Transfection of *Escherichia coli* Spheroplasts with a Bacteriophage Mu DNA-Protein Complex

CHRISTINE D. CHASE† AND ROLF H. BENZINGER*

Department of Biology, University of Virginia, Charlottesville, Virginia 22901

Received 3 August 1981/Accepted 3 December 1981

We disrupted bacteriophage Mu particles by freeze-thaw treatment and recovered the DNA by CsCl density gradient centrifugation. This CsCl-purified DNA had a buoyant density which was indistinguishable from that of phenol-extracted Mu DNA. It was, however, 10^3 times more infective than phenol-extracted DNA for spheroplasts of *exoV endI Escherichia coli*. Infectivity was destroyed by proteinase K as well as by pancreatic DNase, indicating that the infective form was a DNA-protein complex. The infective properties of the complex demonstrated that the protein protects Mu DNA against degradation by exonuclease V and that it serves at least one other function in bacteriophage Mu infection. The infectivity of the CsCl-purified DNA was due to a small class of highly infective molecules which sedimented 1.2 times faster than phenol-extracted Mu DNA on neutral sucrose gradients. This change in sedimentation rate is best explained by the formation of protein-linked circular monomers or linear dimers of Mu DNA. In vitro labeling of the DNA-protein complex, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, showed that the CsCl-purified DNA contained a noncovalently associated 65,000-dalton polypeptide. A 65,000-dalton protein was also found to be a minor component of the bacteriophage Mu particle. No protein was found in phenol-extracted Mu DNA. These results suggest that the 65,000-dalton protein is necessary for successful phage infection and is normally injected into the host cell with the Mu genome.

The DNA of the temperate bacteriophage Mu is a double-stranded, linear molecule of 38 kilobases (26). Mu DNA is unique in that heterogeneous host DNA sequences are covalently linked to both ends of the Mu sequences. There are approximately 150 base pairs of host DNA at the left or immunity end of the molecule and about 1,200 base pairs at the right end (4). This DNA is poorly infective for Ca^{2+} -treated cells of *Escherichia coli* strains which plate intact Mu particles with high efficiency (17, 49). Mu DNA is 50 times more infective for Ca^{2+} -treated cells which lack exonuclease V and endonuclease I, but transfects even these strains with low efficiency (10^{-3} that of lambda standard DNA) (17). The efficiency of transfection can be further improved 100-fold by the expression of a Mu early gene (*sor*) in the cells before transfection (49). It is hypothesized that phenol extraction of Mu DNA removes proteins which are necessary for successful infection and which are normally injected into the host with the Mu genome (17, 49).

Injected proteins clearly function in other viral systems. In bacteriophage T4 infection, the

product of gene 2 is injected in noncovalent association with the phage genome and protects it from degradation by exonuclease V (42). The gene H protein of bacteriophage ϕX174 is noncovalently associated with the penetrating phage DNA. Once inside the cell, this protein functions in the synthesis of the parental replicative form of DNA (15, 16). Proteins which are covalently linked to the genomes of bacteriophage ϕ29 (12), poliovirus (21, 27), and adenovirus (32, 43) also function in viral nucleic acid replication.

To characterize proteins which might be injected with the Mu genome, we used transfection to assay the recovery of a DNA-protein complex from purified phage particles. In our most enriched preparations, the complex was 5×10^3 times more infective than phenol-extracted Mu DNA for spheroplasts of wild-type *E. coli*. The infective properties of the complex indicated that the protein protects Mu DNA against exonuclease V and that it serves at least one other function in bacteriophage Mu infection. A minor phage protein of 65,000 daltons was the major one present in the infective preparation. The infective DNA sedimented 1.2 times faster than phenol-extracted Mu DNA on neutral sucrose gradients, suggesting that it was a circular monomer or linear dimer of Mu DNA.

† Present address: Department of Plant Pathology, University of Florida, Gainesville, FL 32611.

MATERIALS AND METHODS

Bacteria and phage strains. *E. coli* K-12 strain 594 was used as a phage indicator and to make wild-type spheroplasts. JC5183 (*exoV*, *endl*, *sbcA*), JC6720 (*exoV*, *endl*), and HF4733 (*exoV*+, *endl*) were obtained from A. J. Clark. We obtained wild-type Mu phage from A. Toussaint and isolated a spontaneous, clear-plaque mutant which we used throughout this work. Lambda phage *cI857ts* was obtained from H. Drexler.

Bacteriophage techniques. Phage indicator was grown to 2×10^9 cells per ml in LB (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 4 ml of 1 N NaOH/liter). CaCl_2 and MgSO_4 were added to final concentrations of 2 mM and 5 mM, respectively. Phage were plated on LB plates in LB soft agar (10 g and 8 g of agar per liter, respectively). Both hard and soft agar contained 1 mM CaCl_2 and 2.5 mM MgSO_4 .

Mu phage stocks were grown on SB plates (32 g of tryptone, 20 g of yeast extract, 5 g of NaCl, 5 ml of 1 N NaOH, and 15 g of agar per liter). Indicator bacteria (less than 12 h old) and phage were incubated together at 37°C for 20 min and plated in SB soft agar; 10^5 phage and 10^8 cells were plated per 150-mm petri dish. Plates were incubated at 37°C for 18 h. After incubation, the plates were flooded with cold SM buffer (20 mM Tris [pH 7.5]–10 mM MgSO_4 –90 mM NaCl). The buffer was poured off after 4 h, and the resulting crude lysate was centrifuged at $11,500 \times g$ for 30 min. Supernatant phage were pelleted by centrifugation at $37,000 \times g$ for 1 h. The pellets were soaked in 0.5 ml of SM buffer for 8 h and resuspended. This preparation was centrifuged at $4,500 \times g$ for 10 min. The resulting supernatant was layered onto a step gradient formed with CsCl solutions of 1.6, 1.5, 1.4, 1.3, and 1.2 g/ml. Centrifugation was for 6 h at $52,000 \times g$ at 15°C. The phage particles, which had a buoyant density of 1.45 g/ml, were removed through the side of the tube with a syringe, mixed with an equal volume of SM buffer, and centrifuged on a second gradient identical to the first. Phage recovered from the second gradient were dialyzed against SM buffer.

