Electron Microscopic Evidence for Linear Insertion of Bacteriophage MU-1 in Lysogenic Bacteria

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Temperate bacteriophage Mu-1 was used to generate a lysogenic derivative of the F'*lac* episome of *Escherichia coli*. Intact, covalently circular molecules of F'*lac* and lysogenic F'*lac* Mu⁺ deoxyribonucleic acid (DNA) were isolated and examined by electron microscopy. The mean contour lengths of F'*lac* and F'*lac* Mu⁺ molecules were $37.6 \pm 0.4 \mu\text{m}$ and $53.2 \pm 0.4 \mu\text{m}$, respectively. The mean difference, $15.6 \mu\text{m}$, is similar to the mean contour length of $12.9 \pm 0.1 \mu\text{m}$ obtained for linear DNA molecules released by osmotic shock from mature phage Mu-1 virions. These results provide direct physical evidence that phage Mu-1 integrates by linear insertion of its genome into the DNA of lysogenic host bacteria. Chemical and physical analyses of phage Mu-1 DNA indicate that it is similar to *E. coli* DNA in respect of gross base composition, buoyant density, and melting temperature.

Bacteriophage Mu-1 is a temperate phage endowed with the distinctive attribute of inducing mutations in the genome of its host bacterium, *Escherichia coli* K-12 (34). Several lines of genetic evidence, to be reported separately, indicate that the prophage Mu-1 can integrate at many different sites in the bacterial chromosome and that these sites coincide with the loci of phage-induced mutational events. Other genetic experiments (21, 37; Martuscelli and Taylor, *in preparation*) suggest that Mu-1 becomes integrated by linear insertion of its genome into the host chromosome, perhaps in a manner similar to that proposed by Campbell (7) for phage λ .

Although suggestive of insertion, the genetic data for Mu-1 are necessarily indirect and they cannot completely rule out certain alternative models in which integration occurs by synaptic attachment of circular or linear viral deoxyribonucleic acid (DNA) to host DNA (7). The experiments described in this report provide a direct physical test of the Campbell insertion model for bacteriophage Mu-1. Electron microscopic evidence will be presented to show that a prophage which is genetically linked to the small episome, F'*lac* (19), of *E. coli* is inserted into and covalently continuous with circular molecules of F'*lac* DNA. This report also presents experimental data on the chemical composition and molecular size of phage Mu-1 DNA.

MATERIALS AND METHODS

Bacteria and bacteriophages. The genotypes of bacteria derived from strain K-12 of *E. coli* are listed in Table 1. Strain EN12, containing the F-merogenote F'*lacI78* (*i*⁺), was the generous gift of John R. Sadler.

The mutagenic phage used in this investigation was isolated from the American Type Culture Collection (ATCC) strain 9637 by plating cell-free culture medium with indicator cells of *E. coli* K-12 (strain AB259). Since the phage from ATCC 9637 is indistinguishable from the phage Mu-1 originally isolated from a chance lysogen of strain K-12 (34) in respect of its morphology (35; Fig. 1), lysogenic immunity, bacterial host range, and ability to induce mutations, we presume that the two phages are closely related and that ATCC 9637 may be the natural reservoir of phage Mu-1. Phage Mu-1c is a clear-plaque mutant used in replica plating tests for host immunity to Mu-1 (34). Purified stocks of phage T4, wild-type phage λ , and the double deletion mutant $\lambda b2b5$ (22) were obtained from F. A. Eiserling. Male-specific phage MS-2, kindly provided by Alvin J. Clark, was propagated on strain AB259 and used to test for the presence or absence of sex factor, F.

Growth and purification of phage Mu-1. The medium for growth of batch lysates (T broth) contained per liter of distilled water: 10 g of Casamino Acids (Difco no. 0230), 2.42 g of tris(hydroxymethyl)aminomethane (Tris), 1.2 g of NH₄Cl, 1 g of NaCl, 0.5 g of KCl, 20 mg of L-tryptophan, and hydrochloride to adjust to pH 7.4. After autoclaving, separately sterilized solutions were added to give final concentrations of 4 mM MgSO₄, 0.01% gelatin, 0.5% glucose, 0.2 μg of thiamine hydrochloride per ml, and 0.6 mM CaCl₂, the last being added at the time of infection. Fernbach flasks (2.8 liter, equipped with

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TABLE 1. *Bacterial strains*

Strain	Sex	Relevant markers ^a	Source
AB259	Hfr	Hfr Hayes, <i>thi-1</i>	From E. A. Adelberg
C600	F ⁻	<i>thr-1</i> , <i>leu-6</i> , <i>thi-1</i>	From M. L. Morse
EN12	F'	F' <i>lacI78 (i^s)/lacI78 (i^s)</i>	F' <i>lac</i> from strain 200P (19)
AB705	F ⁻	<i>lac Δ 175 (i⁻)</i> , Str ^r	From M. L. Morse
AT3144	F ⁻	Nal ^r	
AT3152	F ⁻	<i>lacY1</i> , Nal ^r , (Mu-1 <i>Δ6</i>) ⁺	
AT3155	F'	F' <i>lacI78 (i^s)/lac Δ 175 (i⁻)</i> , Str ^r	EN12 X AB705
AT3156	F'	F' <i>lacI78, lacI176 (i⁻)</i> Mu ⁺ / <i>lac Δ 175 (i⁻)</i> , Str ^r	Mu-1 lysogen of AT3155
AT3158	F ⁻	<i>thyA8</i> , <i>lacY1</i> , Nal ^r , (Mu-1) ⁺	

