

## Postinfection Control by Bacteriophage T4 of *Escherichia coli* *recBC* Nuclease Activity

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Received for publication 6 October 1975

Infection by bacteriophage T4 has previously been shown to cause a rapid inhibition of the host *recBC* DNase, an ATP-dependent DNase that is required for genetic recombination in *Escherichia coli*. We report here the partial purification of a protein ("T4 *rec* inhibitor") from extracts of T4-infected cells and some characteristics of the *in vitro* inhibition reaction with purified inhibitor and *recBC* nuclease. This inhibitory activity could not be purified from extracts of uninfected *E. coli*. Both the ATP-dependent exonuclease and DNA-dependent ATPase activities of *recBC* DNase are inhibited by T4 *rec* inhibitor. Experiments suggest that the inhibitor interacts with the nuclease in a stoichiometric manner. The biological significance of this inhibition is discussed with respect to control reactions in phage-infected cells.

Bacteriophage development is dependent on a complex interaction between host and phage systems of replication, translation, and transcription. In this work we are dealing with a control mechanism involving a host enzyme, namely *Escherichia coli recBC* DNase, and its interaction with an inhibitory substance induced by bacteriophage T4 infection (16, 18). The ATP-dependent nuclease called *recBC* DNase (exonuclease V), which is controlled by the *recB* and *recC* genes of *E. coli* (2, 15, 23), is involved in the major pathway of genetic recombination in the bacterium. Tomizawa and Ogawa (19) have shown that *recB* and *recC* genes code for *recBC* nuclease. This nuclease is similarly inhibited after phage  $\lambda$  infection (20), and in this case the inhibition has been attributed to  $\gamma$  protein, which is specified by the *gam* gene. The presence of the  $\gamma$  protein is essential for  $\lambda$  growth on certain specific hosts; *gam*<sup>-</sup> mutants fail to grow on *polA*<sup>-</sup> *E. coli*, and *gam*<sup>-</sup>*red*<sup>-</sup> phage grow poorly in *recA*<sup>-</sup>*recB*<sup>+</sup>*recC*<sup>+</sup> strains (24). This report presents evidence that a protein inhibitor of *recBC* nuclease is induced likewise after T4 infection. The partial purification of the inhibitory protein designated as "T4 *rec* inhibitor" is described, and characteristics of the inhibition reaction have been studied.

### MATERIALS AND METHODS

**Bacteriophage and bacteria.** *E. coli* K-12 strains AB1157 and AB2470 (*recB21*) were obtained from the Coli Genetic Stock Center, Yale University. *E. coli* K-12 strain W3110 (*thy*<sup>-</sup>) was received from J. Cairns. Bacteriophage T4D is a laboratory stock originally from R. S. Edgar.

**Reagents.** ATP, 2-mercaptoethanol, dithiothreitol, and glutathione were from Calbiochem. Sephadex G100 and Dextran T500 came from Pharmacia. Polyethylene glycol (Carbowax 6000) was purchased from Union Carbide. DEAE-cellulose (DE-52) is a Whatman product. New England Nuclear was the supplier of [8-<sup>14</sup>C]ATP (specific activity, 52.15 mCi/mmol) and [methyl-<sup>3</sup>H]thymidine (20.0 Ci/mmol). Bovine pancreas trypsin (EC 3.4.4.4) and soybean trypsin inhibitor were obtained from Worthington. All other chemicals were reagent grade.