Lambda *cI857ts* phage were grown and purified in the same manner on LB plates and in soft agar with M9 buffer (7 g of Na_2HPO_4 , 3 g of KH_2PO_4 , 0.5 g of NaCl, and 1 g of NH_4Cl per liter with CaCl_2 and MgSO_4 added to final concentrations of 0.1 mM and 1 mM, respectively).

Mu phage labeled with [^3H]thymidine were grown in liquid cultures in the presence of adenosine (53). We grew *E. coli* 594 to 2.5×10^8 cells per ml in T broth (26). Mu phage were added at a multiplicity of one phage per cell; adenosine and CaCl_2 were each added to a final concentration of 0.6 mM. [^3H]thymidine (10 Ci/mmol from Schwartz/Mann) was added to 5 $\mu\text{Ci}/\text{ml}$. The culture was aerated at 37°C until lysis. Debris was pelleted by centrifugation at $11,500 \times g$ for 30 min. We added pancreatic DNase, at a final concentration of 10 $\mu\text{g}/\text{ml}$, to the supernatant and stored it on ice for 8 h. After a second centrifugation at $11,500 \times g$ for 30 min, the phage were concentrated and purified as described above. The purified preparations contained 3×10^4 cpm/ μg of DNA.

DNA preparation. DNA was prepared by extracting purified phage with phenol which had been saturated with water and neutralized with 1 M Tris, pH 8. After

three extractions, residual phenol was removed by dialysis against 10 mM Tris–1 mM EDTA, pH 8. For preparation of DNA-protein complex, 1-ml portions of purified Mu phage in plastic tubes were immersed in liquid nitrogen for 60 s and incubated at room temperature for 30 min. After three freeze-thaw cycles, each 1-ml sample was layered onto a step gradient formed with CsCl solutions of 1.8, 1.7, 1.6, 1.5, and 1.4 g/ml. Centrifugation was for 12 h at $115,000 \times g$. Fractions of 0.25 ml were collected through the bottom of the tube. UV-absorbing fractions in the density range of 1.65 to 1.75 g/ml (as determined by refractive index) were pooled and dialyzed against 10 mM Tris–1 mM EDTA, pH 8. UV-absorbing fractions at the density of phage particles (1.45 g/ml) were also pooled and dialyzed. These particles could be recentered to yield more DNA-protein complex. The DNA recovered after the second cycle of centrifugation was similar in concentration to that recovered after the first cycle and had identical physical and biological properties.

The integrity of lambda DNA, phenol-extracted Mu DNA, and CsCl-purified Mu DNA was demonstrated by sedimentation in neutral and alkaline CsCl (44). There were no single-strand breaks in 75% of the phenol-extracted DNA molecules and 90% of the CsCl-purified DNA molecules.

Electron microscopy. DNA and phages were mounted for electron microscopy by the drop diffusion method (20). We mixed 40 μl of a solution containing 1 μg of DNA per ml with 160 μl of 0.25 M ammonium acetate (pH 6.8) and 5 μl of 0.021% (wt/vol) cytochrome *c*. From this mixture, 40 μl was removed and dropped onto a clean Teflon surface. After 10 min, a grid coated with carbon-stabilized Formvar was touched to the surface of the drop. The grid was washed in absolute ethanol, dried face down on filter paper, and rotary shadowed with platinum-palladium at an angle of 4°. Grids were examined in a Hitachi HS 8 electron microscope at 75 kV.

Spheroplasts and transfection assays. All spheroplasts were prepared by the lysozyme-EDTA technique described for *recBC* strains (2), with one modification. Each liter of Fraser and Jerrel medium was supplemented with 1 ml of 1% (wt/vol) tryptophan (filter sterilized), 1 ml of 1% (wt/vol) thiamine, and 5 ml of a solution containing the 20 common amino acids at 0.1% (wt/vol) each. These supplements were necessary to achieve an efficiency of transfection of 10^{-4} PFU per lambda DNA molecule.

Phenol-extracted Mu DNA or CsCl-purified DNA was diluted to 1.25 $\mu\text{g}/\text{ml}$ in 10 mM Tris, pH 8. Lambda DNA was diluted to 1.25×10^{-3} $\mu\text{g}/\text{ml}$ in the same buffer. (Saturation for lysozyme-EDTA spheroplasts occurred at Mu or lambda DNA concentrations of 2 $\mu\text{g}/\text{ml}$.) Transfection was performed by incubating 0.4 ml of DNA with 0.4 ml of spheroplasts at 30°C for 10 min and plating the mixture in 3 ml of indicator soft agar on an LB plate.

Enzymes and reagents. Electrophoretically purified pancreatic DNase was purchased from Worthington Diagnostics and dissolved at 0.01 mg/ml in 0.1 M magnesium acetate, pH 6.8. We digested 100 μl of DNA (25 $\mu\text{g}/\text{ml}$ in 10 mM Tris, pH 8) for 15 min at 37°C with 0.1 μg of enzyme. The DNA was then diluted into 10 mM Tris, pH 8, and used for transfection. RNase type 1-A (purchased from Sigma Chemical Co.) was dissolved at 5 mg/ml in 0.1 M sodium acetate, pH 5,

and boiled for 30 min. RNase was then diluted to 0.01 mg/ml in 0.1 M magnesium acetate and incubated with DNA under conditions identical to those described for DNase digestion.

Proteinase K was purchased from Merck & Co., Inc. The protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was from Calbiochem. Lambda exonuclease was a generous gift from the laboratory of Charles Radding.

Radioactive labeling of protein in vitro. 125 I-labeled Bolton and Hunter reagent (*p*-hydroxyphenylpropionic acid, *N*-hydroxysuccinimide ester [3]; 1,500 Ci/mmol) was purchased from New England Nuclear Corp. CsCl-purified Mu DNA was dialyzed into 10 mM sodium borate (pH 8.5)–0.1 mM EDTA–0.01 mM PMSF. The DNA was then concentrated 10-fold by evaporation under a nitrogen stream. A volume of 50 μ l, containing 10 μ g of DNA, was allowed to react with 0.2 mCi of dried Bolton and Hunter reagent at 0°C for 2 h. We then added 0.25 ml of 0.2 M glycine, pH 7, and dialyzed the reaction mixture exhaustively against 10 mM Tris, pH 7. Phenol-extracted Mu DNA was treated in the same manner. Bacteriophage Mu particles were dialyzed into 100 mM sodium borate (pH 8.5)–1 mM EDTA–0.1 mM PMSF and disrupted by heating at 60°C for 30 min. A volume of 50 μ l, containing 3×10^{11} Mu particles, was allowed to react with 0.2 mCi of Bolton and Hunter reagent as described above.