^a Genotypic symbols *thr*, *leu*, *thi*, and *thy* denote auxotrophic requirements for threonine, leucine, thiamine, and thymine, respectively. The symbols *lacY* and *lacI* refer to structural genes for lactose permease and lactose repressor, respectively; the phenotypes associated with mutations in *lacI* are identified by the symbols *i⁻* for constitutivity and *i^s* for *trans* dominant repression (39). The mutation *lac Δ 175* is a deletion of the *lac* operon which appears to cover part of the *lacI* gene as well. The symbols Str^r and Nal^r denote resistance to streptomycin (200 μg/ml) and nalidixic acid (40 μg/ml), respectively

FIG. 1. Bacteriophage Mu-1 (two particles) and T4 (one particle), negatively stained with uranyl acetate. Magnification 206,000; scale 130 nm.

aeration baffles) containing 1.4 liters of T broth were inoculated with 50 ml of an overnight T-broth culture of strain C600. Cultures were grown to 10^8 bacteria per ml with vigorous rotary shaking at 37 C and then inoculated with Mu-1 at a multiplicity of 4 to 5 phage per bacterium. As a rule, lysis was complete after 2 hr of additional shaking at 37 C, and the phage yields ranged from 10^{10} to 2×10^{10} plaque-forming units (PFU)/ml.

The pooled lysates from five flasks were gently stirred with 5 ml of chloroform and 0.5 mg each of deoxyribonuclease and ribonuclease. The phage particles, and other particulate matter in the lysates, were concentrated by a modification (10) of Albertson's polyethylene glycol-dextran sulfate two-phase partition method (3). The concentrated suspension was centrifuged at $14,000 \times g$ for 15 min to remove large debris, and the resulting supernatant was then centrifuged at $56,000 \times g$ for 90 min to sediment the phage. The phage pellets were covered with 2 ml of Tris-buffered saline (0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Tris, pH 7.2) and resuspended by storing them at 4 C for 15 to 18 hr. After one additional low-speed centrifugation, the concentrated phage suspension contained about 50% of the PFU present in the unpurified lysates. Additional cycles of high- and low-speed centrifugation resulted in severe losses.

A CsCl density-step gradient was used for final purification of the phage. Gradients were formed by introducing 4.5 ml of solution (density 1.22 g/ml) into a centrifuge tube. Additional 4.5-ml volumes of density 1.38, 1.49, 1.57, 1.69, and 1.81 g/ml were introduced successively beneath the preceding layers. Because the phage is highly sensitive to CsCl, the salt concentrations were minimized by preparing solutions in deuterium oxide (D_2O , 99%, density 1.105 g/ml) containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM Tris-hydrochloride to make pH 7.2. One milliliter of phage suspension was layered onto the gradient and centrifuged for 60 min at 20,000 rev/min in a Spinco SW25 rotor. The phage formed a sharp band which was recovered by collecting drops from the tube bottom. Fractions containing phage were pooled and carefully dialyzed against Tris-buffered saline at 4 C, starting with a 10:1 volume ratio for the first 12 hr and followed by 50:1 and 100:1 ratios for the next two 12-hr periods. These preparations, which contained 10 to 20% of the initial PFU in the crude lysate, were devoid of extraneous particles (see below) and were stable at 4 C (approximately 50% survival after 60 days of storage).

Preparation of phage DNA. Phage Mu-1 was disrupted by gently dissolving 1.1 g of guanidine hydrochloride (Schwarz/Mann ultrapure grade) in 5 ml of purified phage suspension containing 8×10^{11} PFU/ml. The resulting clear viscous solution was supplemented with ethylenediaminetetraacetic acid (EDTA) at 4 mM, held for 30 min at 4 C, and then diluted to 12 ml with DNA buffer (0.02 M potassium phosphate, 0.1 M NaCl, 3 mM EDTA, pH 7.0). The solution was gently mixed with 13.54 g of CsCl (optical grade) to yield a final density of 1.63 g/ml and then transferred

to centrifuge tubes, taking necessary precautions to avoid shearing the DNA. After centrifugation in a Spinco SW39 rotor for 40 hr at 29,000 rev/min and 15 C, fractions were collected dropwise from the bottom of the tubes. Fractions containing DNA were detected spectrophotometrically, pooled, and dialyzed against DNA buffer. DNA concentration was calculated, assuming that a solution of optical density 1.0 at 260 nm in a 1-cm pathlength cuvette contains 50 μ g of DNA per ml. Bacterial DNA was prepared by the procedure of Marmur (29), except that CsCl density-gradient centrifugation was employed in lieu of isopropanol precipitation as the final purification step.

