New Deoxyribonuclease Activity After Bacteriophage P22 Infection

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Extracts from P22-infected and uninfected cultures of *Salmonella typhimurium* were subjected to deoxyribonucleic acid (DNA)-cellulose and diethylaminoethyl-cellulose chromatography. Comparison of the elution patterns revealed that in infected cells there is a decrease in the amount of nuclease activity specific for denatured DNA and an increase in the amount of nuclease activity specific for native DNA. The latter activity was shown to differ from a similar host enzyme in Mg^{2+} , Mn^{2+} , and pH optima. This new activity is not found after infection of a lysogen with $\sim 10^2$ a nonvirulent phage or after infection under nonpermissive conditions with $\times \rightarrow$ P22ts25.1 (a mutant in gene 25 that carries out no known functions other than adsorption and injection) and thus appears to be specified by the phage genome.

Much is known about the genetics of phage P22 as well as the control mechanisms involved in infections by this temperate phage. However, virtually nothing is known about the enzymes synthesized after phage P22 infection. Since most of the phage-controlled enzymes which have been described in other phage systems are involved in deoxyribonucleic acid (DNA) synthesis, we have searched for such enzymes in the P22-*Salmonella typhimurium* system. A comparison of crude extracts from uninfected and infected cells shows only small differences in enzyme activity. Some of these differences can be amplified by a purification scheme which uses DNA-cellulose chromatography to select for DNA-binding proteins (1). We present evidence here for the appearance of a new deoxyribonuclease activity after phage infection which is not detected in extracts of uninfected cultures of *S. typhimurium*.

MATERIALS AND METHODS

Bacterial and phage strains. *S. typhimurium* LT2, strain 18 cured of PB1 prophage (12), was used as the sensitive host. Strain 210, which is a derivative of strain 18 carrying a *sie*⁻ mutant prophage, was used as a lysogenic host. The *sie*⁻ mutant lysogen does not exclude superinfecting phages, but phage production remains repressed (9, 11). P22c₁ is defective in the ability to lysogenize and undergoes only the lytic response in nonlysogenic cells (5). The conditional lethal mutant P22ts25.1c₁ (6) is negative for phage DNA synthesis at 37 C.

Assay of DNA polymerase activity. The DNA po-

lymerase assay measures the incorporation of ³H-deoxythymidine triphosphate (³H-dTTP) into trichloroacetic acid-insoluble material. The reaction mixture contained, per 0.25 ml: 0.01 μ mole each of deoxycytidine triphosphate, deoxyguanosine triphosphate, deoxyadenosine triphosphate, and dTTP including 0.75 μ Ci of ³H-dTTP (Schwarz BioResearch; specific activity approximately 10⁴ counts per min per μ mole); 0.015 mmole of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.5; 0.24 μ mole of mercaptoethanol; 1.6 μ mole of MgCl₂; 40 μ g of calf thymus DNA; 125 μ g of bovine serum albumin; 0.0625 μ mole of sodium-ethylenediaminetetraacetic acid (Na₂EDTA); and 40 μ liters of enzyme extract. After 5 min at 37 C, duplicate 50- μ liter samples of the reaction mixture were placed on filter-paper strips and immersed for at least 60 min in cold 5% trichloroacetic acid containing 1% Na₂P₂O₇. The strips were washed twice with 5% trichloroacetic acid, twice with 95% ethanol, and twice with ether. The dried strips were placed in 15 ml of scintillation fluid (PPO, 19 g; dimethyl-POPOP, 1.14 g; toluene to 3.8 liters) and counted in a Packard TriCarb liquid scintillation spectrometer.

Assay of deoxyribonuclease activity. The nuclease assay measures the conversion of ³²P-labeled P22 DNA to acid-soluble nucleotides. The reaction mixture contained, per 0.3 ml: 30 μ moles of Tris-hydrochloride, pH 8.7; 100 μ g of bovine serum albumin; 0.3 μ mole of MgCl₂; 2 to 5 nmoles of ³²P-DNA (specific activity approximately 10⁴ to 5 $\times$ 10⁴ counts per min per μ mole); and 5 to 50 μ liters of enzyme extract. The reaction was stopped after incubation at 37 C for 30 min by addition of 0.2 ml of cold calf thymus DNA (2.5 mg/ml) and 0.3 ml of cold 7% perchloric acid. After centrifugation at 12,500 $\times$ g for 10 min in a Sorvall SM-24 rotor, 0.5 ml of the acid-soluble fraction was placed in 10 ml of *p*-dioxane scintillation

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medium (3) and counted in a Packard TriCarb scintillation spectrometer. This assay is used with either native or denatured ^{32}P -labeled P22 DNA.