Discontinuous pH, SDS-polyacrylamide gel electrophoresis. The 125 I-labeled preparations were analyzed by discontinuous pH, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Laemmli (19). A fraction of the dialyzed reaction mixture was mixed with an equal volume of sample buffer (100 mM Tris, 60 mM sodium phosphate, 20% [wt/vol] SDS, 1% [vol/vol] mercaptoethanol, 20% [vol/vol] glycerol, and 0.1% bromophenol blue, pH 7). Protein molecular weight standards (purchased from Pharmacia Fine Chemicals, Inc.) were hydrated in 50 μ l of the above sample buffer mixed with 50 μ l of water; a 5- μ l amount was added to the labeled sample.

The entire mixture was boiled for 5 min and applied to the top of a tube gel. On one occasion, CsCl-purified DNA was digested for 30 min at 37°C with 1 mg of pancreatic DNase per ml before it was mixed with the sample buffer and boiled. The resolving gels (10 cm long) were 10% or 15% polyacrylamide. The stacking gels (1 cm long) were 3% polyacrylamide. Electrophoresis was at constant voltage (200 V) until the bromophenol blue tracking dye reached the bottom of the gels. Gels were removed from the tubes and stained in Coomassie blue to visualize the molecular weight standards. After destaining, they were frozen, sliced, and counted in a Gamma 4000 counter (Beckman Instruments, Inc.).

RESULTS

Preparation of DNA-protein complex. We found that three freeze-thaw cycles resulted in a 9-log decrease in Mu phage titer. After freeze-thaw treatment, a preparation of 10^{10} Mu particles per ml contained no detectable infectivity for intact *E. coli* cells, but was infective for spheroplasts of both wild-type and *exoV endI* strains of *E. coli* (Table 1). Infectivity is expressed as the ratio of PFU per Mu DNA molecule to PFU per lambda DNA molecule (17). Lambda DNA was chosen as a standard because it transfects spheroplasts of wild-type and *exoV endI E. coli* with equal efficiency (2). Mu phage particles were visualized by electron microscopy after the freeze-thaw procedure. We observed that the DNA was released from the disrupted phage through the tail but remained attached to the particle.

To determine which Mu proteins might be responsible for the infectivity of our preparation for wild-type spheroplasts, we needed to detach and separate the DNA molecules from the phage particles. After centrifugation on a CsCl density

TABLE 1. Efficiency of transfection of Mu DNAs for wild-type and nuclease-deficient spheroplasts of *E. coli*

Source of Mu DNA	PFU per Mu molecule/PFU per lambda molecule ^a			
	594 <i>exoV</i> ⁺ <i>endI</i> ⁺	JC5183 <i>exoV</i> <i>endI</i>	HF4733 ^b <i>exoV</i> ⁺ <i>endI</i>	JC6720 ^b <i>exoV</i> <i>endI</i>
Phage after freeze-thaw	2.5×10^{-3}	1.3×10^{-3}		
CsCl-purified DNA	2.2×10^{-4}	2.8×10^{-4}	5.6×10^{-5}	1.1×10^{-4}
Phenol-extracted DNA	2.9×10^{-7}	1.3×10^{-5}	1.5×10^{-7}	4.5×10^{-6}

^a The efficiency of transfection for lambda DNA was 1×10^{-4} PFU per DNA molecule on 594, 5×10^{-5} PFU per DNA molecule on JC5183, 3×10^{-4} PFU per molecule on HF4733, and 6×10^{-5} PFU per molecule on JC6720. The values in the table are averages of three to five experiments with 594 and JC5183 spheroplasts and the results of a single experiment with HF4733 and JC6720 spheroplasts. Phage after freeze-thaw treatment were diluted to 0.125 μ g of DNA per ml. CsCl-purified DNA and phenol-extracted Mu DNA were diluted to 1.25 μ g/ml. Lambda DNA was diluted to 1.25×10^{-3} μ g/ml. Assays were performed in triplicate, except 6 to 10 plates were used for assays with phenol-extracted Mu DNA. We counted a minimum of 150 plaques per plate with 594 spheroplasts, 65 plaques per plate with JC5183 spheroplasts, 200 plaques per plate with HF4733 spheroplasts, and 80 plaques per plate with JC6720 spheroplasts. However, for phenol-extracted Mu DNA, we counted a minimum of 0.5 plaques per plate on 594 and HF4733, 6 plaques per plate on JC5183, and 3 plaques per plate on JC6720. No plaques were observed on control plates which contained indicator bacteria but no spheroplasts.

^b JC6720 is an *exoV* transductant (via P1) of HF4733.

FIG. 1. Separation of Mu DNA from disrupted phage particles by CsCl density gradient centrifugation. Purified phage particles were disrupted by three freeze-thaw cycles, layered onto a CsCl step gradient, centrifuged, and recovered as described in the text. Absorbance at 260 nm was plotted for each fraction. The material in the denser peak (fractions 5, 6, and 7) had a buoyant density of 1.7 g/ml, as determined by refractive index, and an absorbance ratio (absorbance at 260 nm/absorbance at 280 nm) of 1.8. The material in the lighter peak (fractions 13, 14, and 15) had a buoyant density of 1.45 g/ml and an absorbance ratio of 1.6. The contents of both peaks were dialyzed and spread for electron microscopy. The lighter peak contained disrupted phage particles and the denser peak contained free DNA molecules. OD, Optical density.

gradient, we recovered two peaks of UV-absorbing material (Fig. 1). One peak was recovered at the density of phage particles (1.45 g/ml). The absorbance ratio (absorbance at 260 nm/absorbance at 280 nm) of this material was 1.6, a typical value for Mu particles. The second peak was recovered at the density of free DNA (1.7 g/ml). The absorbance ratio of this material was 1.8, which is characteristic of DNA. We visualized the contents of both peaks by electron microscopy. The former contained disrupted phage particles as described above. The latter contained linear, duplex DNA molecules, and no phage structures were observed among the 100 molecules we examined. DNA recovered in this manner will be referred to as CsCl-purified DNA.