Chemical analyses. Tests for the presence of ribonucleic acid (RNA) or protein in the DNA preparations were those of Dische (12) and Lowry et al. (27), respectively. The base compositions of phage Mu-1 DNA and of calf thymus DNA (Sigma Chemical Co.) were determined by using the methods of Wyatt (40). Phage DNA was dialyzed against water, precipitated with two volumes of cold absolute ethanol, washed successively with absolute ethanol, 70% ethanol, ethyl ether, and brought to constant dry weight over anhydrous $CaCl_2$. Samples of 0.8 to 0.9 mg of dry DNA were hydrolyzed at 175 C for 30 min in sealed glass ampoules containing 80% formic acid. The hydrolysates were brought to pH 6.0, spotted on Whatman no. 1 paper, and run by descending chromatography for 20 to 22 hr with an *n*-butanol-0.6 N NH_4OH solvent system (9). A mixture of known adenine, thymine, guanine, and cytosine was hydrolyzed similarly and run on the same papers as the hydrolyzed DNA samples. Individual bases were detected as ultraviolet (UV)-absorbing spots, cut from the paper and eluted quantitatively in 0.1 N HCl. Bases were identified from their absorption spectra over the 220 to 310 nm range and the concentration of each eluted base was calculated by the differential extinction method of Bendich (5).

DNA melting curves. Solutions of purified DNA from Mu-1 and *E. coli* were dialyzed against saline-citrate buffer (15 mM NaCl, 1.5 mM trisodium citrate, pH 7.0) and placed in Teflon-stoppered quartz cuvettes of 1 cm pathlength. Absorbance at 260 nm was monitored with a Zeiss PMQII spectrophotometer as the solutions were heated by pumping water from a thermostatically controlled water bath through the chambers of a heatable cuvette holder. Temperature was monitored continuously with a thermistor inserted into the DNA solution through a hole in the Teflon cap. *E. coli* and phage DNA species were run simultaneously in adjacent cuvettes. The heating rate was held to an average of 0.75 deg/min from 25 to 70 C and reduced to 0.25 deg/min from 70 to 85 C.

Density-gradient analysis. Density-gradient equilibrium sedimentation was performed in a four-place rotor with the necessary precautions taken to prevent shear degradation (11, 14). The flotation densities of the Mu-1 DNA in Cs_2SO_4 and CsCl were determined by using *E. coli* K-12 DNA as a reference marker (density: 1.426 g/cm³ in Cs_2SO_4 and 1.703 g/cm³ in CsCl). Approximately 2 μ g of DNA was added di-

rectly to solid CsCl or Cs₂SO₄ to yield a final density of 1.70 g/cm³ and 1.43 g/cm³, respectively. Equilibrium was established by centrifugation at 122,000 × *g* for 22 hr in CsCl and at 67,000 × *g* for 40 hr in Cs₂SO₄ at 25°C. Denaturation of the Mu-1 DNA at 2 μg/ml was carried out by heating at 101°C for 4 min in one-third strength DNA buffer, followed by rapid cooling in crushed ice. The sample was then added to the solid cesium salt (11). Heating for less than 3.5 min led to incomplete denaturation as evidenced by the presence of DNA at the native density position.

Sedimentation analysis. The sedimentation coefficient of Mu-1 DNA was determined by using the analytical zone sedimentation method (38) as described by Studier (33). A 20-μl amount of DNA at 50 to 100 μg/ml in DNA buffer was layered onto 0.50 M NaCl, 50% D₂O (pH 7.7) in a band-forming centerpiece, and the sample was centrifuged at 53,000 × *g* at 20 to 25°C. Pictures were taken at 16-min intervals with monochromatic UV optics by using Ilford Rapid Process experimental plates (11). The plates were analyzed on either a Nikon two-dimensional comparator model 6C or a Beckman microdensitometer. The calculated sedimentation coefficients were corrected to a water basis at 20°C (*S*_{20,w}). DNA samples obtained from T2 and λ bacteriophages were used as standards and these *S*_{20,w} values agreed to within 5% with those previously reported (33).

Electron microscopy of phage DNA. The DNA of phages Mu-1, λ⁺, and λb2b5 was released and spread by the osmotic shock technique of Kleinschmidt et al. (24), as modified by Caro (8). Samples of the DNA-containing protein film were transferred to copper specimen grids (200 mesh) coated with carbon-supporting films (26). A solution of 0.05 M uranyl acetate in 0.05 M HCl was diluted 100-fold in absolute ethanol and used immediately to dehydrate and stain the specimen in one step. Specimens were rotary shadowed by evaporating a 4-cm length of 80% Pt-20% Pd wire (8 mil diameter) placed at 7.5 cm from the grid and at an angle of 3°. The micrographs were taken with a Siemens Elmiskop IA with double-condenser illumination. The magnification was repeatedly calibrated with a carbon replica of a cross-ruled diffraction grating (E. Fullam, Inc., 2,160 squares/mm), and only visibly long molecules were photographed. The contour lengths of DNA molecules were measured on photographic prints with a map-measuring curvimeter (Keuffel and Esser Co.).

Genetic procedures. Media and experimental conditions for obtaining Mu-1 lysogens, scoring for lysogeny and immunity to phage Mu-1, conjugation crosses, and the nutritional characterization of bacteria have been described elsewhere (1, 34). The lactose phenotype of F'*lac* strains was scored on MacConkey agar (Difco) plates supplemented with nalidixic acid (40 μg/ml) as required. Mutants resistant to 40 μg of nalidixic acid per ml were selected by the procedure of Hane and Wood (17). Conditions for the elimination of F-merogenotes with acridine orange were those described by Hirota (18). The thymine auxotroph used for DNA labeling (strain

AT3158) was isolated in the following way. A thymidylate synthetase mutant (*thyA*⁻), which required exogenous thymine at 40 μg/ml for growth, was grown overnight in a thymine-poor medium (0.8% Difco tryptone, 0.7% NaCl) and plated on minimal medium containing only 2 μg of thymine per ml to select for modified auxotrophs that can grow on low levels of thymine. A colony from these plates was designated as strain AT3158 and we presume that its low thymine requirement stems from a secondary mutation at the *dra* (deoxyriboaldolase) or *drm* (deoxyribomutase) locus (2).

