

Partial Purification and Properties of an Exonuclease Inhibitor Induced by Bacteriophage Mu-1

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From an induced lysogen of bacteriophage Mu-1, we partially purified a substance of high molecular weight that blocks the action of several exonucleases on double-stranded DNA. The presence of the inhibitor in cell-free extracts is dependent on induction of a Mu prophage. The Mu-related inhibitor acts by binding to double-stranded DNA rather than by interacting with the DNase. The inhibitor protects linear duplex DNA of Mu, P22, and ϕ X174*am3* from exonucleolytic degradation by recBC DNase and λ exonuclease. Single-stranded DNA, however, is not protected by the inhibitor from degradation by either recBC DNase or exonuclease I. The inhibitor preparation contains a protein that binds to linear duplex DNA, but not to circular duplex DNA; ends are required for binding to occur. Single-stranded DNA is not a substrate for the binding protein. These and other results suggest that the binding protein and the inhibitor are the same activity.

RecBC DNase of *Escherichia coli* K-12 is involved in cell viability, in DNA repair, and in genetic recombination (5). However, if not controlled, recBC DNase can have harmful effects on the cell and on the growth of bacteriophage. In *recA*⁻ mutants of *E. coli*, recBC DNase causes extensive degradation of chromosomal DNA (34). There is evidence that the *recA* gene product plays a direct role in the inhibition of recBC DNase in vivo (23), probably by binding to single-stranded DNA (J. G. K. Williams, T. Shibata, and C. M. Radding, *J. Biol. Chem.*, in press). Under certain conditions, recBC DNase can block the growth of phages λ and T4 (see below). Phages T2, T3, T4, T5, T6, T7, P1, ϕ 80, and λ all cause the inactivation of recBC DNase after infection of the host cell (2, 19, 22). In the case of phage λ , the λ *gam* gene product inhibits all three nuclease activities (10) of recBC DNase by forming a complex with the DNase (12). In a λ *gam*⁻ mutant, recBC DNase blocks the transition from the early "circle" mode of replication to the late "rolling circle" mode. Rolling circle replication generates concatamers of DNA (27) which are required for encapsidation (8). Since concatamers can also be formed by recombination between λ circles, the inhibition of recBC DNase by *gam* protein is not essential for phage growth except when recombination is blocked. Thus, λ *red*⁻ *gam*⁻ phage do not grow on a *recA*⁻ host (36).

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The induction of a *Mu*ts prophage in a *recA*⁻ cell stimulates the growth of λ *red*⁻ *gam*⁻ phage in this host. This suggests that Mu encodes a *gam*-like protein (30). The Mu activity has been termed *gam* and has been mapped to the region between genes *B* and *C* on the Mu genetic map (9).

In this communication, we report the partial purification and characterization of a Mu-related exonuclease inhibitor.

MATERIALS AND METHODS

Materials. Calf thymus DNA, cytochrome *c*, dithiothreitol (DTT), 2-mercaptoethanol, agarose, Brij-58, and ethidium bromide were purchased from Sigma Chemical Co. Pentex bovine serum albumin (BSA) was obtained from Miles Laboratories and was found to be free of endonuclease activity on double-stranded DNA. Ovalbumin was purchased from Worthington, ultrapure ammonium sulfate was from Schwarz/Mann Research Laboratories, Polymin P was from the Aldrich Chemical Co., Sephacryl S200 was from Pharmacia Fine Chemicals, phosphocellulose P11 and DEAE DE52 were from Whatman, hydroxylapatite HTP was from Bio-Rad Laboratories, and ATP was from PL Biochemicals. From New England Nuclear Corp., we purchased ³²P_O₄, [*methyl*-³H]thymidine, Econofluor, Liquifluor, and Triton X-100. Triton fluor is 140 ml of Liquifluor, 1,200 ml of Triton X-100, and 2,000 ml of toluene.

Bacteria, phage, and culture medium. All bacteria are derivatives of *Escherichia coli* K-12. BUHM8358 Δ (*pro-lac*) *his met* Str^r Mu^r/F^r *pro-lacZ*::(*Mu*ts62) was a gift from A. Bukhari. D110 *polA1 endI* (18) was obtained from C. Richardson.

D110(Mucts62) was constructed by the method of Bukhari and Ljungquist (3) from phage obtained by thermal induction (see below) of BUHM8358. We verified that D110(Mucts62) contains a Mu prophage by preparing phage DNA from a lysate of this strain as described below. The DNA was cleaved with restriction endonuclease *EcoRI* and analyzed by agarose gel electrophoresis. The phage DNA was cleaved into three fragments of sizes consistent with the positions of the *EcoRI* cleavage sites in Mu DNA (1). SK1447 *recA1 str31 leu-6 thr-1/pVK10* (31) was the gift of S. Kushner. H560 *polA endI thy* (32) was from A. Worcel. Phages P22, ϕ X174*am3*, and fd were from stocks in our laboratory. Phage M13*Gori1*, phage M13 which contains the cloned replication origin of phage G4 (11), was the gift of D. Ray.

Hershey broth contains, per liter, 8 g of Difco nutrient broth, 5 g of Difco peptone, and 5 g of NaCl, supplemented after autoclaving with 5 ml of 20% glucose (33).

Determination of acid-soluble DNA. Unless noted otherwise, the following procedure was used to measure acid-soluble DNA nucleotides. The sample was diluted to 200 μ l by the addition of 25 mM EDTA. Acid-soluble DNA was determined by mixing 150 μ l of each sample with 350 μ l of calf thymus DNA (1 mg/ml) and adding 500 μ l of 10% trichloroacetic acid. Incubation was at 0°C for 10 min. The precipitate was removed by centrifugation at 10,000 rpm for 5 min in a Sorvall SE-12 rotor at 4°C. The supernatant (800 μ l) was counted in 7 ml of Triton fluor. The amount of total DNA in each sample was determined by mixing, in this order, 30 μ l of sample, 370 μ l of water, 7 ml of Triton fluor, and 400 μ l of 10% trichloroacetic acid.

Agarose gel electrophoresis. For electrophoresis we used a vertical slab gel apparatus (17 by 16 by 0.3 cm; Blair Craft, Cold Spring Harbor, N. Y.). The running buffer was 40 mM Tris-acetate (pH 7.9)–5 mM Na acetate–1 mM EDTA. After electrophoresis, gels were stained in ethidium bromide (0.5 μ g/ml), and the DNA was visualized under UV light. Gels were photographed on Polaroid film (type 55 or type 57).

DNA substrates. DNA concentrations are given in moles of nucleotide residues. Abbreviations used for the DNA of phages ϕ X174*am3*, fd, and M13*Gori1* are as follows: form I is negatively superhelical DNA, form II is circular duplex DNA that contains one or more single-strand breaks, and form III is full-length linear duplex DNA.