Preparation of DNA-cellulose. DNA-cellulose was prepared by the method of Alberts (1) with cellulose (Munktell no. 410, 4 g) mixed with 12 ml of native calf thymus DNA (2 mg/ml). The suspension was dried overnight at room temperature, lyophilized, washed with 0.01 M Tris-hydrochloride and 0.001 M Na_3EDTA , pH 7.4, and stored (in the same buffer plus 0.15 M NaCl) as a frozen slurry. Two 4-ml packed columns were poured from each preparation.

Other methods. ^{32}P -DNA was prepared from ^{32}P -labeled phage P22 as previously described by Botstein (2). ^{32}P -labeled P22 DNA was denatured by heating at 100 C for 10 min in 0.01 M Na_3EDTA and 0.01 M Tris-hydrochloride, pH 8, followed by rapid cooling. Protein concentrations were determined by the method of Lowry et al. (7) with bovine serum albumin as a standard. Glycerol gradients contained 5 to 20% glycerol, 0.3 M NaCl, 0.02 M Tris-hydrochloride, pH 7.4, and 0.01 M mercaptoethanol. After centrifugation, the tubes were punctured, and 0.35-ml fractions were collected.

Purification of enzyme. The purification results appear in Table 1. Throughout the procedure, infected and uninfected cell extracts were run in parallel. All procedures were carried out at 0 to 4 C unless otherwise indicated.

(i) **Preparation of crude extracts.** Cells were grown in 1 liter of M9CAA (10) to a concentration of 5×10^8 cells per ml. Magnesium sulfate was added to a final concentration of 0.015 M (8), and one-half of the culture was infected at a multiplicity of 5. Fifteen minutes after infection, the cells were rapidly chilled and centrifuged. The packed cells were suspended in

6 ml of buffer (0.01 M MgCl_2 , 1 mM mercaptoethanol, 0.2 mM Na_3EDTA , and 0.04 M Tris-hydrochloride, pH 8.1) and then disrupted in a MSE sonifier using three 60-sec bursts at 1 amp per 2 ml of sample. The temperature of the sample was always less than 8 C during sonic oscillation. The extract was centrifuged at $105,000 \times g$ for 60 min at 4 C in a Spinco 50 rotor, and the supernatant fluid was collected (fraction 1).

(ii) **DNA-cellulose chromatography.** DNA-cellulose columns (0.96 by 6.5 cm) were equilibrated with 0.02 M Tris-hydrochloride, pH 8.1, containing 0.1 M NaCl, 1 mM mercaptoethanol, 1 mM Na_3EDTA , 10% glycerol, and 0.5 mg of bovine serum albumin (Calbiochem, A grade) per ml.

Fraction 1 was treated for 15 min at 23 C with 0.02 mg of pancreatic deoxyribonuclease I (Worthington Biochemical) per ml and then dialyzed overnight against two changes of 500 ml of buffer (0.1 M NaCl, 1 mM mercaptoethanol, 1 mM Na_3EDTA , and 0.02 M Tris-hydrochloride, pH 8.1). The dialyzed extract was centrifuged for 30 min at $105,000 \times g$, and the supernatant fluid was diluted with an equal volume of the equilibration buffer and applied to a DNA-cellulose column at a flow rate of 0.2 ml/min. The column was washed with 20 ml of the equilibration buffer and then eluted with 10-ml volumes of the same buffer containing 0.15 M, 0.6 M, and 2.0 M NaCl. Fractions of 0.4 ml were collected. The majority of the DNA-binding proteins were found in 2 to 3 ml of the 0.6 M NaCl eluate. These fractions were pooled (fraction 2).