DNA labeling and extraction. The procedures of Bazaral and Helinski (4) were generally followed for the isolation of F'*lac* DNA. Bacteria to be labeled were grown in TM medium which contains, per liter of water: 2.5 g of Casamino Acids (Difco 0288), 5 g of NaCl, 2 g of NH₄Cl, 370 mg of KCl, 26 mg of Na₂SO₄, 10 mg of MgCl₂·6H₂O, 0.9 mg of FeCl₃, 4 g of glycerol, 0.2 mg of thiamine hydrochloride, 4 mg of thymine, and 100 ml of 1 M Tris (pH 7.3). For each experiment, 112 ml of TM medium was inoculated with 8 ml of overnight TM culture and grown at 37°C with rotary shaking to 10⁸ bacteria per ml. At this time, 0.8 ml of [*methy*-³H] thymidine (1 mCi/ml, specific activity 6.7 Ci/mmol) was added, and incubation was continued until the culture reached approximately 10⁹ bacteria per ml. Labeled cells were harvested by centrifugation at 4°C, washed twice with TES buffer (50 mM NaCl, 50 mM Tris, 5 mM EDTA, pH 8.0) at 4°C, and finally resuspended in 8 ml of spheroplast-forming mixture which contains 1 mg of lysozyme per ml, 0.5 mg of ribonuclease per ml, and 100 mg of sucrose per ml in TES buffer. After incubation at 37°C for 10 min, followed by 5 min of chilling in ice, 4 ml of a 2% aqueous solution of sodium lauryl sarcosinate (Sarkosyl, Geigy Chemical Co.) was added to the mixture to effect lysis. The resulting viscous solution was diluted with 8 ml of TES buffer, mixed briefly, and brought to room temperature. Bulk (chromosomal) DNA in the lysate was sheared by drawing up the solution into a conventional 1-ml serological pipette 40 times in succession over a period of 2 min.

Dye-buoyant-density centrifugation. The technique of Radloff et al. (31), as modified for bacterial DNA (4), was used to separate supercoiled F'*lac* DNA from broken chromosomal DNA. The sheared lysate was transferred to a 250-ml screw-cap flask which contained 104.8 g of CsCl, 60.8 ml of water, and 32 ml of ethidium bromide (prepared as a separate solution at 1.4 mg/ml of 0.1 M sodium phosphate buffer, pH 7.0). After gentle mixing of the flask contents, the DNA-CsCl solution was dispensed into eight polyallomer centrifuge tubes which were then topped off with light mineral oil. The tubes were centrifuged in a fixed-angle rotor (Spinco type 50.1) for 38 to 40 hr at 35,500 rev/min at a temperature of 17°C. On completion of the run, 0.35-ml fractions were collected dropwise from the bottom of the tubes. Radioactivity was assayed by precipitating a 40-μl sample of each fraction onto 2-cm-square pieces of Whatman no. 3 paper, as described by Bollum (6). The papers were put in vials containing toluene-2,5-diphenyloxa-

zole-1,4-bis-2(5-phenyloxazolyl)-benzene and counted in a Beckman LS-100 liquid scintillation spectrometer with a counting efficiency of 6%. Fractions which contained episomal DNA were pooled and dialyzed for 48 hr at 4°C against modified TES buffer (containing 0.3 M NaCl instead of 0.05 M) to facilitate removal of ethidium bromide from the DNA. This was followed by 24 hr of dialysis against standard TES buffer.

Electron microscopy of F'lac DNA. Episomal DNA from the dye-buoyant density gradient was prepared for electron microscopy by dialyzing it against 10 mM sodium phosphate buffer (pH 7.0) for 60 min at 4°C and then treating it with deoxyribonuclease (DNase: Sigma Chemical Co. stock no. DN-C) to promote unwinding of the supercoiled molecules (20). The reaction mixture contained 0.2 ml of DNA solution, 4 μ liters of 0.1 M $MgCl_2$, and 5 μ liters of deoxyribonuclease solution (20 ng of deoxyribonuclease/ml in 10 mM sodium phosphate buffer, pH 7.0, containing 0.1% bovine serum albumin). After incubation for 10 min at 20°C, the reaction was stopped by adding 10 μ liters of 0.2 M sodium citrate to the mixture.