CsCl-purified and phenol-extracted Mu DNAs were identical in their UV-absorbing properties (absorbance at 260 nm/absorbance 280 nm = 1.8), buoyant densities as measured by analytical ultracentrifugation (1.710 g/ml), and appearance in electron micrographs (linear, duplex

molecules). However, infective properties of the two DNA samples were quite different (Table 1). CsCl-purified Mu DNA was 10^3 times more infective than phenol-extracted Mu DNA for spheroplasts of wild-type *E. coli*. The CsCl-purified DNA was equally infective for spheroplasts of wild-type and *exoV endI E. coli*, whereas phenol-extracted Mu DNA was 50 times more infective for spheroplasts of the nuclease-deficient strain. We obtained similar values in an experiment using closely related *exoV+* and *exoV* strains (Table 1).

The infectivity of CsCl-purified DNA was destroyed by pancreatic DNase, but was unaffected by RNase (not shown). The infectivity was also destroyed by incubation with proteinase K, but was unaffected by protease inhibited with PMSF (Fig. 2). Proteinase K had no effect on the infectivity of lambda DNA. As the concentrations of enzyme and inhibitor were constant in all assays, the inactivation was not due to any effect of proteinase K or PMSF on the spheroplasts. The infectivity of CsCl-purified Mu DNA was, therefore, due to the presence of a DNA-protein complex.

A special class of DNA molecules. The DNA recovered from CsCl gradients was equally infective for wild-type and *exoV endI* spheroplasts, at an efficiency 2×10^{-4} that of lambda DNA (Table 1). This preparation might contain a homogeneous population of molecules resistant to host nucleases (but lacking in other factors required to achieve infectivity equal to that of lambda DNA) or a small class of highly infective molecules and many uninfected molecules. To distinguish between these two alternatives, we prepared phenol-extracted DNA and CsCl-purified DNA from Mu phage grown in the presence of [3 H]thymidine and digested them with lambda exonuclease in vitro. We detected no nuclease-resistant molecules in either DNA sample (Fig. 3). (Half of the 3 H remained acid precipitable because of the enzyme's requirement for a double-stranded substrate [22, 31]). Incubation of the CsCl-purified DNA in a reaction mix without enzyme had no effect on its infectivity, indicating that the complex remained intact under the reaction conditions. Therefore, we postulated a special class of highly infective DNA molecules.

Sedimentation of [3 H]thymidine-labeled, CsCl-purified Mu DNA on neutral sucrose gradients confirmed this hypothesis (Fig. 4). The DNA sedimented with an apparent $s_{20,w}$ of 30S, the same value we obtained for phenol-extracted Mu DNA. The infectivity for wild-type spheroplasts, however, was recovered at the 36S position, with minor peaks at the 46S and 50S positions. None of the infectivity for wild-type spheroplasts was found at the 30S position.

The material in the 36S peak contained DNA

FIG. 2. Sensitivity of transfecting activity to proteinase K. CsCl-purified Mu DNA and lambda DNA were dissolved at 60 $\mu\text{g}/\text{ml}$ in 10 mM Tris-1 mM EDTA, pH 8. Proteinase K was dissolved at 100 $\mu\text{g}/\text{ml}$ in 10 mM Tris-1 mM CaCl_2 , pH 8, or in 10 mM Tris-1 mM CaCl_2 -0.1 mM PMSF, pH 8. Reaction tubes contained 30 μl of DNA and 30 μl of enzyme. Control and zero-time tubes contained 30 μl of DNA and 30 μl of enzyme inhibited with PMSF. Tubes were incubated at 37°C. At 0, 10, 20, and 30 min, a tube was removed to an ice bath, and 1.5 ml of cold buffer (10 mM Tris-0.1 mM PMSF, pH 8) was added. Lambda DNA was further diluted 1:30,000 in 10 mM Tris-0.1 mM PMSF containing 20 μg of proteinase K per ml, thereby keeping the concentrations of proteinase K and PMSF constant in all transfection assays. The DNA was used to transfect spheroplasts of wild-type *E. coli*. Three assays and a control (with indicator bacteria only) were plated for each time point. The total number of plaques counted for each time point was plotted against time of incubation with the enzyme.

which was 1.1×10^{-3} times as infective as lambda DNA for wild-type spheroplasts (Fig. 4). The infective form in this peak was enriched eightfold over that applied to the sucrose gradient. A similar preparation from another sucrose gradient was 1.7×10^{-3} times as infective as lambda DNA for *exoV endI* spheroplasts.

Identification of Mu proteins by in vitro labeling. To identify the proteins present in the DNA-protein complex, we reacted CsCl-purified Mu DNA with ^{125}I -labeled Bolton and Hunter reagent. We analyzed the labeled reaction products by SDS-polyacrylamide gel electrophoresis and found that a 65,000-dalton polypeptide was the major one present in the preparation (Fig. 5). When we digested the ^{125}I -labeled DNA-protein complex with pancreatic DNase before electro-

phoresis, we observed no new polypeptides in SDS gels. We also tested the reaction of phenol-extracted Mu DNA with ^{125}I -labeled Bolton and Hunter reagent. This sample incorporated 25 times less ^{125}I than an equal amount of CsCl-purified DNA, and we detected radioactivity only at the position of the tracking dye on our gels.

We analyzed the proteins in Mu phage particles by the same techniques and determined that a 65,000-dalton polypeptide was a minor component of the phage (Fig. 6). We also observed major phage structural polypeptides of 52,000, 48,000, and 36,000 daltons. We consistently detected other minor phage proteins of 76,000, 72,000, 41,000, 39,000, 30,000, 27,000, 25,000, 23,000, and 20,100 daltons.