Mu phage were prepared by thermal induction of BUHM8358 grown to a density of 10^8 cells per ml, as described by Bukhari and Ljungquist (3). For the preparation of radioactive DNA we used the Casamino Acids medium of Martuscelli et al. (16) without thymine. For labeling with 32 P, we added 32 PO₄ (10 μ Ci/ml) 5 min after induction. For labeling with 3 H, we added adenosine to a concentration of 0.3 mM (35) at the time of induction, followed by the addition of [methyl- 3 H]thymidine (5 μ Ci/ml) 5 min after induction. The lysate was clarified by centrifugation, and the phage were recovered by centrifugation at 22,000 rpm for 3 h in a Beckman SW27 rotor. The pellets were suspended in Mu buffer (100 mM NaCl, 20 mM MgCl₂, 10 mM Tris-hydrochloride [pH 7.5]), and the suspension was layered onto a CsCl step gradient (2-

ml layers each of CsCl at 1.3, 1.4, and 1.6 g/cm³ in 10 mM Tris-hydrochloride [pH 7.5], 40 mM MgCl₂, 5 mM EDTA). Centrifugation was at 22,000 rpm for 2.5 h in a Beckman SW27 rotor. The phage were recovered in the bottom 3 ml of the gradient, diluted to 30 ml with Mu buffer, and centrifuged through a second step gradient as described above. The phage were dialyzed against two 1-liter changes of 50 mM Tris-hydrochloride (pH 7.5)–20 mM MgCl₂–10 mM EDTA, and extracted three times at room temperature with phenol equilibrated in this buffer. The DNA was dialyzed for 24 h against three 1-liter changes of 10 mM Tris-hydrochloride (pH 7.5)–0.1 mM EDTA. One to two micromoles of DNA nucleotide was obtained from 250 ml of lysate.

Single-stranded DNA from phage fd and form I DNA of the phages ϕ X174*am3*, fd, and M13*Gori1* were prepared as described previously (6). A mixture of fd form II and form III DNA was prepared by the cleavage of fd form I DNA with S1 nuclease as described previously (7) and was a gift from R. Cunningham. ϕ X174*am3* form III [3 H]DNA with 5' single-stranded ends four nucleotides long was prepared by the cleavage of form I [3 H]DNA with restriction endonuclease *XhoI*. The reaction mixture (50 μ l) contained 100 μ M DNA, 150 mM NaCl, 6 mM MgCl₂, 8 mM Tris-hydrochloride (pH 7.5), 6 mM 2-mercaptoethanol, and 30 U of *XhoI* (Bethesda Research Laboratories). Incubation was at 37°C for 3 h. The DNA was extracted with phenol and dialyzed against 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA. ϕ X174*am3* form III [3 H]DNA with 3' single-stranded ends four nucleotides long was prepared by the cleavage of form I [3 H]DNA with restriction endonuclease *PstI* and was the gift of A. Sheyette-Weiss. M13*Gori1* form III [3 H]DNA with blunt ends was prepared by the cleavage of form I [3 H]DNA with restriction endonuclease *PvuII*. P22 [3 H]DNA was prepared as described previously (4). M13*Gori1* form III DNA and P22 DNA were the gifts of C. DasGupta.

Enzymes. RecBC DNase was purified 1,500-fold from *E. coli* H560 by a modification of the procedure of Goldmark and Linn (10). Assay conditions were 10 mM Tris-hydrochloride (pH 7.9), 10 mM 2-mercaptoethanol, 1 mg of BSA per ml, 10 mM MgSO₄, 0 or 220 μ M ATP, and 15 μ M Mu [32 P]DNA in a volume of 100 μ l. One unit of recBC DNase catalyzes the ATP-dependent conversion of 1 nmol of Mu DNA to acid-soluble nucleotides in 20 min at 37°C. The most pure fraction had a specific activity of 14,700 U/mg of protein. The enzyme preparation had ATP-dependent exonuclease activity on both single- and double-stranded linear DNA and ATP-stimulated endonuclease activity on single-stranded DNA, but did not have endonuclease activity on duplex DNA. These properties are characteristic of recBC DNase (10). Details of the purification and characterization of recBC DNase were as described by Williams (Ph.D. thesis, Yale University, New Haven, Conn., 1981). RecBC DNase was diluted in recBC diluent (10 mM Tris-hydrochloride [pH 7.5], 1 mM DTT, 1 mg of BSA per ml).

Exonuclease I was purified from the overproducing strain SK1447 by the procedure of Lehman and Nussbaum (14). Under their conditions, 1 U of exonuclease I degrades 10 nmol of single-stranded DNA to acid-

soluble nucleotides in 30 min at 37°C. The specific activity of the most pure fraction (hydroxylapatite step) was 339,000 U/mg of protein. The preparation of exonuclease I was able to degrade linear single-stranded DNA, but did not attack circular single-stranded DNA. Details of the purification and characterization of exonuclease I were as described by Williams (Ph.D. thesis).

λ exonuclease (phosphocellulose fraction α) was prepared and assayed as described by Radding (20) and was a gift from C. DasGupta. One unit of λ exonuclease degrades 10 nmol of *E. coli* DNA to acid-soluble nucleotides in 30 min at 37°C. The enzyme preparation had a specific activity of 20,000 U/ml.

Proteinase K was purchased from EM Laboratories, egg white lysozyme was from Worthington, restriction endonuclease *Xho*I was from Bethesda Research Laboratories, *Pst*I and *Pvu*II were from New England Biolabs, and both *Eco*RI and S1 nuclease were from Miles Laboratories.

Protein concentrations were determined by the method of Lowry et al. (15); we used BSA as the standard.

Purification of the Mu-related exonuclease inhibitor. The Mu-related exonuclease inhibitor was purified from thermally induced D110(Mucts62) (Table 1). All operations were carried out at 0 to 4°C and centrifugations were at 27,000 $\times g$ unless noted otherwise.

(i) **Inhibitor assay.** The inhibition of recBC exonuclease activity on Mu DNA was used to monitor inhibitor activity during purification. Reaction mixtures (100 μ l) contained 10 mM Tris-hydrochloride (pH 7.9), 10 mM 2-mercaptoethanol, 1 mg of BSA per ml, 10 mM MgSO₄, 0 or 220 μ M ATP, 1.5 nmol of Mu [³H]DNA, and 0.2 U of recBC DNase. Protein samples to be assayed were diluted in diluent G (10 mM Tris-hydrochloride [pH 7.9], 10 mM 2-mercaptoethanol, 1 mg of BSA per ml, 10 mM MgCl₂). Incubation was at 37°C for 30 min. Acid-soluble DNA was determined by adding to each sample 100 μ l of calf thymus DNA (1 mg/ml) and 200 μ l of 10% trichloroacetic acid, incubating at 0°C for 10 min, and centrifuging at 10,000 $\times g$ for 10 min at 4°C. The entire supernatant was counted in Triton fluor. The amount of acid-soluble DNA was calculated from the specific radioactivity of the DNA. One unit of inhibitor inhibits the ATP-dependent degradation of the DNA by 50%.