Spreading of the relaxed DNA molecules by the method of Kleinschmidt and Zahn (25) followed immediately after the deoxyribonuclease treatment because citrate did not completely arrest the enzymatic reaction. A mixture containing 0.1 ml of treated DNA solution, 0.2 ml of 2 M ammonium acetate, and 30 μ liters of an 0.2% aqueous solution of cytochrome *c* was spread onto the surface of 0.15 M ammonium acetate solution in a Teflon-coated Langmuir trough. The DNA-protein film was not compressed and samples of the film were transferred to carbon-coated copper grids (athene type, 200 mesh) immediately after spreading and at 2-min intervals thereafter. The deoxyribonuclease-treated molecules continued to unwind after spreading and were found to be broken after 15 to 16 min. Specimens were dehydrated with absolute ethanol and rotary shadowed with a 9-cm length of Pt-Pd wire as described above for phage DNA. The micrographs were taken at a magnification of about 8,000 with an RCA model EMU4 equipped with a reentry-type grid cap. The molecules were photographed on Dupont COA-7 film. Complete pictures of fully extended DNA molecules often required two or three 3.75 by 4 inch photographs. These photographs were taken with sufficient overlap to allow for accurate superimposition, as well as to minimize image distortion at the periphery of the picture. The magnification for each series of DNA pictures was calibrated by photographing a standard preparation of phosphotungstic acid-stained phage T2 particles at the same time.

Photographic negatives of molecules whose circular contours could be followed as a continuous closed loop were projected onto paper sheets at a magnification of about 120,000 and traced with a pen. At the same time, the corresponding phage T2 micrographs were projected onto the papers and the distance from tail collar to the tip of the median tail plate spike was marked off for not less than 10 separate particles located in both central and peripheral areas of the field. The mean value of this interval was taken to

represent 100 nm for calibration purposes. Contour lengths of the traced molecules were measured with a curvimeter.

RESULTS

Purification of phage Mu-1 and characterization of its DNA. Our attempts to purify phage Mu-1 from batch lysates by two-phase polymer partition and differential centrifugation usually yielded preparations that were grossly contaminated with both ribosomes and isometric virus-like particles of unknown genetic origin (Fig. 2). Hence the D_2O -CsCl step gradient described above was employed to obtain phage preparations sufficiently pure for chemical analysis. The isometric particles do not seem to be related to Mu-1, as they were found just as readily in lysates of strain C600 infected with phage λ . These contaminants may be related to "petit λ " particles (23), which they resemble morphologically, or they may stem from a different and as yet unknown defective prophage of *E. coli* K-12.

DNA was extracted from the purified phage and banded in a CsCl density gradient. The

FIG. 2. Empty, virus-like particles found in both λ and Mu-1 lysates of strain C600. A phage Mu-1 "ghost" is indicated by the arrow. Negatively stained with phosphotungstic acid. Magnification 110,000; scale 100 nm.

resulting DNA preparations contained no detectable protein or RNA as determined by the Lowry and the orcinol tests, respectively, and the ratios of absorbance at 260/280 nm were equal to or greater than 1.85. The purified Mu-1 DNA was hydrolyzed and chromatographed as described above to determine its base composition. Four UV-absorbing spots were detected on the chromatograms and their positions matched those of the four standard bases (adenine, thymine, guanine, and cytosine). The UV spectra of the materials eluted from these spots confirmed that the four bases in Mu-1 DNA correspond to the four normal major bases. The base composition of Mu-1 DNA (in moles %) was: adenine, 24.4; thymine, 24.2; guanine, 26.3; and cytosine, 25.1. The values obtained for a sample of calf thymus DNA, which was run concurrently with Mu-1 DNA as a control, were: adenine, 29.9; thymine, 27.5; guanine, 20.8; and cytosine, 21.8.

The DNA melting curves obtained for samples of purified Mu-1 and *E. coli* DNA are shown in Fig. 3. The curves, essentially identical, have the shape characteristic for double-stranded DNA molecules. There was no increase in absorbance before 72°C and the overall hyperchromicity achieved was 36%. The results indicate that the guanine plus cytosine content of Mu-1 DNA is similar to that of *E. coli* DNA, about 51% (30).

Further confirmation of the chemical similarity of phage Mu-1 DNA to host DNA was obtained from density-gradient equilibrium sedimentation analysis. As illustrated in Fig. 4A, the density of native Mu-1 DNA was identical to that of *E. coli* DNA in both CsCl and Cs₂SO₄ density gradients. Heat-denatured phage Mu-1 DNA produced a single symmetric band when brought to equilibrium in CsCl, but the same denatured DNA repeatedly produced two separate bands in Cs₂SO₄. The difference in density between the bands in Cs₂SO₄ was 0.007 g/cc, with the heavier band comprising roughly 90% of the DNA in the gradient (Fig. 4B).

The molecular weight of Mu-1 DNA extracted by the guanidine hydrochloride procedure was estimated from its sedimentation coefficient as described above. The *S*_{20,w} determined for this DNA was 30.7S, which corresponds to an approximate molecular weight of 22×10^6 to 23×10^6 daltons.

To obtain a direct measure of the size of phage Mu-1 DNA, individual molecules were extracted from the phage by osmotic shock and photographed in the electron microscope. The released DNA molecules were always linear (Fig. 5), and the distribution of contour lengths obtained for 35 molecules ranged from 12.21 to 13.63 μ m (Fig. 6). The mean contour length of

FIG. 3. Melting curves for *E. coli* DNA (●) and phage Mu-1 DNA (○). Values for optical density at 260 nm were corrected for thermal expansion.

phage Mu-1 DNA was $12.9 \pm 0.07 \mu$ m (standard error of the mean), which corresponds to an approximate molecular weight of 25×10^6 daltons (Table 2). For comparison, DNA molecules of the well-known phages λ^+ (wild type) and its deletion mutant $\lambda b2b5$ were similarly prepared and measured. The lengths of molecules from Mu-1 and from the λ phages were compared by measuring DNA of both types in mixed samples on the same grid as well as in separate preparations. The lengths obtained for λ^+ and $\lambda b2b5$ DNA (Fig. 6, Table 2) were in excellent agreement with values previously reported by several other laboratories (8, 15, 28).