**Preparation of DNA.** <sup>3</sup>H-labeled bacteriophage T4 DNA (7,510 counts/min per nmol of nucleoside) was prepared as follows. *E. coli* W3110 (*thy*<sup>-</sup>) was grown to  $8 \times 10^8$  cells/ml in M9 medium (11) supplemented with 0.025% Casamino Acids, 20  $\mu$ g of thymidine per ml, and 0.001% gelatin. Harvested cells were suspended at  $6 \times 10^8$  cells/ml in fresh M9 medium supplemented as above, except with 5  $\mu$ g of thymidine and 2  $\mu$ Ci of [<sup>3</sup>H]thymidine per ml. After aeration at 37 C for 30 min, bacteriophage T4D was added at a multiplicity of 5 followed in 5 min by a second addition of an equal amount of T4D. Aeration at 37 C was continued for 6 to 8 h when lysis was complete. Labeled phage were purified by differential centrifugation, and DNA was prepared by two phenol extractions of the phage. Alkaline and neutral sucrose gradient analyses indicated that the <sup>3</sup>H-labeled T4 DNA was somewhat heterogeneous in size and contained approximately one double-strand cut per molecule. <sup>3</sup>H-labeled *E. coli* DNA (864 counts/min per nmol) was prepared by the method of Miura (12).

**Preparation and assay of *recBC* DNase.** The *recBC* DNase used to monitor the activity of *rec* inhibitor was made in a manner similar to that of Nobrega et al. (14). All steps were performed at 0 to 4 C. Freshly harvested cells (45 g) of *E. coli* AB1157 were disrupted by sonic treatment (four 20-s periods) with a W140 Branson sonifier at 60 W, and the

preparation was centrifuged at  $27,000 \times g$  for 3 h. The optical density of the supernatant at 260 nm was adjusted to 200 with TMS (0.01 M Tris-hydrochloride [pH 7.8]-0.01 M  $MgCl_2$ -5 mM 2-mercaptoethanol-0.2 mM EDTA) (14), and then 2% protamine sulfate neutralized with KOH was added to a final concentration of 0.54%. The mixture was pelleted, suspended in 10 ml of 0.05 M  $K_2HPO_4$ -10 mM mercaptoethanol-0.1 mM EDTA-10% glycerol buffer, and pelleted. The active fraction was extracted twice with 10 ml of 0.25 M  $K_2HPO_4$  with additions as noted. Solid ammonium sulphate (25.8 g/100 ml) was slowly added, and the precipitate was centrifuged out 30 min later. The pellet was dissolved in 5 ml of 20 mM Tris-hydrochloride (pH 7.5) containing 30% glycerol, 10 mM mercaptoethanol, and 0.1 mM EDTA and dialyzed against the same buffer overnight. The sample was applied to a 2.5 by 14 cm column of DEAE-cellulose equilibrated with 10 mM Tris-hydrochloride (pH 7.8)-10 mM mercaptoethanol-0.1 mM EDTA-10% glycerol and eluted with a concave 0 to 1 M NaCl gradient (total volume, 600 ml) over 12 h. Active fractions were pooled and concentrated with an Amicon UM-10 filter. The activity of the nuclease preparations was typically around 600 U/mg of protein, where 1 U is defined as the amount required to produce 1 nmol of acid-soluble T4 DNA nucleotide in 20 min at 37 C. This definition is based on enzyme activity with T4 DNA as a substrate and may not be directly comparable with units defined using *E. coli* DNA (14, 15). *recBC* nuclease activity was assayed according to Nobrega et al. (14) using 30 nmol of  $^3H$ -labeled T4 DNA per ml; under these assay conditions, nuclease activity varied linearly with the amount of *recBC* nuclease added. The acid-soluble counts were determined by transferring an aliquot of the trichloroacetic acid supernatant to Aquasol (New England Nuclear) and counting with a Nuclear Chicago Isocap 300. The DNA-dependent ATPase activity of the *recBC* enzyme was assayed using [ $^3H$ ]ATP and analyzed for ADP production by thin-layer chromatography (4). Protein determinations were done according to Lowry et al. (10) after trichloroacetic acid precipitation to remove thiol compounds.