The polypeptides in Mu phage heads were also labeled and analyzed on SDS-polyacrylamide gels (not shown). These heads were recovered from preparations of wild-type phage

FIG. 3. Sensitivity of phenol-extracted and CsCl-purified Mu DNA to lambda exonuclease. We digested 0.25 μg of phenol-extracted Mu DNA or CsCl-purified Mu DNA with various concentrations of lambda exonuclease at 37°C for 30 min. Each reaction mixture contained 10 μl of substrate (0.04 mM deoxyribonucleotide as ^3H -labeled, phenol-extracted Mu DNA or CsCl-purified Mu DNA in 20 mM Tris [pH 7.5]-20 mM MgCl_2) and 10 μl of enzyme diluent (10 mM Tris, pH 7.5, 2 mM dithiothreitol, and 1 mg of bovine serum albumin per ml) containing lambda exonuclease in various concentrations. The reaction was terminated by the addition of 0.9 ml of calf thymus DNA dissolved at 1 mg/ml. We analyzed 0.12 ml of each mixture for total ^3H radioactivity. Another 0.6 ml was analyzed for acid-soluble radioactivity. The percentage of total radioactivity solubilized was plotted against enzyme concentration. Fifty percent of the total counts per minute was 3,750 cpm. TCA, Trichloroacetic acid.

FIG. 4. Sedimentation of Mu DNA-protein complex. We sedimented 2.5 μg of [^3H]thymidine-labeled, CsCl-purified Mu DNA, or phenol-extracted Mu DNA through a linear, 10 to 30%, neutral sucrose gradient at $151,000 \times g$ for 4.5 h. Fractions of 0.24 ml were pumped from the bottom of the tube. The number of trichloroacetic acid-precipitable counts per minute in 0.1 ml of each fraction is plotted against fraction number in the lower panel. Another 0.1 ml of each fraction was diluted 1:10 and used to transfect spheroplasts of the *exoV*⁺ *endI*⁺ strain 594. (These dilutions contained from 0.02 to 0.5 μg of DNA per ml). Two assays and a control (with indicator bacteria only) were plated for each fraction. The total number of PFU counted is plotted against the fraction number in the upper panel. We recovered 60% of the infectivity and 90% of the acid-precipitable radioactivity which was applied to the gradient. The CsCl-purified Mu DNA applied to this gradient was 1.3×10^{-4} times as infective as lambda DNA. The DNA in the 36S peak (fraction 22) was 1.1×10^{-3} times as infective as lambda DNA (eight times more infective than the starting preparation). This fraction contained a total of 1,500 acid-precipitable cpm, 2% of the applied radioactivity. S values were determined from sw^t charts (Beckman Instruments, Inc.).

and banded at a buoyant density greater than that of intact particles. The major head components were the 36,000- and 48,000-dalton polypeptides. We also observed a very minor 65,000-dalton component.

DISCUSSION

Protein-stimulated transfection. It is hypothesized that Mu phage proteins are required for efficient expression of Mu DNA and are injected into the host during normal bacteriophage infection (17, 49). The low efficiency of transfection obtained with phenol-extracted Mu DNA may be due to the loss of these proteins. Our CsCl-purified DNA contained such proteins, as it was more infective than phenol-extracted Mu DNA in transfection assays and this infectivity was destroyed by proteinase K.

Protein-stimulated transfection has been observed in other viral systems. The infectivity of ϕX174 DNA for *E. coli* spheroplasts is stimulated 15- to 37-fold when the DNA is noncovalently complexed with the ϕX gene H protein (15, 16). The DNA of the *Bacillus* phage $\phi 29$ contains a 31,000-dalton protein covalently linked to the 5' ends (12, 14, 55), and the transfecting activity of $\phi 29$ DNA is also destroyed by protease (13). The DNA of adenoviruses has a 55,000-dalton protein covalently linked to the 5' ends (32), and adenovirus 5 DNA-protein complex is 100 times more infective than pronase-treated adenovirus DNA in the transfection of HeLa cells (40).

Infective form of Mu DNA. The infectivity of CsCl-purified Mu DNA was primarily due to a small class of molecules which sedimented 1.2 times faster than the main band of DNA on neutral sucrose gradients (Fig. 4). Therefore, the infective form might be a protein-linked, circular monomer or linear dimer of Mu DNA. Interaction of the ends of Mu DNA would also result in higher multimers which could account for the minor, faster-sedimenting forms we observed. We visualized DNA from the 36S and 50S peaks of our sucrose gradients by electron microscopy. Among 22 DNA molecules, we observed only linear monomers; however, these results do not rule out the presence of labile circles or dimers.

Mu DNA which sediments two times faster than phenol-extracted phage DNA can be recovered from superinfected Mu lysogens (23). Phenol extraction converts this form to a normally sedimenting species (23).

The abortive transducing DNA of bacteriophage P1 is maintained in recipient cells as a protein-linked, circular molecule (36). Protease-sensitive, circular molecules are also observed in DNA preparations from *Bacillus* phages $\phi 29$ (30) and GA-1 (1) and from adenoviruses (34). In the case of bacteriophage Mu DNA, both ends of the Mu DNA sequences are required for DNA replication, integration, and transposition (6, 7, 39, 48). Molecular mechanisms have been proposed which involved the direct interaction of the ends of the Mu sequences during these events (46, 48). If our infective form of Mu DNA

FIG. 5. SDS-polyacrylamide gel electrophoresis of ^{125}I -labeled proteins associated with CsCl-purified Mu DNA. CsCl-purified Mu DNA was allowed to react with ^{125}I -labeled Bolton and Hunter reagent as described in the text. We applied 20 μl of the dialyzed reaction mixture, containing 1.5×10^{10} DNA molecules and 48,000 acid-precipitable cpm, to the 15% gel shown here. ^{125}I radioactivity was plotted for each 2-mm gel slice. Solid triangles indicate the positions of stained molecular weight standards. (The exact size determination for the 65,000-dalton polypeptide was made on a 10% polyacrylamide gel). The 65,000-dalton peak contained 15% of the total acid-precipitable radioactivity applied to this gel.

is a protein-linked, circular monomer, the proteins might join the physical ends of the Mu DNA, bringing the host-Mu junctions within 1,300 bases of each other. Alternatively, the proteins might bind at the host-Mu junctions; the interaction of proteins would then bring these sites into direct contact.

