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New Method for Large-Scale Preparation of Covalently Closed λ DNA Molecules

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A combination of mutations in bacteriophage λ and its host *Escherichia coli* K-12 provides a convenient system for the isolation of large quantities of covalently closed circular DNA molecules. We describe two procedures for the large scale preparation of λ DNA in the duplex circular form.

In vitro analysis of the biochemical events involved in the metabolism of DNA requires DNA substrates of defined structure. The DNA of bacteriophage λ is an especially useful substrate for such study for several reasons: (i) it is small enough to be relatively shear resistant; (ii) it is of the unique class in that each molecule of mature linear DNA is like every other in the population; and (iii) its cohesive ends, which are of known structure and sequence, permit the formation of hydrogen-bonded monomeric circles or higher polymers, depending upon the DNA concentration at which the annealing occurs. Furthermore, the hydrogen-bonded circles can be treated with polynucleotide ligase to form covalently closed molecules. Other advantages derive from the ability to isolate characterizable DNA fragments (either single stranded [9] or double stranded [1, 7]) and from the extensive genetic knowledge of this bacteriophage and its hosts.

Although one can readily obtain large quantities of mature linear DNA and modest quantities of the by-products listed above, it has, up to now, been fairly difficult to obtain large quantities of covalently closed molecules. Two methods that have been employed to isolate such molecules have been the in vitro sealing of hydrogen-bonded Hershey circles by polynucleotide ligase, and superinfection of an immune lysogen. The former procedure necessitates the purification of the enzyme and, in addition, results in molecules of varying degrees of superhelicity depending upon the conditions employed in the reaction mixture (3); the product, therefore, is not necessarily the same as that occurring in vivo. The yield of covalently closed circles formed in vivo by superinfection, on the other hand, is limited to a maximum of 10 to 20 molecules per cell. We report here a new procedure for the preparation of covalently closed circles formed in vivo and therefore of natural superhelicity. This procedure employs genetic manipulations which restrict λ to a circular mode of replication and results in the accumulation of large quantities of covalently closed circles (2; P. Dawson, A. Skalka, and L. D. Simon, J. Mol. Biol., in press).

Normally, replication of phage λ proceeds in two stages. At early times following infection or induction of a lysogen, the phage DNA replicates as a circle. At late times, concatemers are formed, presumably as products of rolling circle replicative intermediates. A mutation or deletion of the phage *gam* gene prevents the transition from the early to the late mode of replication, and results in the production of circular molecules throughout the lytic cycle. Additional mutations in the phage and host general recombination functions *red* (*exo* or *bet*) and *recA*, respectively, prevent concatemer formation mediated by recombination with a resultant defect in progeny phage (i.e., mature linear DNA) production (2). Inclusion of another phage mutation (gene S [6]) which prevents the shut-off of phage DNA synthesis and cell lysis results in the accumulation of as many as 300 circular DNA molecules per cell. Finally, a heat inducible lysogen of the multiply mutant phage can be prepared to further increase the convenience of this system.

The circular phage DNA molecules which accumulate in such induced lysogens can be quantitatively separated from the host DNA either by a modification of the procedure described by Hirt (3, 4) or of that described by Stonington and Pettijohn (8, 11). The covalently closed molecules can be separated from relaxed circular DNA either by velocity sedimentation in neutral sucrose at low ionic

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strength or by equilibrium centrifugation in ethidium bromide CsCl density gradients. Our adaptation of combinations of both procedures are described below.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. *Escherichia coli* 204 *thy⁻ recA⁻su⁻* lysogenized with either λ cI_{857} red_{am270} gam_{am210} S_{am7} or λ cI_{857} $red_{113}gam_5$ S_{am7} were the strains employed in both of our procedures. *E. coli* 204 was obtained from M. Meselson and the *red⁻* and *gam⁻* mutants from J. Zissler. The S_{am7} mutation was crossed into the phage by standard techniques.