**Preparation of bacteriophage T4-infected *E. coli*.** M9 medium (11) supplemented with 0.05% Casamino Acids and 0.25% yeast extract (Difco) was used for preparing infected cells. *E. coli* AB2470 were grown at 37 C to a cell density of  $10^9$ /ml, harvested, and suspended in 1/2 volume of 37 C M9 medium supplemented with 0.05% Casamino Acids, 0.025% yeast extract, and 5  $\mu$ g of tryptophan per ml. After aeration for 7 min, T4D was added at a multiplicity of 5, and the culture was gently agitated for 2 min. After addition of an equal volume of 37 C medium and raising the yeast extract concentration to 0.25%, the cells were vigorously aerated for 8 min and then rapidly cooled in the presence of 100  $\mu$ g of chloramphenicol per ml. Harvested cells were suspended in 1/50 volume of 0.05 M Tris-hydrochloride (pH 7.8) containing 20% sucrose and 1 mM EDTA. The pelleted cells were frozen in dry ice-ethanol and stored up to 2 weeks. Only those cell preparations that were infected 90% or more on the basis of bacterial

survival were used for cell extracts. Uninfected bacteria were prepared in an identical manner with the omission of T4D.

**Preparation of T4 *rec* inhibitor.** All steps were carried out at 0 to 4 C. Thawed T4-infected cells (3 g) were suspended in 10 ml of 0.2 M Tris-hydrochloride (pH 8.0) containing 5 M NaCl, 2 mM glutathione, and 10 mM  $MgCl_2$  after Alberts (1), crushed three times in a French pressure cell at 12,000 lb/in<sup>2</sup>, and centrifuged at  $27,000 \times g$  for 2 h. The supernatant (fraction I, Table 1) was treated with polyethylene glycol and dextran for phase extraction as described by Alberts (1). The top phase was dialyzed against 10 mM Tris-hydrochloride (pH 7.5) containing 10 mM mercaptoethanol and 10% glycerol (fraction II) and then applied to a 1 by 10 cm DEAE-cellulose column equilibrated with the same buffer. Fractions (9 ml) of increasing NaCl concentration were used to develop the column and subsequently concentrated against 0.02 M Tris-hydrochloride (pH 7.6) containing 10 mM mercaptoethanol, 0.5 mM EDTA, 20% glycerol, and 25% polyethylene glycol and then suspended in 1 ml of 0.05 M Tris-hydrochloride (pH 7.6)-10 mM mercaptoethanol-1 mg of albumin per ml-20% glycerol. The inhibitor activity of these fractions for a typical preparation is shown in Fig. 1. A similar preparation of inhibitor which was more concentrated was used for the data presented in Fig. 2 and 3. The T4 *rec* inhibitor eluted by 0.3 M NaCl from DEAE-cellulose (fraction III, Table 1) was stable after concentration for at least 3 weeks when stored at -90 C in 30% glycerol.

**Assay of T4 *rec* inhibitor.** Inhibitor activity was determined by assaying for remaining *recBC* nuclease activity after preincubation of inhibitor and nuclease. Appropriate amounts of *recBC* nuclease and T4 *rec* inhibitor were mixed at 0 C in 0.09 ml of 0.05 M Tris-hydrochloride (pH 7.8)-0.01 M dithiothreitol-0.02 M  $MgCl_2$ -1 mg of bovine serum albumin per ml-20% glycerol. An aliquot (usually 10  $\mu$ l) was removed for the 0-min assay immediately before placing the preincubation mixtures in a 30 C water bath. Aliquots were removed at various times, cooled in ice, and diluted 1/10 in 0.05 M Tris (pH 7.8)-0.01 M mercaptoethanol-1 mg of albumin per ml-20% glycerol and assayed for *recBC* nuclease

TABLE 1. Purification of T4 *rec* inhibitor<sup>a</sup>

Fraction	Protein (mg)	Inhibitor units	Sp act (units/mg of protein)
I. Cell extract	390	- <sup>b</sup>	-
II. Polyethylene glycol-dextran phase extract	250	8,600	35
III. DEAE-cellulose	1.5	1,770	1,180

<sup>a</sup> Assays for *rec* inhibitor were done as described in Materials and Methods. The activity in fraction II is a rough estimate, since high levels of ATP-independent nucleases make accurate determinations difficult.