If the protein bound to the physical ends of the DNA molecule, binding could not be sequence specific, as the heterogeneous host sequences are at the ends of the Mu genome (4). A protein which protects P22 and Mu DNA from exonuclease V and lambda exonuclease has been partially purified from a Mu lysogen after 30 min

FIG. 6. SDS-polyacrylamide gel electrophoresis of ^{125}I -labeled Mu phage proteins. Mu phage particles were disrupted by heating at 60°C for 30 min and allowed to react with ^{125}I -labeled Bolton and Hunter reagent. We applied 20 μl of the dialyzed reaction mixture, containing 3×10^{10} Mu particles and 100,000 acid-precipitable cpm, to the 15% gel shown here. ^{125}I radioactivity was plotted for each 1-mm gel slice. Solid triangles mark the positions of stained molecular weight standards. Open triangles mark the positions of the 13 Mu polypeptides which we consistently detected in our gels. Size determinations for proteins of greater than 48,000 daltons were made on 10% gels. The 65,000-dalton peak contained 1.6% of the total acid-precipitable radioactivity applied to the gel shown here.

of thermal induction (52). This activity elutes from a gel filtration column in the 40,000- to 80,000-dalton range and accomplishes protection by binding to DNA. The binding of this protein is not sequence specific, as P22 DNA is circularly permuted and therefore also has heterogeneous sequences at its ends (33). The protective protein may be identical to the one in our DNA-protein complex. If this is true, this protein should also cause phenol-extracted Mu DNA to sediment more rapidly on neutral sucrose gradients and should increase infectivity for wild-type spheroplasts. We note that this exonuclease inhibitor slows the migration of phage DNAs in agarose gels (52), suggesting a possible change in DNA conformation.

Protein associated with CsCl-purified Mu DNA. We detected only one major protein associated with CsCl-purified Mu DNA; its molecular weight was 65,000 (Fig. 5). We observed that a 65,000-dalton protein was a minor component of the bacteriophage Mu particle (Fig. 6). As this phage protein was the major one present in the CsCl-purified DNA, it was most probably responsible for the infective properties of this DNA.

The 65,000-dalton protein was noncovalently associated with Mu DNA, as it was removed from Mu DNA by phenol extraction and by boiling in SDS. The protein responsible for enhancing the infectivity of Mu DNA is also removed by phenol extraction. In contrast, there are several viral nucleic acid-protein complexes which are covalent in nature. In these cases, the DNA-protein complex is recovered intact after phenol or chloroform-isoamyl alcohol extraction (5, 9, 12, 21, 32). Furthermore, proteins which are covalently linked to nucleic acids do not enter SDS-polyacrylamide gels unless the complex is first digested with nuclease (8, 12, 32, 37).

Roles of associated protein in Mu infection. Unlike phenol-extracted Mu DNA, CsCl-purified DNA transfected *exoV*⁺ *endI* and *exoV* *endI* spheroplasts with equal efficiency. As we obtained the same result with spheroplasts which differed only at the *exoV* locus, the associated protein must protect Mu DNA against exonuclease V. (It is not clear that the protein provides protection against endonuclease I since this is a periplasmic enzyme which is released from *E. coli* by lysozyme-EDTA treatment [28] and is not highly active in lysozyme-EDTA spheroplasts [55]). Exonuclease V is a powerful inhibitor of transfection by double-stranded, linear DNA molecules; the DNAs of T4, T5, and P22 are all 50 to 100 times more infective for exonuclease V-deficient spheroplasts (2). All of these phages (35, 45) encode proteins similar to the *gam* protein of bacteriophage lambda (47). This protein is synthesized early in phage infec-

tion and inactivates exonuclease V by interacting directly with the enzyme (18). The early region of the Mu genome also encodes a *gam* protein (38, 50). However, double-stranded, linear genomes are potentially vulnerable to exonuclease V until the *gam* protein is synthesized. In bacteriophage T4 infection, the product of gene 2 is injected into the host with the phage DNA and provides protection against exonuclease V in the earliest stages of infection (41, 42). A protein in our DNA-protein complex apparently serves the same purpose in early bacteriophage Mu infection.

Our most enriched preparations of DNA-protein complex (Fig. 4) transfected *exoV*⁺ *endI*⁺ spheroplasts 100 times more efficiently than phenol-extracted Mu DNA transfected *exoV* *endI* spheroplasts. Therefore, the associated protein must provide at least one function in addition to protection against exonuclease V. Possibly, the protein in our complex functions in the early replication of the Mu genome. Mu DNA replication is not well understood at this time, but is known to be dependent upon enzymes which are also required for Mu integration and transposition events (6, 29, 51). The gene H protein of bacteriophage ϕ X174 is noncovalently bound to the penetrating phage DNA and is required for the synthesis of the double-stranded replicative form of DNA (15, 16). Injected proteins also function in the replication of bacteriophage ϕ 29 DNA (12, 54) and adenovirus DNA (32, 43), presumably by allowing the priming of daughter strands.

Two Mu genes which might encode an injected protein. The structural proteins of phage particles are generally encoded by late genes. Gene H directs the synthesis of a 64,000-dalton polypeptide in minicells (11). In a cell-free transcription-translation system, one of the genes in the M-Y-N region directs the synthesis of a 65,000-dalton protein (24); in minicells of *E. coli*, genes Y and N direct the synthesis of 12,500- and 60,000-dalton polypeptides (11). Therefore, gene M probably encodes a 65,000-dalton polypeptide. Thus, gene M or H could encode the 65,000-dalton protein in our DNA-protein complex.

The product of gene A (which is a Mu early gene) is a polypeptide of 65,000 (M. S. DuBow and A. I. Bukhari, personal communication) or 70,000 daltons (25). Mutations in gene A eliminate Mu DNA synthesis (51), and the gene A protein is thought to be directly involved in the integration of Mu DNA into the host chromosome (29) as well as in Mu-mediated transposition events (6). We cannot rule out a role for the gene A protein in transfection.

The expression of another Mu early gene (*sot*) in the host cell stimulates the efficiency of

transfection of Mu DNA 100-fold (49). The product of the *sot* gene is probably a protein of 20,500, 11,500, or 5,000 daltons (10), much smaller than the 65,000-dalton protein in our complex. Thus, more than one gene product is probably required to achieve maximum efficiency of transfection with Mu DNA.