The foregoing measurements predict that the total linear insertion of a phage Mu-1 genome into a host DNA molecule of known size should increase the contour length of the host DNA by about 13 μ m. The remainder of this report

FIG. 4. Densitometer tracings of DNA bands obtained after equilibrium centrifugation in CsCl or Cs_2SO_4 . (A) Superimposed tracings of native phage Mu-1 DNA and native *E. coli* reference DNA; (B) heat-denatured phage Mu-1 DNA.

describes a direct test of this prediction, in which the molecular lengths of F'/lac DNA and of F'/lac DNA containing Mu-1 prophage are compared.

Isolation of mutant F'/lac episome containing prophage. Strain AT3155 is an F'/lac merodiploid of the genotype $F'/lacZ^+$, Y^+ , $I78$ (i^s)/ $lac\Delta I75$ (i^-). It is phenotypically Lac^- (lactose non-fermenting) because the i^s character of the $lacI78$ mutation in the episome is dominant over the endogenetic i^- trait (39). We expected that integration of prophage at the $lacI$ locus of the F -merogenote would produce an i^- mutation which would in turn change the phenotype of the merodiploid from Lac^- to constitutive Lac^+ . Accordingly, surviving lysogens from a Mu-1-infected culture of AT3155 were plated on MacConkey agar to detect Lac^+ mutants. Two Lac^+ colonies were found in a total sample of about 10^4 colonies plated and one of these, strain AT3156, was lysogenic for Mu-1 and still pos-

sessed F , as judged by its sensitivity to phage MS-2.

Preliminary evidence for linkage of prophage to F'/lac came from crosses of AT3156 $\times$ AT3144 ($Mu-1^-$, Nal^r , F^-) in which the number of infective centers resulting from the transfer and subsequent zygotic induction of prophage was determined at intervals after the onset of mating. The number increased rapidly from zero time and by 60 min reached a plateau value of about 0.8 infective centers per input donor cell (Fig. 7). This highly efficient transfer of prophage from strain AT3156 is consistent with its being located in the F'/lac episome.

Further evidence that the prophage resides in the F -merogenote was obtained from a 60-min cross of AT3156 $\times$ AT3152 ($lacY1$, Nal^r , $Mu-1d6$, F^-) in which Lac^+ Nal^r exconjugants were recovered by plating the mating mixture on MacConkey agar containing nalidixic acid. The defective prophage of the F^- parent is unable to form active PFU of Mu-1, but it does render the F^- immune to lysis by zygotic induction of wild-type phage. Of 88 Lac^+ recombinants tested, all were found to possess both F (sensitive to MS-2) and active wild-type Mu-1 prophage. The simultaneous inheritance of F and prophage by all of the Lac^+ recombinants again suggests that Mu-1 is integrated into the F'/lac episome.

Final verification of linkage was achieved by growing one of the Lac^+ heterogenotes of AT3152 from the preceding experiment in the presence of acridine orange under conditions known to favor elimination of F and F -merogenotes (18). Presumptive clones of F'/lac cured strains were detected as Lac^- colonies on MacConkey agar. Of 40 Lac^- colonies tested, 36 scored as F^- (resistant to MS-2) and nonlysogenic for wild-type Mu-1; these 36 were Mu-1-immune, however, indicating that acridine orange did not eliminate the $Mu-1d6$ prophage located in the endogenote. The remaining four Lac^- colonies retained both F and wild-type prophage; presumably these are homogenetic for the $lacY1$ mutation. We conclude that prophage Mu-1 can be eliminated concomitantly with F'/lac by acridine orange and that prophage must therefore be integrated in the F'/lac episome of strain AT3156.

Isolation and purification of F'/lac DNA. Standard F'/lac and the lysogenized F'/lac , hereafter designated F'/lac Mu^+ , were transferred by conjugation from strains AT3155 and AT3156, respectively, to F^- strain AT3158 ($thyA8$, $lacY1$, Nal^r , $Mu-1^+$). F'/lac Mu^+ (i^-) heterogenotes were detected as red Lac^+ colonies on MacConkey agar plus nalidixic acid. Standard F'/lac (i^s) heterogenotes were detected as pure

FIG. 5. Linear molecule of phage Mu-1 DNA extracted by osmotic shock. Rotary shadowed with Pt-Pd. Magnification 62,500; scale 1 μ m.

white colonies on MacConkey agar containing 0.001 M isopropyl- β -thiogalactoside and nalidixic acid; colonies of the parental F^- strain AT3158 were dark pink on this medium.

The DNA of the thymine-requiring F'_{lac} strains was labeled, extracted, and sheared as described above. Closed circular episomal DNA was then separated from the fragmented chromosomal DNA by centrifugation to equilibrium in CsCl and ethidium bromide (4, 31). Fractions collected from the bottom of the centrifuge tubes were assayed for radioactivity as described. Figure 8 illustrates the results typically obtained for DNA from both F'_{lac} and F'_{lac} Mu $^+$ derivatives of AT3158. The episomal DNA appears as a minor peak comprising roughly 2% of total activity, and it is well separated from the bulk of less dense sheared DNA. The very small peak of activity present at the top of the gradient was seen reproducibly and may consist of membrane-bound DNA fragments. When carried

through the same procedure, DNA from strain AT3158 (F^-) produced no visible peak corresponding to episomal DNA. Initially, the peak fractions of episomal DNA were pooled and rebanded by additional centrifugation; later on, the rebanding step was omitted with no apparent adverse effect on visualization of the molecules.