<sup>b</sup> Because of the presence of nonspecific inhibitory substances in crude extracts coupled with low levels of T4-specific *rec* inhibitor, activity units for fraction I are not meaningful.

activity. The 0-min samples were treated in an identical manner. One unit of T4 *rec* inhibitor is defined as the amount required to inhibit one unit of *recBC* nuclease after 30 min of preincubation at 30 C. Control reactions omitting ATP were assayed concurrently to correct for nucleases not dependent on ATP.

**Trypsin treatment of *rec* inhibitor.** Inhibitor (98 units) was incubated with 0.5 pmol of trypsin in 0.05 M Tris-hydrochloride (pH 8.0)-0.01 M CaCl<sub>2</sub> for 5 min at 23 C. One half of this mixture was removed to a tube containing 0.5 pmol of soybean trypsin inhibitor and sampled 5 min later for *rec* inhibitor activity, which was assayed as above.

**Sephadex chromatography of *rec* inhibitor.** Purified *rec* inhibitor (2,500 units of fraction III, Table 1) was applied to a 0.9 by 30 cm column of Sephadex G100 equilibrated with 0.05 M Tris-hydrochloride (pH 7.6) containing 10 mM mercaptoethanol and 20% glycerol. The column was standardized with bovine serum albumin and cytochrome *c* that were detected spectrophotometrically. *rec* inhibitor was detected by assaying fractions for inhibitory activity as described above.

## RESULTS

To investigate the inhibition of *recBC* DNase observed after T4 infection (18), preliminary experiments were done with crude extracts of T4-infected *recB*<sup>-</sup> (AB2470) cells. The use of *recB*<sup>-</sup> cells eliminates the presence of endogenous *recBC* nuclease, allowing the study of effects on the defined amount of added nuclease. In several tests a weak inhibitory activity was observed in extracts from T4-infected cells but not from uninfected bacteria. Figure 1 shows the remaining nuclease activity after incubation with DEAE-cellulose fractions from infected and uninfected cells. The experiment shown is representative of three independent purifications where infected and uninfected bacteria were treated in parallel. These results indicate that bacteriophage T4 induces the inhibitory activity.

The activity of T4 *rec* inhibitor was destroyed by preincubation with trypsin as described above. Trypsin-treated inhibitor produced only 7% inhibition, whereas a similarly treated control without trypsin gave 100% inhibition. Thus the inhibitor is probably protein in nature. Consistent with this is the chromatographic behavior of the inhibitor on DEAE-cellulose (Fig. 1). The *rec* inhibitor is nondialyzable and has an estimated molecular weight of 12,000 determined in a preliminary experiment using Sephadex G100 as described above. The inhibitor is very heat stable and is approximately 50% inactivated by 5 min of incubation at 100 C. Also, as noted above, the isolated inhibitor is stable on prolonged storage at -90 C.

*recBC* DNase possesses several activities;

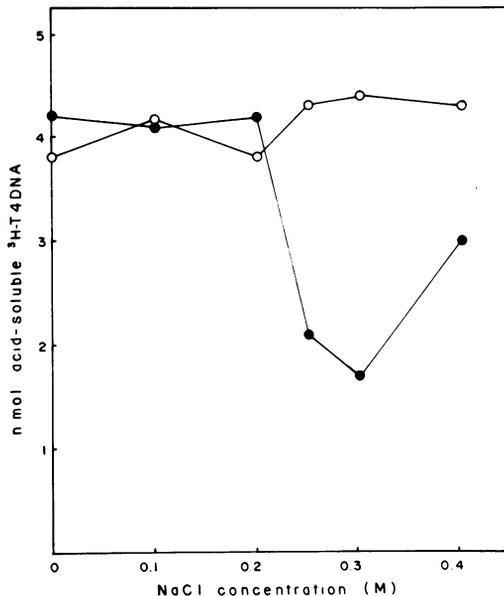


FIG. 1. Elution of *rec* inhibitor from DEAE-cellulose by fractions of increasing NaCl concentration. *recBC* nuclease activity was determined after incubation with DEAE-cellulose fractions from T4-infected (●) and from uninfected cell extracts (○). Conditions for elution of the DEAE-cellulose column and assay of inhibitory activity are given in Materials and Methods. For each incubation, 99 units of *recBC* DNase and 0.03 ml of the concentrated fractions were held at 30 C for 30 min in a total volume of 0.09 ml. Activities shown are those determined for *recBC* nuclease assay (0.5-ml reaction volume, 15 nmol of <sup>3</sup>H-labeled T4 DNA) of diluted enzyme-inhibitor mixtures corrected for ATP-independent nuclease activity.