Conclusions. It is clear that a phage structural protein, in noncovalent association with bacteriophage Mu DNA, enhances the infectivity of that DNA for spheroplasts of *E. coli*. The associated protein renders Mu DNA exonuclease V resistant, but must have at least one other function in Mu infection. The protein responsible for the infectivity of CsCl-purified Mu DNA is probably injected into the host cell during normal phage infection. Proof of the injected protein hypothesis requires the recovery of a DNA-protein complex from Mu-infected cells. Recently, a 65,000-dalton protein has been found in noncovalent association with DNA recovered from bacteriophage Mu-infected minicells of *E. coli* (A. Puspurs and J. Reeve, personal communication). By fully understanding the nature of such proteins and their association with Mu DNA, we may gain a better understanding of the early events of bacteriophage Mu infection.

ACKNOWLEDGMENTS

This work was supported by Public Health Service training grant GM-01450, Public Health Service grant AI-08572 from the National Institute of Allergy and Infectious Diseases, and a University of Virginia Institutional Grant.

We gratefully acknowledge the generous gifts of bacterial and phage strains from A. J. Clark, H. Drexler, M. Howe, and A. Toussaint and the gift of lambda exonuclease from J. Williams and C. Radding. We thank M. Howe, R. Huskey, J. Murray, J. Reeve, and J. Williams for helpful discussions. We also thank A. Dunn for help with the ^{125}I labeling, C. Mitchell for performing the analytical centrifugation, and T. Keller for help with the SDS-polyacrylamide gel electrophoresis.

LITERATURE CITED

1. Arnberg, A. C., and F. Arwert. 1976. DNA-protein complex in circular DNA from *Bacillus* bacteriophage GA-1. *J. Virol.* 18:783-784.
2. Benzinger, R., L. Enquist, and A. Skalka. 1975. Transfection of *Escherichia coli* spheroplasts. V. Activity of the *recBC* nuclease in *rec+* and *rec-* spheroplasts measured with different forms of bacteriophage DNA. *J. Virol.* 15:861-871.
3. Bolton, A. E., and W. M. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I -containing acylating agent. *Biochem. J.* 133:529-539.
4. Bukhari, A. I., S. Froshauer, and M. Botchan. 1976. The ends of bacteriophage Mu DNA. *Nature (London)* 264:580-583.
5. Daubert, D. S., G. Breuning, and R. C. Najarian. 1978. Protein bound to the genome RNAs of cowpea mosaic virus. *Eur. J. Biochem.* 82:45-51.
6. Faalen, M., O. Huisman, and A. Toussaint. 1978. Involvement of phage Mu-1 early functions in Mu-mediated chromosomal rearrangements. *Nature (London)* 271:580-582.
7. Faalen, M., A. Toussaint, and A. Resibois. 1978. Mini-Mu: an insertion element derived from temperate phage Mu-1. *Cold Spring Harbor Symp. Quant. Biol.* 43:1169-1177.
8. Flanagan, J. B., R. F. Patterson, V. Ambros, H. J. Hewlett, and D. Baltimore. 1977. Covalent linkage of a protein to a defined nucleotide sequence at the 5' terminus of virion and replicative intermediate RNAs of poliovirus. *Proc. Natl. Acad. Sci. U.S.A.* 74:961-965.
9. Gerlich, W., and W. S. Robinson. 1980. Hepatitis B virus contains protein attached to the 5' terminus of its complete DNA strand. *Cell* 21:801-810.
10. Giphart-Gassler, M., J. Reeve, and P. van de Putte. 1981. Polypeptides encoded by the early region of bacteriophage Mu synthesized in minicells of *Escherichia coli*. *J. Mol. Biol.* 145:165-191.
11. Giphart-Gassler, M., C. Wijffelman, and J. Reeve. 1981. Structural polypeptides and products of late genes of bacteriophage Mu: characterization and functional aspects. *J. Mol. Biol.* 145:139-163.
12. Harding, N. E., J. Ito, and G. S. David. 1978. Identification of the protein firmly bound to the ends of bacteriophage $\phi 29$ DNA. *Virology* 84:279-292.
13. Hirokawa, H. 1972. Transfecting deoxyribonucleic acid of *Bacillus* bacteriophage $\phi 29$ that is protease sensitive. *Proc. Natl. Sci. U.S.A.* 68:1555-1559.
14. Ito, J. 1978. Bacteriophage $\phi 29$ terminal protein: its association with the 5' termini of the $\phi 29$ genome. *J. Virol.* 28:895-904.
15. Jazwinski, S. M., A. A. Lindberg, and A. Kornberg. 1975. The gene H spike protein of bacteriophage $\phi X174$ and S13. I. Functions in phage receptor recognition and transfection. *Virology* 66:283-293.
16. Jazwinski, S. M., R. Marco, and A. Kornberg. 1975. The gene H spike protein of bacteriophage $\phi X174$ and S13. II. Relation to synthesis of the parental replicative form. *Virology* 66:294-305.
17. Kahmann, R., D. Kamp, and D. Zipser. 1976. Transfection of *Escherichia coli* by Mu DNA. *Mol. Gen. Genet.* 149:323-328.
18. Karu, A. E., Y. Sakaki, H. Echols, and S. Linn. 1975. The gamma protein specified by bacteriophage lambda. *J. Biol. Chem.* 250:7377-7387.
19. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
20. Lang, D. 1971. Individual macromolecules: preparation and recent results with DNA. *Philos. Trans. R. Soc. London* 261:151-158.
21. Lee, Y. F., A. Nomoto, B. M. Detjen, and E. Wimmer. 1977. A protein covalently linked to poliovirus genome RNA. *Proc. Natl. Acad. Sci. U.S.A.* 74:59-63.
22. Little, J. W. 1967. An exonuclease induced by bacteriophage lambda. II. Nature of the enzymatic reaction. *J. Biol. Chem.* 242:679-686.
23. Ljungquist, E., and A. I. Bukhari. 1979. Behavior of bacteriophage Mu DNA upon infection of *Escherichia coli* cells. *J. Mol. Biol.* 133:339-347.
24. Magazin, M., and B. Allet. 1978. Synthesis of bacteriophage Mu proteins *in vitro*. *Virology* 85:84-97.
25. Magazin, M., J. N. Reeve, S. Maynard-Smith, and N. Symonds. 1978. Bacteriophage Mu encoded polypeptides synthesized in infected minicells. *FEMS Microbiol. Lett.* 4:5-9.
26. Martuscelli, J., A. L. Taylor, D. J. Cummings, V. A. Chapman, S. S. Delong, and L. Canedo. 1971. Electron microscopic evidence for linear insertion of bacteriophage Mu-1 in lysogenic bacteria. *J. Virol.* 8:551-563.
27. Nomoto, A., B. Detjen, R. Pozzatti, and E. Wimmer. 1977. The location of the poliovirus genome protein in viral RNAs and its implication for RNA synthesis. *Nature (London)* 268:208-213.
28. Nossal, N., and L. A. Heppel. 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. *J. Biol. Chem.* 241:3055-3062.
29. O'Day, K., D. Schultz, W. Ericson, L. Rawluck, and M. Howe. 1978. Search for integration-deficient mutants of bacteriophage Mu, p. 48-51. *In* D. Schlessinger (ed.),