(iii) **Ammonium sulfate precipitation.** Solid ammonium sulfate was added to bring fraction 2 to 85% saturation. The precipitate was collected by centrifugation at $31,000 \times g$ for 30 min, dissolved in 1.5 ml of 0.02 M Tris-hydrochloride, pH 7.4, containing

TABLE 1. Purification of nuclease activity which degrades native P22 DNA

Fraction		Specific activity ^a (units/ mg of protein)		Total units ^b	
No.	Type	Uninfected ^c	Infected ^c	Uninfected ^c	Infected ^c
1	Sonic extract ($100,000 \times g$ supernatant fluid)	75	72	3,591	2,447
2	DNA-cellulose	394	515	312	415
3	$(\text{NH}_4)_2\text{SO}_4$ precipitation	758	803	114	120
4	DEAE-cellulose				
	Peak I	3,880	5,056	15	20
	Peak II	1,802	1,214	36	85
	Peak III	1,770	1,965	7	8

^a The calculations of specific activity are subject to some error due to the effect of combined nucleases in the crude sonic extract. In addition, protein values for the $(\text{NH}_4)_2\text{SO}_4$ and DEAE-cellulose fractions are based on A_{280} readings and include albumin eluted along with enzyme. Albumin and glycerol are used in the equilibration buffer to decrease nonspecific protein binding to the DNA-cellulose and to help stabilize the enzyme. Albumin elutes with enzyme during both DNA-cellulose and DEAE-cellulose chromatography as shown by acrylamide gel electrophoresis. The protein in the sonic extract and DNA-cellulose extract was determined by the procedure of Lowry et al. (7).

^b One unit of nuclease activity is defined as the amount of protein which catalyzes the release of 1.0 nmole of nucleotide in 30 min at 37 C.

^c Assayed at pH 8.7 with 0.3 μmole Mg^{2+} and native P22 DNA substrate.

10 mM mercaptoethanol and 10% glycerol, and dialyzed overnight against two changes of 500 ml of the above buffer. The dialyzed fraction was diluted to 10 ml with the same buffer (fraction 3).

(iv) **DEAE-cellulose chromatography.** DEAE-cellulose columns (0.75 by 4.5 cm) were equilibrated with 0.02 M Tris-hydrochloride, pH 7.4, 0.01 M mercaptoethanol, and 10% glycerol. Fraction 3 was applied to the DEAE-cellulose column at a flow rate of 0.5 ml/min. Elution was accomplished with successive 1.5 column volumes of the above buffer containing, in sequence, no salt, 0.05 M, 0.10 M, 0.20 M, 0.30 M, 0.40 M, and 0.50 M NaCl. Four protein peaks were obtained, and from their order of elution were designated peak I (eluted with 0.1 M NaCl), peak II (0.2 M NaCl eluate), peak III (0.3 M NaCl eluate), and peak IV (0.4 M NaCl eluate).

RESULTS

Elution of enzyme activity from DEAE-cellulose.

Four protein peaks were separated by chromatography on DEAE-cellulose (Fig. 1A) by the purification procedure outlined above. Approximately the same amount of protein was eluted from uninfected and *P22c₁*-infected strain 18 (nonlysogenic) cell extracts. Most of the protein eluted in peak II.

Partial separation of several enzyme activities was detected when the DEAE fractions were assayed. The elution pattern of enzyme activity in uninfected cell extracts is described first. The majority of the nuclease activity which degrades native P22 DNA is found in peaks I and II (Fig. 1B). When denatured DNA is used as the substrate (Fig. 1C), the predominant peaks of nuclease activity are peaks II and III. DNA polymerase activity also elutes in peaks II and III (Fig. 1D), with most of the activity in peak II and only small amounts in peak III.

Infection with *P22c₁* affected some of these enzyme activities. Much less nuclease activity specific for denatured DNA substrate is seen in peaks II and III after phage infection (Fig. 1C). In the accompanying paper by Israel, Woodworth-Gutai, and Levine (4), this decrease in nuclease activity which degrades denatured DNA after phage infection has also been studied in crude sonically disrupted extracts. The elution patterns of polymerase activity in uninfected and infected cell extracts are similar (Fig. 1D). No further studies have been done to distinguish the polymerase activities in the two extracts. Nuclease activity from phage-infected cells specific for native DNA substrate elutes in peaks I and II with approximately twice as much activity as in uninfected cell extracts (Fig. 1B).