Electron microscopy of F'_{lac} DNA. Peak fractions of episomal DNA were pooled and dialyzed exhaustively to remove as much residual ethidium bromide as possible. DNA prepared in this way invariably failed to show the expected characteristic convoluted loops of circular double-stranded DNA in the electron microscope. Instead, the DNA had the appearance of small, amorphous, spindle-shaped blobs; occasionally, thick rope-like strands of supercoiled DNA were seen projecting from one end of the spindles. Simple storage of the DNA at 4 C for 2 to 3 weeks effected some additional relaxation of the spindle structures to yield loops of untwisted double-

FIG. 6. Distribution of contour lengths, to the nearest 0.5 μm , of linear DNA molecules released by osmotic shock from phages Mu-1, $\lambda\text{b}2\text{b}5$, and λ^+ .

TABLE 2. Molecular size of phage DNA

Phage	No. of molecules measured	Mean contour length (μm)	Variance (S^2) ^a	SEM ^a	Molecular weight (daltons) ^b
Mu-1	35	12.9	0.17	0.07	25
$\lambda\text{b}2\text{b}5$	16	13.0	0.23	0.12	25
λ^+	5	16.6	0.30	0.25	32

^a Variance (S^2) and standard error of the mean (SEM) are indicated for each set of measurements.

^b Assuming a linear density of 192×10^4 daltons per μm (28). Values to be multiplied by 10^6 .

stranded DNA, but regions of supercoiling were still too abundant to permit measurement of contour lengths. The unwinding process could be accelerated by dialyzing the DNA against 0.01 M phosphate buffer, pH 7.0, to remove EDTA and by storing it at room temperature. The process was difficult to control, however, and many preparations were degraded excessively to short fragments of linear DNA.

The best results were obtained with DNA which had been treated briefly with deoxyribonuclease prior to spreading (20), as described above. By taking samples of the DNA-containing

FIG. 7. Kinetics of infective center formation in an uninterrupted conjugation cross of F' -lac donor AT3156 $\times$ F^- recipient AT3144. Initial cell inputs were 1.56×10^7 donors/ml and 3.2×10^8 recipients/ml. Samples were removed from the mating mixture at the times indicated, diluted, and plated for infective centers with AT3144 indicator cells on broth-agar containing nalidixic acid (to counterselect parental donor bacteria).

protein film at intervals after spreading, we were always able to get one or two specimen grids which contained a high proportion of unbroken circular molecules devoid of twisted regions. In addition to photographing molecules from the individual F' -lac and F' -lac Mu⁺ preparations, a mixture of the two episomes was also examined to insure that any observed differences in contour length would also be evident in a mixed sample on the same grid. Micrographs of molecules in the mixed preparation were taken consecutively as they were encountered on the specimen grid.

Because of their large size, most of the circular molecules chosen for length measurement contained a number of overlapping loops. At least four or five molecules each of F' -lac and F' -lac Mu⁺ DNA were observed, however, in which loops comprised less than 4% of the total contour length. One of these is shown in Fig. 9. The topological evidence is thus compatible with the view that integrated phage Mu-1 DNA is inserted and continuous with F' -lac DNA.

Contour lengths of the molecules were measured and calibrated against a standard preparation of bacteriophage T2 as described above. The

FIG. 8. Equilibrium centrifugation in cesium chloride-ethidium bromide of sheared DNA from a strain carrying the lysogenic episome *F'*lacMu⁺. Tube no. 1 represents the first fraction collected from the bottom of the centrifuge tube. The radioactivity shown is that found in 40- μ l portions of each fraction. Circular molecules of episomal DNA are found in the small peak on the left.

distribution of lengths obtained for pure *F'*lac DNA, for pure *F'*lac Mu⁺ DNA, and for a mixture of both molecules prepared on the same grid is given in Fig. 10. It is evident that integration of Mu-1 prophage into *F'*lac produces a substantial increase in the contour length of this episome. Moreover, the bimodal distribution of lengths obtained from the mixed DNA sample confirms that the observed difference in contour lengths is real. The mean contour lengths and estimated molecular weights of *F'*lac and *F'*lac Mu⁺ are presented in Table 3.

DISCUSSION

Studies on the physical and chemical properties of phage Mu-1 DNA indicate that Mu-1 shares with λ and several other temperate coliphages

the property of having double-stranded DNA which is grossly similar in base composition to the DNA of *E. coli*. The possibility remains, however, that Mu-1 DNA may contain small amounts of unusual bases because the methods employed here would not have detected minor components. The base composition obtained for Mu-1 derived from ATCC 9637 is in close agreement with previously reported values for K-12-derived phage Mu-1 (35, 36). The peculiar separation of denatured phage Mu-1 DNA into two distinct bands in Cs₂SO₄ equilibrium density gradients is reminiscent of the similar behavior of several other coliphage DNA species (11). Although the physical basis for this is not understood at present, the presence of multiple bands may reflect base compositional heterogeneity in the phage Mu-1 DNA.