(ii) **Cultivation and storage of cells.** D110(Mucts62) was grown at 30°C in 1-liter batches of Hershey broth (supplemented with 10 μ g of thymine per ml) to a density of 2×10^8 cells per ml. The culture flasks were transferred to a 43°C water bath and shaken vigorously for 32 min. The cells were harvested at room temperature by centrifugation at 8,000 rpm in a Sorvall GS3 rotor, suspended with 1.2 ml of 50 mM Tris-hydrochloride (pH 7.5)-10% sucrose, frozen in liquid nitrogen, and stored at -70°C. Each 1-liter culture yielded 2.2 g (wet weight) of cell paste (before suspension in 50 mM Tris-hydrochloride [pH 7.5], 10% sucrose).

(iii) **Lysis.** Frozen cells (a volume of 73 ml containing 50 g of cell paste) were thawed at 0°C and then were mixed with 730 μ l of 1 M DTT, 730 μ l of 100 mM EDTA, and 8.1 ml of lysozyme (2 mg/ml). After in-

TABLE 1. Purification of the Mu-related exonuclease inhibitor

Fraction	Vol (ml)	Protein (mg)	U	U/mg
I. Polymin P supernatant	26	442	44,600	101
II. Gel filtration	10	208 ^a	26,600 ^a	128
III. DEAE	32	22	24,000	1,091
IV. Hydroxylapatite	7.9	5.4	21,400	3,963
V. Phosphocellulose	1.2	0.1 ^b	12,000	120,000 ^b

^a Only one-half of fraction I was taken for this and subsequent steps; to correct for this, we multiplied the experimental values by a factor of 2 to get the tabulated values.

^b Because the protein concentration in fraction V was low, this value, which is based on small differences between samples and blanks in the Lowry assay, is inaccurate.

cupation at 0°C for 30 min, we added to the cell suspension 5.33 ml of 3.5 M KCl and 4.67 ml of 8% Brij-58. Incubation was continued at 0°C for 30 min. The lysed cells were transferred to screw-cap polycarbonate tubes and centrifuged in a Beckman 45 Ti rotor at 35,000 rpm for 90 min. The supernatant (70 ml) was recovered.

(iv) **Polymin P.** To the 70-ml supernatant we added 2.54 ml of 10% Polymin P (pH 7.5) over a 15-min period; stirring was continued for 30 min. The precipitate was collected by centrifugation, and the supernatant (68 ml) (fraction S1) was saved. The precipitate was homogenized in 18 ml of 500 mM NaCl in buffer A (50 mM Tris-hydrochloride [pH 7.5], 1 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol), stirred for 60 min, and centrifuged. The supernatant (17 ml) (fraction S2) was saved. The concentration of protein was measured in both fractions S1 and S2. Solid ammonium sulfate was added to 60% saturation (390 mg/ml of supernatant) in a single step to fractions S1 and S2. After stirring for 30 min, the precipitates were collected by centrifugation, homogenized in 20 ml of 60% saturated ammonium sulfate in buffer A, and stored at 0°C.

As judged from the protein concentrations in fractions S1 and S2 measured before precipitation with ammonium sulfate, we removed from each fraction a volume that contained 50 mg of protein. The protein was recovered by centrifugation. The pellets were dissolved in 1 ml of buffer A that contained 0.5 mg of cytochrome *c*. To determine which fraction, S1 or S2, contained the Mu-related inhibitor, we fractionated each 1-ml protein solution by gel filtration on Sephacryl S200 (see Fig. 1 for gel filtration procedure). We used this procedure to identify the Mu-related inhibitor according to its elution position on the column. Fraction S1 (Fig. 1C) contained an inhibitor of recBC DNase which eluted at the position of the Mu-related inhibitor (see below and Fig. 1A and B). Fraction S2 did not contain this inhibitor (data not shown). To verify that the inhibitor in fraction S1 from induced D110(Mucts62) is related to Mu, we prepared fraction S1 from uninduced D110(Mucts62). This material was analyzed by chromatography on Sephacryl S200 as described above. Uninduced cells did not contain the inhibitor (Fig. 1D). Thus, the Mu-related inhibitor is not precipitated by Polymin P; it remains in the su-

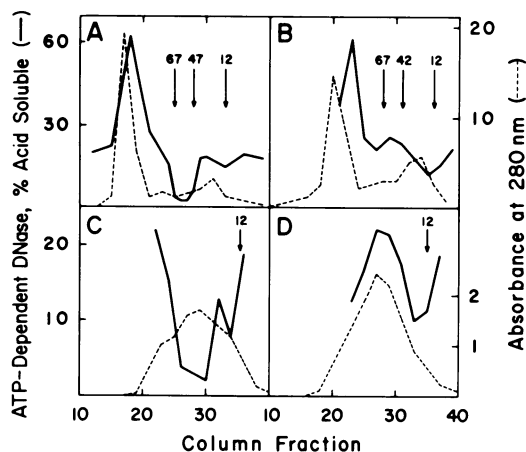


FIG. 1. Fractionation of crude extracts by gel filtration. A and B, Extracts of induced (A) and uninduced (B) D110(Mucts62) were prepared as described in the legend to Fig. 2. Protein was precipitated from each extract by adding ammonium sulfate to 70% saturation (472 mg of ammonium sulfate per 1 ml of extract) and stirring at 0°C for 1 h. The precipitate was recovered by centrifugation and dissolved in buffer A (see text) at a concentration of 50 mg of protein per ml. The protein concentration (50 mg/ml) was estimated from the protein concentration measured in the extract before precipitation with ammonium sulfate. One milliliter of each extract was fractionated on a column of Sephacryl S200 (1.6-cm diameter by 25-cm length) at a flow rate of 5 ml/h at 4°C. The elution buffer was 0.3 M ammonium sulfate in buffer A. Sixty fractions (1.0 ml) were collected. Samples (10 μ l) of selected fractions were assayed for inhibitor activity against recBC DNase as described in the text. Inhibitor activity was not apparent unless ammonium sulfate was first removed from the fractions by dialysis against buffer A. To estimate the size of the inhibitor, we determined the elution positions on the column of three proteins of known molecular weights. A solution (1 ml) of 2 mg of BSA (67,000 daltons), 2 mg of ovalbumin (42,000 daltons), and 0.5 mg of cytochrome c (12,000 daltons) was fractionated on the column as described above. These proteins were located in the column fractions by measuring absorbance at 280 nm (BSA and ovalbumin) or 410 nm (cytochrome c) (see arrows). C and D, Analysis of fraction S1 (see text) from induced (C) and uninduced (D) D110(Mucts62) by gel filtration. Crude extracts were treated with Polymin P as described in the text. The material not precipitated by Polymin P (fraction S1) was fractionated on Sephacryl S200 as described above for panels A and B.

pernatant (fraction I). The specific activity of the inhibitor in the ammonium sulfate suspension of fraction I was determined from a 200- μ l sample which was centrifuged and dissolved in 200 μ l of buffer A before assay.