The nuclease activity which prefers native DNA substrate can be separated from the nuclease activity preferring denatured DNA substrate.

Mixed samples of uninfected and infected cell extracts from DEAE peak II were placed on a 5 to 20% glycerol gradient and centrifuged for 18 hr at $200,000 \times g$ in a Spinco SW50 rotor. The nuclease activity against denatured DNA has

FIG. 1. DEAE-cellulose chromatography of deoxyribonucleases and DNA polymerase from uninfected and infected cells. Extracts partially purified by DNA-cellulose chromatography and ammonium sulfate precipitation (as described in Materials and Methods) were chromatographed on DEAE-cellulose. Fractions of 0.4

a maximal activity in fraction 6, whereas the maximal activity of the nuclease which degrades native DNA appears in fraction 9 (Fig. 2). This clearly indicates that there are two nuclease activities in these extracts which can be physically separated.

Properties of the nuclease activity specific for native DNA from P22_c-infected and uninfected strain 18 cells. The infected-cell activity for native DNA can be distinguished from the host-cell activity by several parameters. The infected-cell activity in peak II has a pH optimum of 7.1, as contrasted with an optimum of 8.7 for the host-cell activity (Fig. 3A). In addition, the host nuclease activity has maximal activity at a Mg^{2+} ion concentration of 1.0 mM, whereas the activity from infected cells increases with increasing concentrations of Mg^{2+} and reaches its maximum at 10.0 mM Mg^{2+} ion (Fig. 3B). Differences in pH optima and Mg^{2+} ion requirements are also seen when the nuclease activity in DEAE peak I is studied (Fig. 3C and D). The nuclease activity in both uninfected and phage-infected cell extracts is enhanced by the Mn^{2+} ion, but again the activity from infected cells prefers a 10-fold higher concentration than does the activity from uninfected cells (data not shown).

The increase in nuclease activity after phage infection is magnified some 15- to 20-fold when the activity in peaks I and II is assayed at conditions optimal for the infected-cell activity (pH 7.1, 10.0 mM Mg^{2+}) (Fig. 4).

Genetic evidence for a new nuclease activity. The above data show that a new nuclease activity is observed after phage infection. If this new activity is coded for by phage P22, it should not be seen after c_1 superinfection of a P22 lysogen. Superinfecting phages only inject DNA into the lysogen, but the DNA is not replicated, and most phage functions are repressed. The nuclease activity specific for native P22 DNA substrate was compared after c_1 infection of a nonlysogenic and a lysogenic host (strains 18 and 210, respectively) (Fig. 5). The pH optimum and Mg^{2+} ion concentration curves from the extracts of the superinfected lysogen are characteristic of the host activity. Thus, repressing the phage genome prevents the appearance of the new activity.

FIG. 2. Separation of native- and denatured-DNA nuclease activity on a 5 to 20% glycerol gradient. A 0.10-ml sample of DEAE peak II containing equal amounts of the two enzyme preparations from uninfected and infected cells was layered on a 5 to 20% glycerol gradient containing 0.3 M NaCl, 0.02 M Tris-hydrochloride, pH 7.4, and 0.01 M mercaptoethanol. The gradient was centrifuged for 18 hr at 50,000 rev/min in a SW-50 swinging-bucket rotor in a Spinco model L ultracentrifuge. Fractions of 0.35 ml were collected, and 50 μ liters of each fraction was assayed at pH 8.7, in the presence of 0.3 μ mole of Mg^{2+} , for 30 min at 37 C. (○---○) Nuclease activity against denatured P22 DNA; (●—●) nuclease activity against native P22 DNA.

Infection at nonpermissive temperature with P22_{ts25}.Ic₁ in strain 18 does not result in the appearance of the new activity. P22_{ts25}.Ic₁ is a DNA-negative mutant, and as far as is known it is unable to carry out any phage functions except adsorption and injection (Hoffman and Levine, unpublished data). These results are consistent with the hypothesis that the new nuclease activity is coded for by the phage genome.