Media and solutions. Minimal medium (5) supplemented with 0.2% glycerol or glucose and 0.2% vitamin free Casamino Acids (containing thymine as indicated below) was used for growth of cells except when induced cells were to be labeled with ^{32}P. In the latter case, cells were grown in buffered peptone broth selected for low inorganic phosphate content (10 g of peptone [Difco] and 5 g of NaCl in 1 liter of 0.1 Tris-hydrochloride, pH 7.4).

Solutions A, B, and C employed in procedure I refer to those described by Worcel and Burgi (11) with minor modifications. Solution A: 0.01 M Tris-acetate, pH 8.2; 0.01 M sodium azide; 20% (wt/vol) sucrose; 0.1 M NaCl. Solution B (prepared fresh each time): 0.1 M Tris-acetate, pH 8.2; 0.1 M EDTA; 4 mg of lysozyme per ml. Solution C: 1% Brij-58; 0.4% deoxycholate; 2.0 M NaCl; 0.01 M EDTA. Solution D: 33% saturated CsCl; 0.01 M Tris-acetate, pH 8.2; 0.04 M EDTA.

Miscellaneous. Dowex-50-H^+ was prepared by boiling in distilled water repeatedly until no more UV-adsorbing material was eluted, and then it was stored under sterile conditions. Dialysis tubing was treated by boiling first a solution of 1 M $NaHCO_3$ and 0.01 M EDTA, and then again in 0.01 M EDTA.

At all steps following lysis of the cells, sterile procedures were employed. All solutions were autoclaved, and only sterile solutions and pipettes were used. Whenever possible during the preparation of the DNA, sterile plastic tissue culture labware was used. For procedure I, extra precautions must be employed. All glassware was cleaned in chromic acid, washed 10 times with distilled water, soaked in aqua regia, and finally rinsed 10 times with distilled water and placed in a 110-C oven overnight.

Preparation of covalently closed circular DNA (procedure I). (i) Growth and induction of cells. One liter of cells in minimal medium with 1 μg of thymine per ml was grown to a density of 3×10^8 cells per ml at 30 C with shaking. The culture was transferred to a 42-C shaking incubator for 30 min and then incubated at 37 C (again with shaking) for 90 min with [^{3}H]thymine present, at a concentration of 1 μCi/ml.

(ii) Preparation of cell extract. The cells were harvested by centrifugation and resuspended in 12 ml of solution A at 0 C. Next, 3 ml of ice-cold solution B were added, and this mixture was kept on ice for 1 min; 15 ml of ice-cold solution C was then added, and the solutions were mixed. The mixture was placed at room temperature (without shaking) for 35 min during which cell lysis was completed. The lysate was then centrifuged at 27,000 × *g* for 30 min.

(iii) Equilibrium density centrifugation in CsCl in the presence of ethidium bromide. The supernatant was carefully pipetted off and adjusted to 0.1% sarkosyl. In reduced illumination, 7 g of solid CsCl and an excess (>200 mg) of solid ethidium bromide were added. The mixture was gently shaken at room temperature for 1 h, after which 23 ml of solution D was added and the refractive index adjusted to 1.3845 with solid CsCl. The entire solution was transferred to one tube of the Ti-35 Spinco rotor and centrifuged for 30 h at 35,000 rpm at 5 C. One-milliliter fractions were collected (under reduced illumination) and the radioactivity of each fraction was determined. The material in the denser band was collected and pooled.

(iv) Removal of ethidium bromide. To remove ethidium bromide, 0.2 ml of a 50% slurry of Dowex-50-H^+ was added to each tube and gently shaken for 5 min. The entire mixture from each tube was passed over a column (0.4 by 0.5 cm) of Dowex-50-H^+ (in a Pasteur pipette). If all the color has not been removed, the material can be passed over a second column. The DNA solution was placed in freshly boiled dialysis tubing and dialyzed overnight against 50 volumes of a sterile solution of 0.02 M Tris-acetate, pH 8.0, and 0.01 M EDTA. The DNA was stored in small frozen portions in sterile plastic tissue culture tubes.