(v) **Gel filtration.** One-half of the fraction I ammonium sulfate suspension, or 220 mg of protein, was centrifuged, and the precipitate was dissolved in 3 ml

of buffer A (see above) which contained 3 mg of cytochrome c as a marker for gel filtration. The sample was loaded onto a column of Sephacryl S200 (3.2-cm diameter by 35-cm length) that had been equilibrated with 0.3 M ammonium sulfate in buffer A. The flow rate was 2.5 ml/h. Eighty fractions (2.8 ml) were collected. The peak fractions of inhibitor activity were pooled, and the protein was precipitated by the addition of ammonium sulfate to 70% saturation (470 mg of ammonium sulfate per ml). The precipitate was held overnight at 0°C. The precipitate was recovered by centrifugation, washed once with 10 ml of 70% saturated ammonium sulfate in buffer B (20 mM potassium phosphate, 0.1 mM DTT, 1 mM EDTA, 10% glycerol [pH 6.8]), and was dissolved in 10 ml of buffer B (fraction II). The specific activity of the inhibitor was determined from a sample of fraction II which had been diluted twofold and dialyzed against buffer B for 1 h.

(vi) **DEAE.** Fraction II was diluted 2.5-fold with buffer B and dialyzed against this buffer until the conductivity was equal to that of buffer B containing 45 mM KCl. The sample was loaded onto a column of DE52 (1.6-cm diameter by 10.7-cm length) that had been equilibrated with buffer B. The flow rate was 16 ml/h. Fractions (5.3 ml) were collected while the column was washed with 30 ml of 40 mM KCl in buffer B. The pass-through fractions were pooled (fraction III). (A small-scale column had shown that the inhibitor does not adsorb to DE52 under these conditions.)

(vii) **Hydroxylapatite.** Fraction III was dialyzed against two changes (90 min each) of 1 liter of buffer D (20 mM potassium phosphate, 10 mM 2-mercaptoethanol, 10% glycerol [pH 6.8]) to give a conductivity equivalent to that of buffer D containing a final potassium phosphate concentration of 25 mM. The sample was loaded onto a hydroxylapatite column (1.6-cm diameter by 4.6-cm length) at a flow rate of 22 ml/h. The column was washed successively with 20-ml volumes of buffer D containing 20, 100, and 200 mM potassium phosphate. Fractions (2 ml) were collected. The inhibitor eluted with the 100 mM potassium phosphate wash. The peak fractions of inhibitor activity were pooled (fraction IV).

(viii) **Phosphocellulose.** To fraction IV we added EDTA to a final concentration of 0.1 mM. After fraction IV was dialyzed for 12 h against buffer E (10 mM potassium phosphate, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol [pH 6.8]), we loaded fraction IV onto a phosphocellulose column (1-cm diameter by 3.8-cm length) at a flow rate of 2.3 ml/h. The column was washed with 3 ml of buffer E that contained 50 mM KCl. A 34-ml gradient from 50 to 800 mM KCl was applied, and 60 fractions (570 μ l) were collected. Inhibitor activity eluted between 300 mM and 400 mM KCl. The peak fractions of inhibitor activity were pooled (3 ml), dialyzed for 12 h against 300 ml of 10 mM potassium phosphate, 1 mM DTT, 0.1 mM EDTA, 10% glycerol (pH 6.8) and concentrated to a volume of 1.2 ml by dialysis against dry Sephadex G-25 (fraction V). Fraction V was stored at 0°C.

RESULTS

RecBC DNase is inhibited by a cell-free extract of an induced Mu lysogen. To detect

a Mu-related inhibitor of recBC DNase, we incubated a cell-free extract from a thermally induced *Mu*cts62 lysogen with purified recBC DNase and double-stranded Mu [³H]DNA in the presence and absence of ATP. The ATP-dependent recBC DNase activity was inhibited by the extract (Fig. 2A). Inhibition was not observed with extracts from an uninduced *Mu*cts62 lysogen, a mock-induced nonlysogen, or an uninduced nonlysogen (Fig. 2B, C, and D); instead, these extracts contributed additional recBC DNase activity. Thus, induction of a Mu prophage seems to induce an inhibitor of the recBC exonuclease activity on double-stranded DNA.

We fractionated extracts of both induced and uninduced *Mu*cts62 lysogens by gel filtration on Sephacryl S200. To assay the column fractions for inhibitor activity, we mixed a sample of each fraction with purified recBC DNase and radioactive Mu DNA, in the presence and absence of ATP. The ATP-dependent DNase activity was measured (Fig. 1A and B). The purified recBC DNase alone made roughly 20% of the DNA acid soluble, as determined from samples containing column fractions that did not have detectable amounts of protein (i.e., fractions 12 and 39). In both extracts, an ATP-dependent DNase activity, probably recBC DNase, eluted just behind the peak of excluded material. However, only the extract of the induced lysogen contained a DNase inhibitor that eluted at a position corresponding to a molecular weight of between 40,000 and 80,000 (Fig. 1A).

Partial purification of the Mu-related inhibitor. The Mu-related inhibitor of recBC DNase was purified about 1,000-fold (Table 1). An extract of an uninduced lysogen, which was fractionated through the gel filtration step (fraction II), did not contain the fraction II exonuclease inhibitor (compare Fig. 1C and D). On this basis we have assumed that the inhibitor purified through subsequent steps is a Mu-related inhibitor. Fractions IV and V were tested for nuclease activity on linear and circular double-stranded DNA, and on linear single-stranded DNA. Ten units of inhibitor was mixed with 0.1 nmol of each substrate in the absence of ATP and incubated at 37°C for 30 min. Under these conditions, 1 U of inhibitor is sufficient to inhibit almost completely the action of recBC DNase on Mu DNA. The results (Table 2) show that fraction IV is free of detectable exonuclease activity on double-stranded DNA, but is contaminated with an exonuclease activity which degrades single-stranded DNA to acid-soluble nucleotides. Fraction IV also contains an endonuclease activity which converts ϕ X174 am 3 form I DNA to a nicked or broken form. Fraction V