inhibition of the double-strand exonuclease activity was used for purifying the inhibitor and for the experiments shown in Fig. 2 and 3. In the presence of sufficient inhibitor, the nuclease reaction was completely abolished. Figure 2 shows the time course of inhibition with varying amounts of inhibitor and is representative of five similar experiments. The degree of reduction in nuclease activity reaches a level roughly proportional to the amount of inhibitor for each case. In the presence of inhibitor, the nuclease activity appears to be inhibited even at 0 min; the inhibition reaction may proceed at significant velocity during the removal of the initial sample or during the subsequent nuclease assay, especially at the higher concentrations of inhibitor. Notably, 10 μl of inhibitor produced approximately 95% inhibition after 30 min of preincubation. These results were reproducible, and the addition of larger amounts of inhibitor (Fig. 3d) routinely produced 100% inhibition of nuclease activity. To further investi-

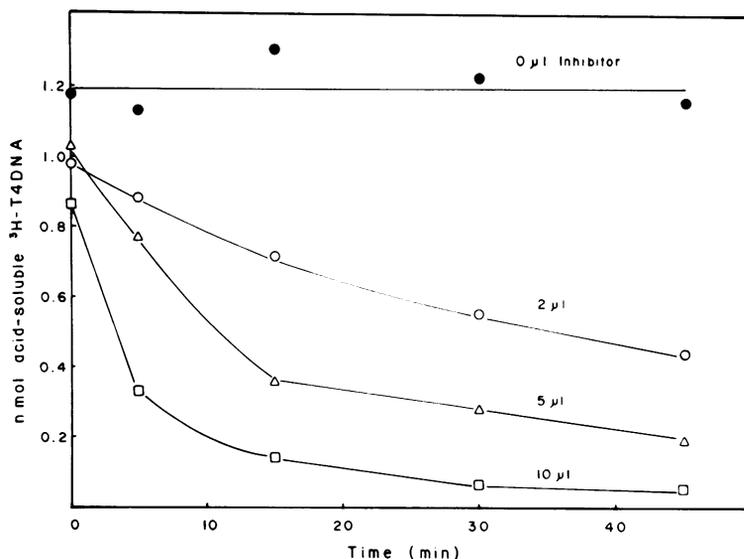


FIG. 2. Reduction of *recBC* DNase activity in the presence of varying amounts of *rec* inhibitor. Times indicated are for minutes of preincubation of nuclease with inhibitor before being sampled for nuclease activity. Aliquots were diluted and assayed for *recBC* nuclease activity using 7.5 nmol of  $^3\text{H}$ -labeled T4 DNA in a reaction volume of 0.25 ml. Each preincubation mixture contained 32 units of *recBC* nuclease and indicated volumes of inhibitor. The inhibitor preparation used is the same as that used for the experiment shown in Fig. 3 and had a specific activity of 840 units/mg of protein and contained 2.5 mg of protein per ml.