DISCUSSION

DNA-cellulose chromatography has been a useful technique for selectively isolating proteins with deoxyribonuclease activity and DNA polymerase activity from crude extracts of infected and uninfected cells. The deoxyribonuclease activities and DNA polymerase activity have been partially separated on DEAE-cellulose columns. Whereas we have recovered only approximately 2 to 5% of the original nuclease activity, the purification

ml were collected. (A) Protein as measured by absorbancy at 280 nm. (B) Nuclease activity with native P22 DNA as substrate. The assay was carried out at pH 8.7 in the presence of 0.3 μ mole of Mg^{2+} , for 30 min at 37 C. (C) Nuclease activity with denatured P22 DNA as substrate at pH 8.7 in the presence of 0.3 μ mole of Mg^{2+} , for 30 min at 37 C. (D) DNA polymerase activity; (●) c_1 -infected cell extracts; (○) uninfected (strain 18) cell extracts.

FIG. 3. Nuclease activity: pH optima and Mg^{2+} ion requirements in uninfected and infected extracts. (A) pH curve of nuclease activity in DEAE-cellulose peak II. Incubations were carried out at 37 C for 30 min with native P22 DNA, 0.3 μ mole of Mg^{2+} , and Tris-hydrochloride buffers (prepared at 37 C). (B) Mg^{2+} requirement of nuclease activity in DEAE-cellulose peak II. Incubations were carried out with varying amounts of Mg^{2+} at pH 8.7 for 30 min at 37 C with native P22 DNA as substrate. (C) pH curve of nuclease activity in DEAE-cellulose peak I. Incubations were carried out at 37 C for 30 min with native P22 DNA, 0.3 μ mole of Mg^{2+} and Tris buffers. (D) Mg^{2+} requirement of nuclease activity in DEAE-cellulose peak I. Incubations were carried out at pH 8.7 for 30 min at 37 C with native P22 DNA as substrate. (●) c₁-infected cells; (○) uninfected (strain 18) cells.

procedure has given consistently reproducible results.

A new nuclease activity which degrades native DNA appears after infection of *S. typhimurium* with P22c₁. Paper chromatography of the acid-soluble products of the nuclease assay reveals that approximately 70% travels as mononucleotides, while the remainder stays at the origin indicating acid-soluble oligonucleotide products. These data suggest that the new nuclease activity we are describing is an exonuclease but further purification is needed to remove the endonuclease activity also present. As compared to the host enzyme activity, the infected-cell activity has a more acidic pH optimum and requires for maximal activity a 10-fold higher Mg^{2+} or Mn^{2+} ion concentration.

The poor recovery of the original activity raises the possibility that the above observations might be artifacts of the purification procedure. Whereas an interpretation of differential recovery cannot be rigorously ruled out at this time, we consider

it unlikely because the appearance of the new nuclease activity is dependent on the genotype of the infecting phage and that of the host cell. When cells carrying prophage are superinfected with

FIG. 4. Nuclease activity in DEAE-cellulose peaks I and II with native P22 DNA substrate, 3.0 μ moles of Mg^{2+} , at pH 7.1, for 30 min at 37 C in c₁-infected cell extracts (●); in uninfected (strain 18) cell extracts (○).

FIG. 5. Nuclease activity: pH optima and Mg^{2+} ion requirements of extracts of c₁-infected lysogenic and nonlysogenic hosts. (A) pH curve of nuclease activity. Assays were done at 37 C for 30 min with native P22 DNA, 3.0 μ moles of Mg^{2+} , and Tris buffers; (B) Mg^{2+} requirement of nuclease activity. Assays were done at 37 C for 30 min with native P22 DNA at pH 7.1. (●) c₁-infected nonlysogen (strain 18); (▲) c₁-infected lysogen (strain 210).

P22c₁, the new nuclease activity is not seen. In addition, the new nuclease activity is not seen after infection with a mutant (*ts25.Ic₁* at non-permissive temperature) which carries out no known phage functions other than adsorption and injection. Therefore, injection alone does not cause the appearance of the pH 7.1 activity in DEAE peaks I and II.

This new nuclease activity appears, then, to be coded for by the phage genome. We are attempting to identify the structural gene and to further purify this enzyme activity in order to characterize it.

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