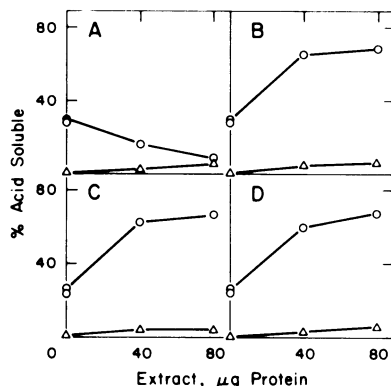


FIG. 2. Extract of an induced *Mu* lysogen inhibits the degradation of *Mu* DNA by recBC DNase. Purified recBC DNase was incubated with *Mu* [³²P]DNA and various amounts of cell-free extract from D110 or D110(*Mu*cts62) which had been either induced or not induced. The percentage of *Mu* DNA made acid soluble was measured. D110 and D110(*Mu*cts62) were grown and harvested as described in the text. Extracts were prepared by thawing 1 ml of cells at 0°C and adding 20 µl of 5 M NaCl and 50 µl of lysozyme (4 mg/ml). After incubating the cells in a Beckman polycarbonate centrifuge tube at 0°C for 30 min, we warmed the cells in a 37°C water bath for 2 min, chilled them on ice, and centrifuged them at 33,000 rpm at 3°C for 30 min in a Beckman 75 Ti rotor. The supernatant was dialyzed against 1 liter of 10 mM Tris-hydrochloride (pH 7.9)–10 mM 2-mercaptoethanol–50 mM KCl for 3 h at 0°C. The protein concentration in the dialyzed extract was measured by the method of Lowry *et al.* (see text). The ATP-dependent recBC DNase activity was measured as described in the text. Panels: A, induced D110(*Mu*cts62); B, uninduced D110(*Mu*cts62); C, mock-induced D110; D, uninduced D110. Symbols: (○) with ATP; (△) without ATP.

is free of exonuclease activity on single-stranded DNA, but contains the same amount of endonuclease activity on double-stranded DNA as fraction IV. Both fractions IV and V were used in the following experiments as indicated in each case.

Nuclease inhibition studies. To learn whether the inhibitor acts by binding to the DNA substrate or through a direct interaction with recBC DNase, we studied the dependence of inhibition on the concentrations of DNA and recBC DNase. When increasing amounts of inhibitor were added to reaction mixtures containing recBC DNase and four different concentrations of *Mu* DNA, the amount of inhibitor required to inhibit the DNase by half was proportional to the DNA concentration (Fig. 3). In contrast, when the DNA concentration was fixed and the recBC DNase concentration was varied, the amount of inhibitor required for half-inhi-

TABLE 2. Nuclease activities associated with inhibitor fractions IV and V^a

Inhibitor fraction	Substrate, % acid soluble		% Nicked or broken circles
	Single-stranded DNA	Double-stranded DNA	
No inhibitor	1.0	0.0	15
IV	11.4	0.1	61
V	1.3	0.1	56

^a Nuclease activity was measured on three different substrates: (i) single-stranded DNA fragments produced by heating fd phage [³H]DNA at 100°C for 10 min, (ii) linear double-stranded Mu [³H]DNA, and (iii) circular double-stranded ϕ X174am3 form I [³H]DNA. Reaction mixtures (20 μ l) contained 12 mM Tris-hydrochloride (pH 7.5), 100 μ g BSA per ml, 10 mM MgCl₂, 5 μ M DNA, and either no inhibitor (line 1), 10 U of fraction IV (line 2), or 10 U of fraction V (line 3). Incubation was at 37°C for 30 min. Exonuclease activity on single-stranded DNA and on Mu DNA was determined by measuring acid soluble DNA nucleotides as described in the text. Endonuclease activity on double-stranded DNA was determined by measuring the percent nicked or broken molecules of ϕ X174am3 DNA by the method of Kuhnlein et al. (13). In this procedure, we mixed the sample with 260 μ l of 25 mM EDTA. The total amount of DNA in each sample was determined by spotting a 50- μ l sample onto a nitrocellulose filter (Sartorius SM 11306, 0.45- μ m pore size). The amount of nicked DNA was determined by mixing a 200- μ l sample of each sample with 200 μ l of 0.3 M potassium phosphate (pH 12.3). Incubation was at room temperature for 5 min. The samples were neutralized with 150 μ l of 1 M potassium phosphate (pH 4.0) were mixed with 5 ml of 1.5 M NaCl and 150 mM sodium citrate at 0°C and were then filtered under suction through a nitrocellulose filter at a flow rate of 4 ml/10 sec. The filters were washed with 10 ml of 1.5 M NaCl and 150 mM sodium citrate, dried, and counted in Econofluor.

bition was not affected by the nuclease concentration (Fig. 4). These results suggest that the inhibitor binds to DNA and protects it from recBC DNase.

If the recBC DNase inhibitor binds to DNA, it should also inhibit other exonucleases. To test this idea, we mixed Mu DNA with various amounts of inhibitor in the absence of Mg²⁺. After a short incubation, we added λ exonuclease and Mg²⁺ to initiate digestion. The inhibitor blocked the action of λ exonuclease (Fig. 5, circular symbols). In a variation of this experiment, we first incubated Mu DNA with λ exonuclease in the absence of Mg²⁺. Under these conditions, λ exonuclease binds to DNA without degrading it (21). Then we added inhibitor and finally Mg²⁺ to initiate degradation. In this case, inhibition was weak (Fig. 5, triangular symbols). Since λ exonuclease acts processively (4), these results

suggest that the inhibitor binds to DNA; the binding of λ exonuclease to DNA appears to block the subsequent binding of the inhibitor.

The specificity of the inhibitor for the DNA substrate was tested by adding various amounts of inhibitor to reaction mixtures containing recBC DNase and both ³²P-labeled Mu DNA and ³H-labeled P22 DNA (Fig. 6). Both substrates were protected to the same extent by the inhibitor, showing that the inhibitor is not specific for Mu DNA.

The inhibitor might act by binding to ends or to internal sites on double-stranded DNA. To learn whether the structure of the ends affects inhibitor activity, we prepared ϕ X174am3 form III DNA with either 3' or 5' single-stranded ends by cleaving form I DNA at a single site with the restriction endonucleases *Pst*I (which yields 3' single-stranded ends four nucleotides long and *Xho*I (which yields 5' single-stranded ends four nucleotides long). When these substrates were incubated with recBC DNase and increasing amounts of inhibitor, both substrates were protected to a similar extent (Fig. 7A). Thus, the inhibitor appears to bind to double-stranded DNA with short 3' or 5' single-stranded ends.

To learn whether the inhibitor protects single-stranded DNA, we incubated increasing amounts of inhibitor with heat-denatured Mu DNA and either recBC DNase or exonuclease I (Fig. 7B). Neither of these nucleases was inhibited. A control showed that the inhibitor protected double-stranded DNA from recBC DNase. Thus, the inhibitor probably does not bind to single-stranded DNA.