Preparation of covalently closed circular DNA, (procedure II). (i) Growth and induction of cells. Cells (200 ml) were grown at 30 C with aeration to a cell density of 2×10^8 per ml. The culture was transferred to 46 C for 15 min (or longer when larger volumes are employed) to inactivate the thermolabile repressor. The culture was then transferred to 37 C and aeration was continued throughout. If cells were to be labeled with [^{3}H]thymidine, minimal medium supplemented with 10 μg of thymine per ml was employed, and [^{3}H]thymidine (Schwarz/Mann, 20 mCi/mmol) was added to a final concentration of 5 μCi/ml between 20 and 25 min after induction. When cells were to be labeled with $^{32}PO_4$, low phosphate peptone broth was employed, and carrier-free $^{32}PO_4$ (Schwarz/Mann) was added immediately after transfer to 37 C. The culture was incubated for 90 min at 37 C.

(ii) Preparation of cell extract. Cells were collected by centrifugation and resuspended in 8 ml of 0.05 M Tris-hydrochloride, pH 8.0; 25% sucrose. A 1.6-ml portion of lysozyme (5 mg/ml) was added and the mixture was incubated for 5 min at 0 C. A 3.2-ml portion of 0.2 M EDTA was added and the mixture was incubated for an additional 5 min at 0 C. The cells were checked by microscopic examination for formation of spheroplasts. If spheroplast formation is not complete (<80%), the cells are incubated for an additional 5 min at 30 C. The suspension was added dropwise to 6.4 ml of 3% sodium dodecyl sulfate at 65 C with gentle swirling and incubated for at least 20 min at 65 C, or until the solution had cleared. A 4.8-ml portion of 5 M NaCl was added (final concentration 1.0 M) and mixed very gently. The mixture was left on ice overnight. The following morning the

extract was centrifuged at 17,000 × g for 60 min; the supernatant fluid was carefully removed with a large bore pipette, and macromolecules were precipitated from it by the addition of 2 volumes of ethanol. After 30 min at −20 C and centrifugation at 17,000 × g for 30 min, the supernatant was discarded and the precipitate was resuspended in 3 ml of 0.01 M Tris-hydrochloride, pH 7.4; 0.01 M NaCl; and 0.01 M EDTA.

(iii) Equilibrium density centrifugation in CsCl. To exactly 3 g of solution containing the phage DNA, 3.820 g of solid CsCl was added, and the solution was centrifuged to equilibrium (50 Ti rotor, 36,000 rpm, 4 C for at least 18 h). Six-drop fractions were collected and portions were tested for radioactive content. The fractions containing the DNA were pooled and dialyzed against 0.01 M Tris-hydrochloride, pH 7.4; 0.01 M NaCl; and 0.01 M EDTA.

(iv) Velocity sedimentation. The dialyzed solution containing the phage DNA was layered onto a 32-ml 5 to 20% sucrose gradient in 0.01 M Tris-hydrochloride, pH 7.4; 0.01 M NaCl; and 0.01 M EDTA with a 2-ml 70% sucrose pad in the same buffer. Sedimentation was in a SW27 rotor for 16.5 h at 15,000 rpm at 11 C. One-milliliter fractions were collected and sampled for radioactive content. Under these conditions (low ionic strength), covalently closed circles sedimented approximately 1.7 times the rate of relaxed circles. Fractions containing the covalently closed circles were pooled, and the DNA was precipitated with ethanol as described above. The DNA was resuspended in the desired buffer and then stored in small portions in liquid nitrogen.

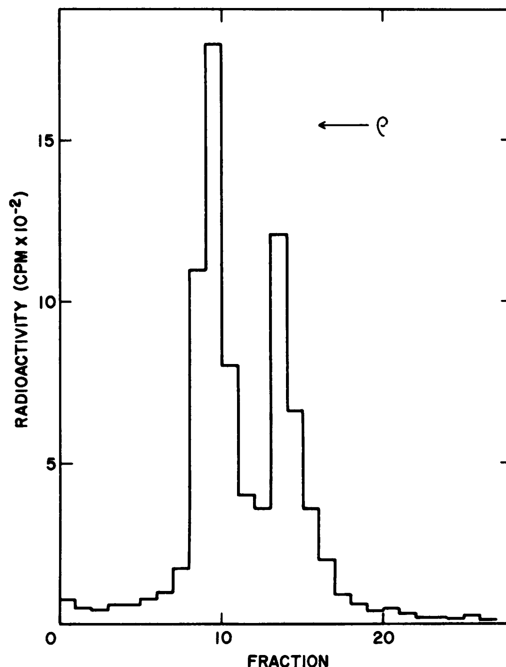

FIG. 1. *Equilibrium density centrifugation of phage DNA in the presence of ethidium bromide. Details are provided in the text. Density increased from right to left; the denser peak contains the covalently closed circles.*