The length of DNA molecules extracted in situ from Mu-1 virions was measured at $12.9 \pm 0.07 \mu\text{m}$, corresponding to an approximate molecular weight of 25×10^6 daltons (Table 2). The Mu-1 genome is thus roughly 22% smaller than the wild-type λ genome and almost identical in size to the DNA of λ b2b5. The molecular weight of λ b2b5 DNA has been shown to be 25×10^6 to 26×10^6 daltons both by contour length measurement (28) and by X-ray target size (15). Our calculated molecular weight of 25×10^6 daltons for λ b2b5 agrees well with both of these independent determinations. The 12.9- μm length obtained for Mu-1 DNA is somewhat shorter, however, than the 14.5- μm figure recently reported in a brief communication by Torti et al. (36).

Although the variance estimate (S^2) for the contour length of pure *F'*lac molecules appears to be abnormally small compared to the other DNA samples (Table 3), a Bartlett's test for homogeneity indicates that the four variance estimates for molecules from pure and mixed DNA preparations do not differ sufficiently to preclude pooling the data. The pooled results (Table 3) yield mean contour lengths of $37.6 \pm 0.4 \mu\text{m}$ for *F'*lac DNA and $53.2 \pm 0.4 \mu\text{m}$ for *F'*lac Mu⁺ DNA. These values correspond to molecular weights of 72×10^6 daltons and 102×10^6 daltons, respectively. Our value for the molecular weight of *F'*lac DNA agrees with and confirms an independent estimate by Freifelder (15) of 74×10^6 daltons, based on X-ray target size.

The mean contour lengths of *F'*lac and *F'*lac Mu⁺ molecules differ by 15.6 μm of DNA (95% confidence interval: 14.4 to 16.8 μm) which must represent linearly inserted prophage DNA. Integrated Mu-1 prophage DNA thus appears to be significantly longer than the 12.9- μm molecules

FIG. 9. Fully extended, closed circular molecule of $F'lacMu^+$ DNA (located by arrows). Two arrows near center field indicate point of juncture of micrographs taken on separate film plates. Rotary shadowed with Pt-Pd. Magnification 9,000; scale 1 μm .

found in mature virions (Table 2). At present, we can only speculate as to possible explanations for this difference. Lang et al. (26) have shown that contour lengths of DNA molecules can vary as much as 20%, depending on the ionic strength of solutions used in the Kleinschmidt spreading procedure. Since the virion DNA and the episomal (prophage) DNA species were prepared and

spread under dissimilar conditions (i.e., spreading from 5 M NH_4NO_3 onto distilled water for the former DNA versus exposure to ethidium bromide and deoxyribonuclease followed by spreading onto 0.15 M ammonium acetate for the latter DNA species), the 17% discrepancy between predicted and observed length of Mu-1 prophage could represent a preparative artifact. A more interesting possibility would be that the prophage is somewhat larger than a normal phage genome since this might offer a clue toward understanding the mechanism of insertion. Experiments to investigate this point in greater detail are currently in progress.

Although there is abundant genetic and physiological evidence for insertion of λ and related phage genomes, reviewed by Dove (13) and Signer (32), direct physical evidence for such insertion is relatively scarce. In an elegant investigation of the X-ray target sizes of lysogenic and nonlysogenic episomes, Freifelder and Meselson (16) have recently provided compelling physical evidence to demonstrate that phage λ genomes are covalently inserted into host DNA molecules. Our electron microscopic evidence that deoxyribonuclease-treated F'/lac Mu^+ DNA yields closed circular molecules containing no fixed loops (Fig. 9), together with the observation that native F'/lac Mu^+ molecules behave like covalently closed supercoiled DNA in CsCl -ethidium bromide density gradients (31; Fig. 8), provides direct proof that chromosomal integration of Mu-1 prophage also occurs by covalent linear insertion. It seems likely, therefore, that insertion will prove to be a universal feature of temperate phages that attach to bacterial genomes, regardless of whether the attachment sites are uniquely determined, as for λ , or randomly distributed, as for Mu-1.

FIG. 10. Distribution of contour lengths, to the nearest micrometer, of circular molecules of F'/lac DNA and lysogenic $F'/lac\text{Mu}^+$ DNA.

TABLE 3. Molecular size of episomal DNA species

Episome	Sample source ^a	No. of molecules measured	Mean contour length (μm)	Variance (S^2) ^b	SEM ^b	Estimated molecular weight (daltons) ^c
F'/lac	Pure	22	37.9	4.30	0.44	72
	Mixed	22	37.2	11.98	0.74	
	Pooled data	44	37.6	8.14	0.43	
$F'/lac\text{Mu}^+$	Pure	23	53.1	8.24	0.60	102
	Mixed	23	53.2	7.02	0.55	
	Pooled data	46	53.2	7.63	0.41	

^a Molecules from the mixed DNA preparation (illustrated in Fig. 10) were classified by linear discriminant analysis as being F'/lac molecules if <44.43 μm in length or $F'/lac\text{Mu}^+$ molecules if >44.43 μm in length.

^b Variance (S^2) and standard error of the mean (SEM) for each set of measurements is indicated.

^c Assuming a linear density of 192×10^4 daltons/μm (28). Values to be multiplied by 10^6 .

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