Complexes between DNA and a protein component in the inhibitor preparation. To define better the interaction between DNA and the inhibitor, we analyzed complexes formed between the inhibitor and various DNA substrates by agarose gel electrophoresis. When increasing amounts of inhibitor were incubated with 1.5 nmol of Mu DNA, the electrophoretic mobility of the DNA gradually decreased as the inhibitor concentration increased (Fig. 8, lanes 1 through 6). Under these conditions, 2 U of inhibitor is sufficient to protect completely 1.5 nmol of Mu DNA from degradation by recBC DNase; the electrophoretic mobility of the DNA was not affected by less than 1.9 U of inhibitor. Incubation with proteinase K before gel electrophoresis restored the mobility of DNA previously treated with inhibitor to the mobility of untreated DNA (Fig. 8, compare lanes 1, 6, and 7). The inhibitor preparation appears to contain a protein which binds to Mu DNA.

When we examined the interaction between the binding protein and Mu DNA by a nitrocellulose filter assay (25) the amount of DNA re-

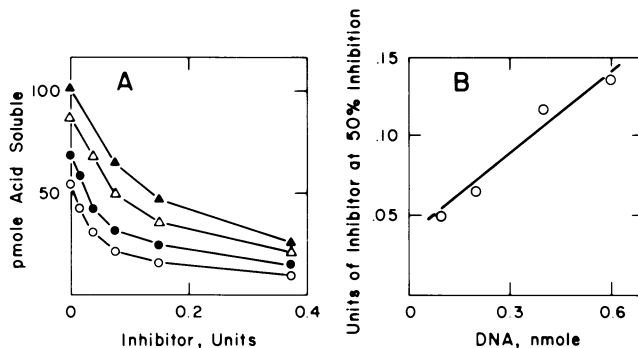


FIG. 3. The amount of inhibitor required to inhibit *recBC* DNase by 50% is related to the DNA concentration. A, Reaction mixtures (20 μ l) contained 17 mM Tris-hydrochloride (pH 7.5), 1.2 mM DTT, 1 mg of BSA per ml, 10 mM $MgCl_2$, 1 mM ATP, inhibitor (fraction V) as indicated, 0.07 U of *recBC* DNase, and (○) 0.1, (●) 0.2, (△) 0.4, or (▲) 0.6 nmol of Mu [3H]DNA. Incubation was at 37°C for 30 min. Acid-soluble DNA was measured as described in the text. B, The amount of inhibitor required to inhibit *recBC* DNase by 50% (determined from the data in panel A) plotted as a function of the amount of DNA.

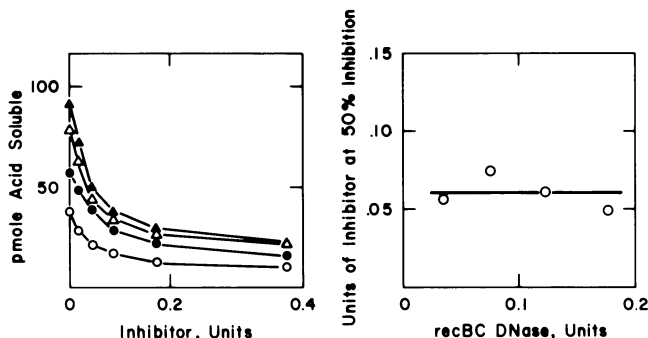


FIG. 4. The amount of inhibitor required to inhibit *recBC* DNase by 50% is independent of the amount of *recBC* DNase. A, Reaction mixtures (20 μ l) contained 17 mM Tris-hydrochloride (pH 7.5), 1.2 mM DTT, 1 mg of BSA per ml, 10 mM $MgCl_2$, 1 mM ATP, 0.1 nmol of Mu [3H]DNA, inhibitor (fraction V) as indicated, and (○) 0.035, (●) 0.075, (△) 0.123, or (▲) 0.176 U of *recBC* DNase. Incubation was at 37°C for 30 min. Acid-soluble DNA was measured as described in the text. B, The amount of inhibitor required to inhibit *recBC* DNase by 50% (determined from the data in panel A) plotted as a function of the amount of *recBC* DNase.

tained by the filter in the presence of inhibitor correlated well with the degree of inhibition of *recBC* DNase measured as described in the legend to Fig. 3. Inhibitor, at a ratio of 0.35 U/nmol of Mu DNA, caused retention of 66% of the DNA by the filter and inhibited 44% of the activity of *recBC* DNase; 3.8 U of inhibitor per nmol of DNA caused 93% retention and 82% inhibition.

When a mixture of phage fd form II and form III DNA was treated with increasing amounts of inhibitor, the migration of the faster-moving linear form III DNA was preferentially retarded (Fig. 8, lanes 8 through 13). This experiment shows that the binding protein binds to double-stranded DNA only if it has ends; circular DNA is not a substrate for binding.

To study the effect on binding activity of the structure at the end of the DNA molecule, we

incubated increasing amounts of inhibitor with phage M13Gori form III DNA with blunt ends, ϕ X174am3 form III DNA with 3' single-stranded ends four nucleotides long, and ϕ X174am3 form III DNA with 5' single-stranded ends four nucleotides long. The inhibitor reduced the electrophoretic mobility of all three substrates to a similar extent (not shown). Thus, a unique structure at an end is not required for binding to occur. This result is in agreement with experiments on nuclease inhibition in which substrates with both 3' and 5' single-stranded ends were protected by the inhibitor (see above).

To look for an activity that binds to single-stranded DNA, we incubated various amounts of inhibitor with fd phage DNA, which consisted of both circular and linear single-stranded DNA. ϕ X174am3 form III DNA with 3' single-stranded ends four nucleotides long was included in each