RESULTS AND DISCUSSION

Figure 1 shows typical results from the CsCl-ethidium bromide banding step of procedure I. In this preparation, approximately 0.5 mg of covalently closed circles (500 counts/min per μg) were obtained from 1 liter of cells. The lighter band in this gradient contained a small percentage of linear phage DNA molecules and a large percentage of relaxed circular DNA, which probably arose by random nicking of the covalently closed circles. Such molecules could be repaired by DNA polymerase and ligase, with approximately the same efficiency as relaxed circles prepared by random (single) nicking of covalently closed circles with pancreatic DNase (L. Enquist, unpublished data). The most serious disadvantage to procedure I resulted from the difficulty in keeping the covalently closed circles intact after exposure to ethidium bromide. Extreme precaution must be taken to prevent nicking due to glassware contaminants or light exposure in the presence of ethidium bromide. Thus, manipulations must take place in the dark and all glassware must be scrupulously cleaned as outlined. If such precautions are inconvenient, covalently closed circles can be separated from the relaxed species as outlined in procedure II. Although procedure II involves an additional centrifugation (velocity sedimentation), it has been our experience that less nicking occurs and the purified DNA is more stable when the second procedure is employed.

Results from the preparative sucrose gradient of covalently closed circles prepared by procedure II are shown in Fig. 2. The yield of covalently closed circles in this experiment was also about 0.5 mg (18,000 counts/min per μg) per liter of culture. The slower sedimenting material in this gradient contained a small percentage of linear phage DNA molecules and a small percentage of relaxed circles, which probably arose by random nicking of covalently closed circles during the purification procedure. The major portion of material in this fraction however consisted of a discrete species of interrupted circles that were not repaired by polynucleotide ligase and polymerase. These molecules, which are currently under study, were preferentially lost when procedure I was employed.

The procedures described above have proven particularly useful for the isolation of large quantities of covalently closed circular DNA

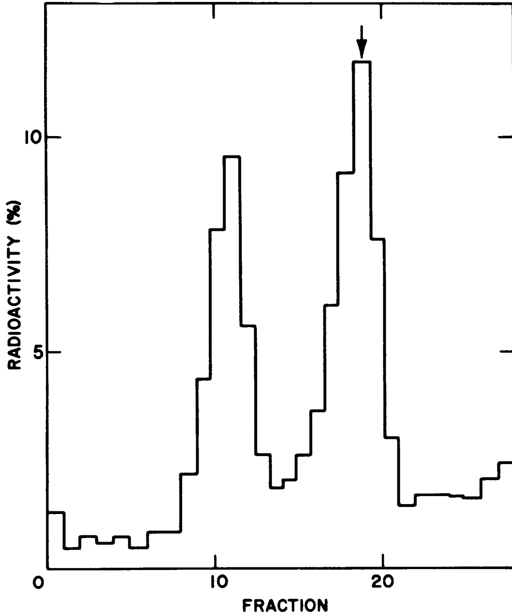

FIG. 2. *Velocity sedimentation of phage DNA in neutral sucrose at low ionic strength. Approximately 100 μg of phage DNA was layered onto a 32-ml gradient. Details are described in the text. Sedimentation was from right to left with covalently closed circles sedimenting more rapidly than interrupted circles or linear molecules. The arrow indicates the position of ^{32}P-labeled λ linear marker DNA.*

molecules. The combination of mutations in phage and host restricts λ DNA replication to the circular mode and results in the accumulation of large quantities of this form of DNA. Thus, sufficient quantities of this defined substrate can be isolated for the in vitro study of duplex circular DNA metabolism.

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