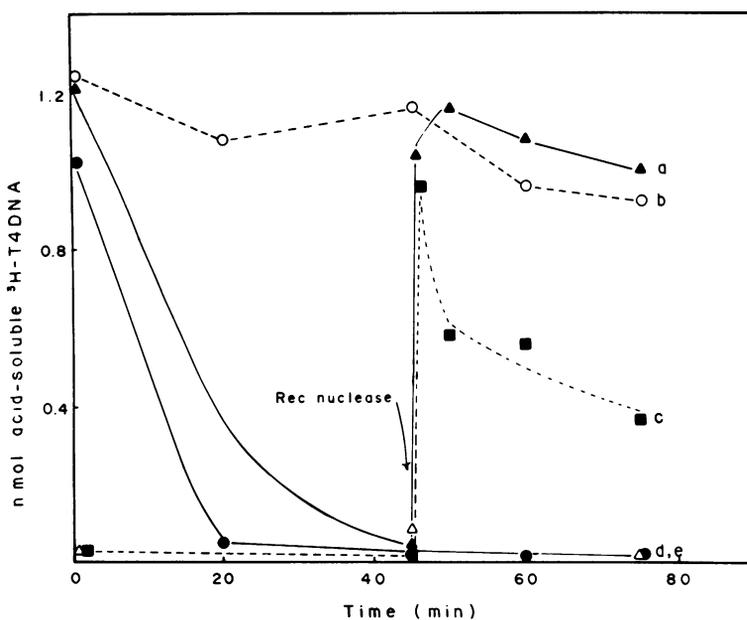


FIG. 3. Noncatalytic interaction of the T4 *rec* inhibitor with *recBC* DNase. Activities are for individual nuclease assays (0.25 ml, 7.5 nmol of  $^3\text{H}$ -labeled T4 DNA) after dilution of aliquots from the incubation mixtures at times of preincubation shown. Each inhibition reaction contained 32 units of nuclease and/or 32 units of inhibitor identical to that used for Fig. 2. Symbols: (a) ▲, *recBC* DNase and *rec* inhibitor with the addition of 32 units of *recBC* DNase at 45 min; (b) ○, *recBC* DNase; (c) ■, *rec* inhibitor with the addition of 32 units of *recBC* DNase at 45 min; (d) △, *rec* inhibitor; (e) ●, *recBC* DNase and *rec* inhibitor. All five conditions were studied as simultaneously as possible with a staggered time protocol.

gate the interaction between inhibitor and nuclease, completely inhibited incubation mixtures of nuclease and inhibitor were challenged with additional nuclease (Fig. 3a). The activity of the *recBC* nuclease added at 45 min was not significantly reduced in comparison to the almost immediate inhibition by unreacted inhibitor shown in Fig. 3c. The control reaction with nuclease alone (Fig. 3b) shows an expected gradual decrease in nuclease activity over 75 min of incubation at 30 C; incubation of *recBC* DNase with inhibitor omitting the addition of nuclease at 45 min indicates that inhibition was essentially complete for these conditions by 20 min (Fig. 3e), and the incubation of inhibitor alone (Fig. 3d) demonstrates the absence of ATP-dependent DNase in the inhibitor preparations. This noncatalytic behavior of *rec* inhibitor was observed in four other similar experiments.

Preliminary experiments to investigate other characteristics of the inhibition reaction produced the following observations (data not shown). Addition of  $5 \times 10^{-5}$  to  $2 \times 10^{-4}$  M ATP to the preincubation mixtures did not enhance the inhibition in tests where *rec* inhibitor concentration was adjusted to give incomplete reduction of nuclease activity; also, complete inhibition of nuclease activity was observed when higher concentrations of inhibitor were present (as in Fig. 3e) without concomitant addition of ATP. Similar levels of inhibitor activity were observed over the pH range 7.2 to 8.8. The purified *rec* inhibitor did not affect the activity of *E. coli* alkaline phosphatase (EC 3.1.3.1) with *p*-nitrophenyl phosphate, suggesting that the inhibitor is not a protease.

Since the *recBC* DNase has several biochemical activities, it is of interest to compare the effect of the T4-induced *rec* inhibitor on each of them. *recBC* nuclease activities with *E. coli* DNA and T4 DNA were decreased to approximately the same level (Table 2); the almost complete inhibition of ATP-dependent single-strand exonuclease activity is an approximation since the inhibitor has a high level of non-specific single-strand exonuclease activity; parallel inhibition was also seen for nuclease and DNA-dependent ATPase activities. The latter observation is somewhat subject to criticism since the inhibitor preparation is contaminated by a significant level of ATPase. However, sufficient ATP was present in the assays to ensure that inhibition was not caused by the trivial mode of depletion of the enzyme substrate. More rigorous studies on the inhibition of the various *recBC* functions must await further purification of the inhibitor.