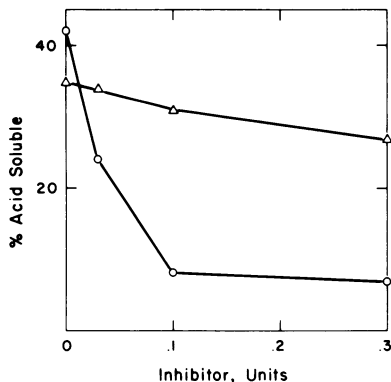


FIG. 5. Protection against λ exonuclease depends on the order of addition of the inhibitor to the reaction mixture. (○) Mu DNA was first incubated with inhibitor, then λ exonuclease was added. Reaction mixtures (14 μ l) contained 21 mM Tris-hydrochloride (pH 7.5), 2.1 mM DTT, 0.7 mM EDTA, 1 mg of BSA per ml, 0.1 nmol of Mu [3 H]DNA, and inhibitor (fraction V) as indicated. The samples were incubated at 37°C for 5 min and then chilled at 0°C for 3 min. Two units of λ exonuclease was added in 4 μ l of recBC diluent (see text), and digestion was initiated by the addition of 2 μ l of 25 mM MgCl₂. Incubation was at 37°C for 15 min. Acid-soluble DNA was measured as described in the text. (△) Mu DNA was first incubated with λ exonuclease, and then the inhibitor was added. Reaction mixtures (14 μ l) contained 21 mM Tris-hydrochloride (pH 7.5), 2.1 mM DTT, 0.7 mM EDTA, 1 mg of BSA per ml, 0.1 nmol of Mu [3 H]DNA, and 2 U of λ exonuclease. The samples were incubated at 37°C for 5 min and then chilled at 0°C for 3 min. Inhibitor (fraction V) was added as indicated in 4 μ l of recBC diluent, and digestion was initiated by the addition of 2 μ l of 25 mM MgCl₂. Incubation was at 37°C for 15 min. Acid-soluble DNA was measured as described in the text.

sample as a positive control. After incubation, the three DNA species were separated by agarose gel electrophoresis (Fig. 9). The single-stranded phage DNA was resolved into two bands (lane 6); the faster-migrating band is linear DNA whereas the slower band is circular DNA (17). The form III DNA (lane 7) migrated faster than did the single-stranded DNA. The inhibitor preparation preferentially retarded the migration of the form III DNA (lanes 1 through 5). Thus, the inhibitor preparation does not contain a prominent activity which binds to single-stranded DNA.

DISCUSSION

We have partially purified an exonuclease inhibitor from an induced Mucts62 lysogen. This inhibitor appears to be related to Mu, since (i) its presence in a crude extract is dependent on the induction of a Mucts62 prophage, and (ii) its

presence after completion of the first two purification steps, Polymin P fractionation and gel filtration, is also dependent on induction.

The amount of inhibitor required to inhibit by half the degradation of Mu DNA by recBC nuclease depended on the concentration of DNA, but not on the nuclease concentration. This result suggests that the inhibitor acts by binding to the DNA substrate rather than through a direct interaction with recBC DNase.

Mu DNA treated with the inhibitor preparation had a lower electrophoretic mobility in an agarose gel than did untreated DNA (Fig. 8). The electrophoretic mobility of Mu DNA previously treated with inhibitor was restored to that of untreated DNA by digestion with proteinase K, which indicates that a protein in the inhibitor preparation binds to DNA and retards its migration in a gel. An excess of inhibitor, greater than the amount required for complete protection, was needed to produce a detectable shift in mobility. Although we do not understand the reason for this, it might mean that many molecules of the binding protein can bind to a single DNA molecule.

Several lines of evidence suggest that the DNA binding protein and the inhibitor are the same activity. (i) The binding protein was not specific for Mu DNA; it also bound to double-stranded linear DNA of phages M13Gori1, fd (both substrates being blunt-ended), and ϕ X174am3 (with 3' or 5' single-stranded ends four nucleotides long). The inhibitor was simi-

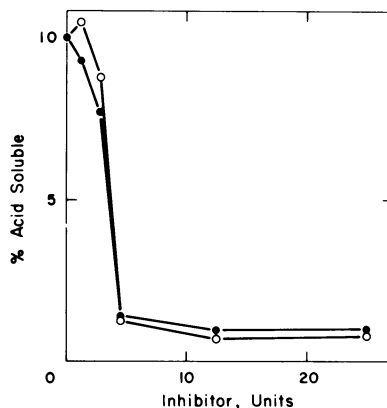


FIG. 6. The inhibitor protects both Mu DNA and P22 DNA from recBC DNase. Reaction mixtures (110 μ l) contained 12 mM Tris-hydrochloride (pH 7.9), 10 mM 2-mercaptoethanol, 1 mg of BSA per ml, 10 mM MgSO₄, 220 μ M ATP, 4 nmol of Mu [32 P]DNA (○) or 4 nmol of P22 [3 H]DNA (●), inhibitor (fraction IV) as indicated, and 1 U of recBC DNase. Incubation was at 37°C for 30 min. Acid-soluble DNA was measured as described in the text.

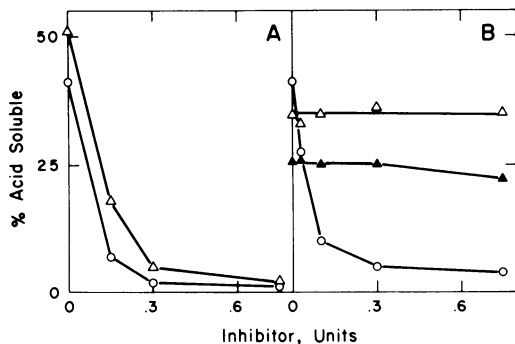


FIG. 7. A, The inhibitor protects double-stranded DNA with 3' or 5' single-stranded ends four nucleotides long. ϕ X174am3 form III [3 H]DNA was prepared by digesting form I DNA with restriction endonucleases (see text). Reaction mixtures (15 μ l) for measuring inhibition contained 16 mM Tris-hydrochloride (pH 7.5), 1.6 mM DTT, 1 mg of BSA per ml, 13 mM MgCl₂, 1.3 mM ATP, 0.1 nmol of form III DNA with either 3' (○) or 5' (△) single-stranded ends, and inhibitor (fraction V) as indicated. Incubation was at 37°C for 10 min. The samples were then chilled at 0°C for 3 min, and 0.08 U of recBC DNase in 5 μ l of recBC diluent (see text) was added to each sample. Incubation was at 37°C for 30 min. Acid-soluble DNA was measured as described in the text. B, The inhibitor does not protect single-stranded DNA from either recBC DNase or exonuclease I. Reaction mixtures contained 17 mM Tris-hydrochloride (pH 7.5) 1.2 mM DTT, 1 mg of BSA per ml, 10 mM MgCl₂, 1 mM ATP, 0.1 nmol of native Mu [3 H]DNA or heat-denatured Mu [3 H]DNA (heated at 90°C for 2 min in 10 mM Tris-hydrochloride [pH 7.5]-0.1 mM EDTA), inhibitor (fraction V) as indicated, and either recBC DNase (0.06 U) or exonuclease I (2 U). (An excess of exonuclease I was required because the conditions in this experiment were suboptimal for this enzyme.) Incubation was at 37°C for 30 min. Acid-soluble nucleotides were measured as described in the text. Symbols: (○) native Mu DNA plus recBC DNase; (△) single-stranded Mu DNA plus recBC DNase; (▲) single-stranded Mu DNA plus exonuclease I.

larly nonspecific. It protected double-stranded linear DNA of phages Mu, P22, and ϕ X174am3, the latter with 3' or 5' single-stranded ends four nucleotides long. (ii) The binding protein did not bind to circular or linear forms of single-stranded DNA. Similarly, the inhibitor failed to protect linear single-stranded DNA from either recBC DNase or exonuclease I. (iii) Although the binding protein bound to linear duplex DNA, it did not bind to circular duplex DNA. An end was required for binding to occur. Consistent with this finding is the failure to inhibit λ exonuclease when the latter bound to the DNA before the addition of inhibitor (Fig. 5). Under the conditions of this experiment, λ exonuclease binds only to single-stranded breaks and to the ends

of double-stranded DNA (21). Since λ exonuclease acts processively, this result suggests that the inhibitor may be unable to bind to DNA which has its ends blocked by λ exonuclease.

