percentage of the double-stranded DNA molecules could be assayed under these experimental conditions. The loss of biological activity could be attributed in part to (i) slight imperfections in the annealed duplexes that could not be demonstrated by other physical-chemical methods, or (ii) the presence of trace amounts of CsCl in the preparations despite the extensive dialysis performed on them. Our previous experience has demonstrated that CsCl has an inhibitory effect on the biological activity of native $\phi 29$ DNA.

Definite conclusions about the biological activity of the individual strands cannot be drawn from these preliminary results. However, it is of interest that the ratio of biological activity of the mixed-annealed preparation to that of the self-annealed L preparation is about 3:1 by the transfection assay. This ratio is approximately equal to the ratio of double-stranded DNA molecules in these two preparations.

Patterns of transcription of the isolated strands. The observation that the poly UG binding sites on the $\phi 29$ DNA strands were asymmetrically distributed led us to investigate transcription of

FIG. 6. Electron micrographs of self-annealed complementary strands of $\phi 29$ DNA. (a) Self-annealed H fraction, $\times 53,000$. (b and c) Self-annealed L fraction, $\times 35,000$.

FIG. 7. Electron micrographs of mixed-annealed *H* plus *L* strands of $\phi 29$ DNA (a and b). $\times 66,000$.

the $\phi 29$ genome. If such binding sites are involved in transcription of DNA, one might predict that both strands of $\phi 29$ DNA would code for phage functions. Pulse-labeled RNA isolated from $\phi 29$

infected cells at various intervals after infection was hybridized to each of the self-annealed $\phi 29$ strands. Table 3 presents data indicating that phage $\phi 29$ -induced messenger RNA (mRNA)

FIG. 8. *Electron micrograph of native ϕ 29 DNA. $\times 66,000$.*

hybridizes to both the L and H DNA fractions. Transcription from the L strand occurs very early in infection, rises to a peak between 24 and 27 min postinfection, and then drops to a low level. Transcription from the H strand begins later in the lytic cycle and peaks at about 33 to 36 min postinfection. It is clear from these preliminary studies that transcription from the ϕ 29 genome is bilateral, and a large quantity of mRNA appears to be made off each strand.

DISCUSSION

Szybalski, Kubinski, and Sheldrick (26) proposed that pyrimidine-rich nucleotide clusters in various viral DNA molecules might act as initiation and termination signals in the process of transcription. It has been demonstrated that whenever such clusters are found in both viral DNA strands, as in coliphage λ (27), ϕ 80 (11), T4 (6), and T5 (Lanni and Szybalski, Bacteriol.

TABLE 1. *Electron microscopic analysis of the self-annealed DNA strands*

Annealed sample	Relative percentage		
	Single-stranded molecules	Partially annealed molecules	Duplex molecules
H fraction.....	90	5	5
L fraction.....	56	16	28

TABLE 2. *Biological activity of the self- and mixed-annealed DNA strands*

Annealed sample	Transfection (infection centers/ μ g of DNA) ^a
H fraction.....	1.0×10^2
L fraction.....	6.4×10^2
H fraction plus L fraction..	20.0×10^2

^a Native ϕ 29 DNA produces about 10^6 infective centers/ μ g of DNA by transfection.

TABLE 3. *Hybridization pattern of 3 H-labeled RNA synthesized after infection with phage ϕ 29*

Time of pulse (min after infection)	Percentage of 3 H-labeled RNA annealing with			
	Expt 1		Expt 2	
	Strand H	Strand L	Strand H	Strand L
3-6	0.00	0.06	0.00	0.00
6-9	0.04	0.20	0.00	0.28
15-18	0.13	0.25	0.00	0.28
24-27	0.55	3.41	0.86	2.58
33-36	3.21	1.86	6.82	1.16
42-45	1.89	0.05	4.02	0.75

Proc., p. 192, 1969), transcription occurs from both strands. When pyrimidine clusters are found on only one DNA strand, as in phage T7, transcription occurs exclusively on that strand (24). Phage ϕ 29 follows the pattern of these results because both of its DNA strands contain pyrimidine-rich (poly UG-binding) clusters and mRNA is made from both strands. Thus, our data support the proposal that pyrimidine-rich clusters are somehow involved in the transcription of DNA molecules.

Our findings on poly UG binding and transcription of ϕ 29 DNA are of additional interest when one considers the small size (11×10^6 daltons) of the ϕ 29 DNA molecule. Phage T7 DNA has a molecular weight of 26×10^6 (22), and the *B. subtilis* phage SPPI contains DNA with a molecular weight of 25×10^6 (17). Both of these phages contain pyrimidine clusters in only one strand, and both make mRNA exclusively from this

strand (16, 24). Thus, ϕ 29 DNA is not like the DNA of other small virulent phages, and it presents a unique opportunity to study selective transcription and translational control on a very small DNA molecule where one or more strand switches must occur during phage development. We have begun studies on ϕ 29 transcription employing temperature-sensitive mutants which have been mapped into 13 distinct complementation groups (Hagen, Zece, and Anderson, *Virology*, *in press*), and we are hopeful that additional work will allow us to gain insight into the developmental cycle of this phage.

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