TABLE 2. Effect of *rec* inhibitor on activities catalyzed by *recBC* DNase<sup>a</sup>

Expt no.	<i>recBC</i> activity-substrate	% Inhibition
1	Double-strand exonuclease-T4 DNA	52
	Double-strand exonuclease- <i>E. coli</i> DNA	63
	Single-strand exonuclease-heated T4 DNA	99
2	Double-strand exonuclease-T4 DNA	68
	DNA-dependent ATPase-T4 DNA	69

<sup>a</sup> In experiment 1, 19.5 units of *recBC* DNase was incubated with 10 units of inhibitor for 30 min at 30 C and analyzed as described. Experiment 2 was carried out in duplicate with 19.2 units of DNase and 13 units of inhibitor.

## DISCUSSION

Our observations on isolated T4 inhibitor provide an explanation for the ability of bacteriophage T4 to inhibit host *recBC* nuclease in vivo (16, 18). The inhibitor appears to be a protein of low molecular weight that acts in a noncatalytic mode. Whether the inhibitor actually forms a complex with the nuclease and/or chemically modifies the nuclease is not known.

The inhibition of *recBC* DNase has also been observed in phage  $\lambda$  infection; studies on  $\gamma$  protein (9, 17) parallel much of the work presented here. Both the T4 and  $\gamma$  inhibitors act in a noncatalytic manner and after the several activities of *recBC* nuclease. Although our assay conditions differ, it may be pointed out that the T4 *rec* inhibitor routinely produces 100% inhibition of the nuclease activity, whereas the  $\gamma$  protein causes a maximum of 65 to 90% inhibition (17). The  $\gamma$  protein is reported to be composed of two subunits of molecular weight 16,500 (9) that migrate through Sephadex columns as a single species of molecular weight approximately 33,000; the T4 inhibitor appears to have a molecular weight of 12,000 according to Sephadex gel filtration. Another difference is noted in the ATP effect on the inhibitor-nuclease interaction;  $\gamma$  protein activity is enhanced by ATP (17), but no such enhancement was seen in the work reported here. However, this does not exclude the possibility that inhibition occurs by phosphorylation or adenylation of the *recBC* nuclease since the inhibitor may be in an activated form or modification may occur subsequent to complex formation.

Regarding the biological significance of the

reaction, such an inhibition of host nuclease may clearly provide a protective function for the bacteriophage DNA. Purified *recBC* nuclease is reported to degrade T4 glucosylated DNA about twice as quickly as nonglucosylated DNA (2, 15). Furthermore, transfection of spheroplasts with T4 DNA is much more effective in *E. coli* lacking *recBC* nuclease (21). Thus the *rec* inhibitor specified by T4 may have a viral function in preventing breakdown of phage DNA during normal infection. A precedent for this hypothesis comes from the finding that  $\gamma$  protein is probably involved in protecting late  $\lambda$  DNA replication (5). Finally, inhibition of a major recombination pathway in the host has obvious implications with regard to genetic recombination in phage T4. The nature of inhibitor function may be clarified when a T4 phage mutant unable to synthesize *rec* inhibitor is available. A search for such mutants as well as screening known mutant strains that are likely candidates is underway.

Early studies on events after phage infection showed that a large number of host activities are altered (3). Some current studies have revealed that the controls for these changes may operate via specific phage proteins interacting with key host enzymes. The inhibitor for the *recBC* nuclease appears to be an example of such a specific control mechanism. Inactivation of *recBC* DNase after infection by phage T7 (22) and other DNA phages (16) has also been reported, indicating widespread distribution of this mode of control for *recBC* nuclease. Likewise, the modification of host RNA polymerase after T4 infection has been well documented (6, 8). Another example is provided by the appearance of modified valyl-tRNA synthetase after T4 infection (13).

#### ACKNOWLEDGMENTS

This work was supported by the Medical Research Council and National Cancer Institute of Canada.

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