- Microbiology—1978. American Society for Microbiology, Washington, D.C.
30. **Ortín, J., E. Vinuela, and M. Salas.** 1971. DNA-protein complex in circular DNA from phage ϕ 29. *Nature (London)* **234**:275–277.
 31. **Radding, C. M.** 1966. Regulation of lambda exonuclease. I. Properties of lambda exonuclease purified from lysogens of lambda T11 and wild-type. *J. Mol. Biol.* **18**:235–250.
 32. **Rekosh, D. M. K., W. C. Russell, A. J. D. Bellet, and A. J. Robinson.** 1977. Identification of a protein linked to the ends of adenovirus DNA. *Cell* **11**:283–295.
 33. **Rhoades, M., L. A. MacHattie, and C. A. Thomas, Jr.** 1968. The P22 bacteriophage DNA molecule. I. The mature form. *J. Mol. Biol.* **37**:21–40.
 34. **Robinson, A. J., H. B. Younghusband, and A. J. D. Bellet.** 1973. A circular DNA-protein complex from adenoviruses. *Virology* **56**:54–69.
 35. **Sakaki, Y.** 1974. Inactivation of the ATP-dependent DNase of *Escherichia coli* after infection with double-stranded DNA phages. *J. Virol.* **14**:1611–1612.
 36. **Sandri, R. M., and H. Berger.** 1980. Bacteriophage P1-mediated generalized transduction of *Escherichia coli*: structure of abortively transduced DNA. *Virology* **106**:30–40.
 37. **Sanger, D. V., D. J. Rowland, T. J. R. Harris, and F. Brown.** 1977. Protein covalently linked to foot-and-mouth disease virus. *Nature (London)* **268**:648–650.
 38. **Schaus, N. A., and A. Wright.** 1980. Inhibition of *Escherichia coli* exonuclease V by bacteriophage Mu. *Virology* **102**:214–217.
 39. **Schumm, J. W., and M. M. Howe.** 1980. Analysis of integration and excision of bacteriophage Mu: a new approach, p. 237–240. *In* D. Schlessinger (ed.), *Microbiology—1980*. American Society for Microbiology, Washington, D.C.
 40. **Sharp, P. A., C. Moore, and J. L. Haverty.** 1976. The infectivity of adenovirus 5 DNA-protein complex. *Virology* **75**:442–456.
 41. **Silverstein, J. L., and E. B. Goldberg.** 1976. T4 DNA injection. I. Growth cycle of a gene 2 mutant. *Virology* **72**:195–211.
 42. **Silverstein, J. L., and E. B. Goldberg.** 1976. T4 DNA injection. II. Protection of entering DNA from host exonuclease V. *Virology* **72**:212–223.
 43. **Stillman, B.** 1981. Adenovirus DNA replication *in vitro*: a protein linked to the 5' end of nascent DNA strands. *J. Virol.* **37**:139–147.
 44. **Studier, W. F.** 1965. Sedimentation studies of the size and shape of DNA. *J. Mol. Biol.* **11**:373–390.
 45. **Tanner, D., and M. Oishi.** 1971. The effect of bacteriophage T4 infection on an ATP dependent deoxyribonuclease in *Escherichia coli*. *Biochim. Biophys. Acta* **228**:767–769.
 46. **Toussaint, A., M. Faelen, and A. I. Bukhari.** 1977. Mu-mediated illegitimate recombination as a part of the Mu life cycle, p. 275–284. *In* A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), *DNA insertion elements, plasmids, and episomes*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 47. **Unger, R. C., and A. J. Clark.** 1973. Interaction of recombination pathways of bacteriophage lambda and its host *E. coli* K12: effects on exonuclease V activity. *J. Mol. Biol.* **70**:539–548.
 48. **van de Putte, P., M. Giphart-Gassler, T. Gosen, A. van Meetern, and C. Wijffelman.** 1978. Is integration essential for Mu replication, p. 33–40. *In* P. Hofschneider and P. Starlinger (ed.), *Integration and excision of DNA molecules*. Springer-Verlag, Berlin.
 49. **van de Putte, P., G. C. Westmaas, and C. Wijffelman.** 1977. Transfection with Mu DNA. *Virology* **81**:152–159.
 50. **van Vliet, F., and M. Couturier.** 1978. Mu-1 directed inhibition of DNA breakdown in *E. coli* rec A cells. *Mol. Gen. Genet.* **164**:109–112.
 51. **Wijffelman, C., M. Gassler, W. F. Stevens, and P. van de Putte.** 1974. On the control of transcription of bacteriophage Mu. *Mol. Gen. Genet.* **131**:85–96.
 52. **Williams, J. G. K., and C. H. Radding.** 1981. Partial purification and properties of an exonuclease inhibitor induced by bacteriophage Mu-1. *J. Virol.* **39**:548–558.
 53. **Yagil, E., and A. Rosner.** 1970. Effect of adenosine and deoxyadenosine on the incorporation and breakdown of thymidine in *Escherichia coli* K-12. *J. Bacteriol.* **103**:417–421.
 54. **Yanofsky, S., F. Kawamura, and J. Ito.** 1976. Thermolabile transfecting DNA from temperature-sensitive mutants of phage ϕ 29. *Nature (London)* **259**:60–63.
 55. **Yehle, C. O.** 1978. Genome-linked protein associated with the 5' termini of bacteriophage ϕ 29 DNA. *J. Virol.* **27**:776–783.