The requirement for ends may reflect a specific interaction between the binding protein and a structural feature at an end. This putative interaction would have to occur with blunt ends and with 3' and 5' single-stranded ends four nucleotides long, since binding occurred with all of these configurations. Alternatively, the requirement for ends may be topological. Binding of the protein to DNA could be analogous to threading beads on a string; the string (DNA) must be in a linear form to accept the beads (binding protein).

The Mu-related exonuclease inhibitor we have purified is clearly different from the *gam* protein of phage λ . The latter inhibits recBC DNase by interacting directly with the DNase, whereas the Mu-related inhibitor binds to the DNA substrate. In spite of this difference, it is possible that the inhibitor we have purified could functionally complement a defective λ *gam* protein in vivo; the Mu-related inhibitor could be the Mu *gam* activity (see above).

The inhibitor could also be related to other

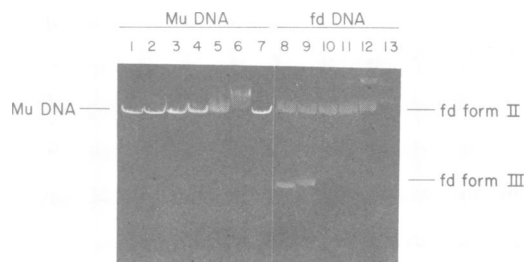


FIG. 8. A protein in the inhibitor preparation forms complexes with linear double-stranded DNA. Lanes 1 through 7, Mu DNA. Increasing amounts of inhibitor (0, 0.63, 1.3, 1.9, 3.8, 6.3, and 6.3 U of fraction IV in lanes 1 through 7, respectively) were added to reaction mixtures (40 μ l) that contained 10 mM Tris-hydrochloride (pH 7.5), 0.1 mM DTT, 1 mM EDTA, and 1.5 nmol of Mu DNA. After incubating the samples at 37°C for 5 min, we added 4.5 μ l of proteinase K (2.75 mg/ml) to one sample (lane 7). Incubation was continued at 37°C for 15 min. The samples were analyzed by electrophoresis in a 1.4% agarose gel at 7 V/cm for 4 h. The gel was stained in ethidium bromide and photographed (see text). Lanes 8 through 13, Circular duplex DNA is not a substrate for the binding protein. Increasing amounts of inhibitor (0, 1.3, 3.1, 6.3, 13, and 25 U of fraction IV in lanes 8 through 13, respectively) were incubated with a mixture of phage fd form II and form III DNA (0.75 nmol total) at 37°C for 5 min. Incubation and electrophoresis conditions were as described above for lanes 1 through 6. Proteinase K was not used in this experiment.

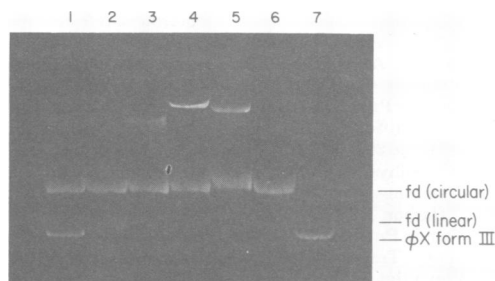


FIG. 9. The DNA binding protein does not bind to single-stranded DNA. Reaction mixtures (40 μ l) contained 10 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA, 0.2 nmol of ϕ X174am3 form III DNA (with 3' single-stranded ends four nucleotides long, see text), 1.5 nmol of fd single-stranded phage DNA, and 0, 0.6, 1.5, 3.0, and 6.0 U of inhibitor (fraction V) in lanes 1 through 5, respectively. Lane 6 contains fd DNA only and lane 7 contains ϕ X174am3 form III DNA only. Incubation was at 37°C for 5 min. The samples were analyzed by electrophoresis in a 1.8% agarose gel at 6.5 V/cm for 4.5 h. The gel was stained with ethidium bromide and photographed (see text).

functions ascribed to Mu. (i) The induction of a Mu_{cts} prophage has been shown to stimulate the growth of a T4 gene 2 amber mutant (24). Growth of the T4 mutant in an amber suppressor host yields normal virions, whereas growth in a nonsuppressor host yields defective virions (T4 2.Su⁻) which plate with reduced efficiency on a *rec*⁺ host, but with normal efficiency on a *recB*⁻ host. Silverstein and Goldberg (26) have suggested that T4 2.Su⁻ virions lack a DNA binding protein which protects phage DNA injected into the cell from recBC DNase. The Mu gene that complements T4 2.Su⁻ phage appears to map between genes *B* and *C*, since the complementing activity is not produced by a Mu_{cts}62 pf7701 prophage (24). The latter has a deletion of 2,800 base pairs between the *B* and *C* genes (Thomas and Howe, personal communication). (ii) Van Vliet et al. (30) and Schaus and Wright (24) have shown that a cell-free extract of an induced Mu_{cts} lysogen contains low recBC DNase activity. Normal recBC DNase activity is present in an extract of an induced Mu_{cts}62 pf7701 lysogen, which suggests that a gene coding for a recBC DNase inhibitor maps between genes *B* and *C* (see above). (iii) Another function, *sot*, which stimulates the transfection of Mu DNA, also maps between genes *B* and *C* (28). Since transfection is stimulated by *sot* in cells that lack an active recBC DNase (29), the effect of *sot* may not be related to the specific inhibition of recBC DNase. (iv) Chase and Benzinger (personal communication) have extracted from Mu virions a DNA-protein complex which is far more efficient in transfection than Mu DNA alone. The major

protein in this complex is a polypeptide of 65,000 daltons. The enhancement of transfection efficiency by the complex is greater in a *rec*⁺ host than in a *recB*⁻ host. These results suggest that the 65,000-dalton protein may protect the Mu DNA from degradation by recBC DNase.

Additional experiments are required to define the relationships between the exonuclease inhibitor purified by us, the Mu *gam* and *sot* functions, the Mu activity that complements T4 2.Su⁻ phage, and the 65,000-dalton Mu virion protein that stimulates transfection of Mu DNA.

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