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 λ infection (20), and in this case the inhibition has been attributed to γ protein, which is specified by the *gam* gene. The presence of the γ protein is essential for λ growth on certain specific hosts; *gam* mutants fail to grow on polA− *E. coli*, and *gam−red−* phage grow poorly in recA−recB−recC+ 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−) 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 [8-14C]ATP (specific activity, 52.15 mCi/mmol) and [methyl-3H]thymidine (20.0 Ci/mmol). Bovine pancreas trypsin (EC 3.4.11.4) and soybean trypsin inhibitor were obtained from Worthington. All other chemicals were reagent grade.

**Preparation of DNA.** 3H-labeled bacteriophage T4 DNA (7,510 counts/min per nmol of nucleoside) was prepared as follows. *E. coli* W3110 (thy−) was grown to 8 × 109 cells/ml in M9 medium (11) supplemented with 0.025% Casamino Acids, 20 μg of thymidine per ml, and 0.001% gelatin. Harvested cells were suspended at 6 × 109 cells/ml in fresh M9 medium supplemented as above, except with 5 μg of thymidine and 2 μCi of [3H]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 3H-labeled T4 DNA was somewhat heterogeneous in size and contained approximately one double-strand cut per molecule. 3H-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 × 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 MgCl2–5 mM 2-mercaptoethanol–0.2 mM EDTA) (14), and then 2% proteamine 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 K2HPO4–10 mM mercaptoethanol–0.1 mM EDTA–10% glycerol buffer, and repelleted. The active fraction was extracted twice with 10 ml of 0.25 M K2HPO4, 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 U-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 [H]-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 [8-'4C]ATP and analyzed for ADP production by thin-layer chromatography (4). Protein determinations were done according to Lowry after tryptic phosphotungstic 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 108/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 μg 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 μg 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 repelleted 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 MgCl2 after Alberts (1), crushed three times in a French pressure cell at 12,000 lb/in2, and centrifuged at 27,000 × 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, 2.0% 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 assayng for remaining recBC nuclease activity after precubation 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 MgCl2–1 mg of bovine serum albumin per ml–20% glycerol. An aliquot (usually 10 μl) was removed for the 0-min assay immediately before placing the precubation 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>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Inhibitor units</th>
<th>Sp act (units/mg of protein)</th>
</tr>
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<tbody>
<tr>
<td>I. Cell extract</td>
<td>390</td>
<td>-a</td>
<td>-</td>
</tr>
<tr>
<td>II. Polyethylene glycol dextran phase extract</td>
<td>250</td>
<td>8,600</td>
<td>35</td>
</tr>
<tr>
<td>III. DEAE-cellulose</td>
<td>1.5</td>
<td>1,770</td>
<td>1,180</td>
</tr>
</tbody>
</table>

*a 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.

*b Because of the presence of non-specific 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 nuclease 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 CaCl2 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– (AB1470) cells. The use of recB– 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; 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 $^3$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 $^3$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.
igate 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 level 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**

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

* 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 λ infection; studies on γ protein (9, 17) parallel much of the work presented here. Both the T4 and γ 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 γ protein causes a maximum of 65 to 90% inhibition (17). The γ 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; γ 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 γ protein is probably involved in protecting late λ 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.

LITERATURE CITED