Isolation of Bacteriophage T4 Mutants Defective in the Ability to Degrade Host Deoxyribonucleic Acid¹

HUBER R. WARNER, D. PETER SNUSTAD, SALLY E. JORGENSEN, AND JAMES F. KOERNER

Department of Biochemistry, Department of Genetics and Cell Biology, College of Biological Sciences, University of Minnesota, St. Paul, Minnesota 55101, and Department of Biochemistry, College of Medical Sciences, University of Minnesota, Minneapolis, Minnesota 55455

Received for publication 30 March 1970

A method was devised for identifying nonlethal mutants of T4 bacteriophage which lack the capacity to induce degradation of the deoxyribonucleic acid (DNA) of their host, *Escherichia coli*. If a culture is infected in a medium containing hydroxyurea (HU), a compound that blocks de novo deoxyribonucleotide biosynthesis by interacting with ribonucleotide reductase, mutant phage that cannot establish the alternate pathway of deoxyribonucleotide production from bacterial DNA will fail to produce progeny. The progeny of 100 phages that survived heavy mutagenesis with hydroxylamine were tested for their ability to multiply in the presence of HU. Four of the cultures lacked this capacity. Cells infected with one of these mutants, designated T4*nd28*, accumulated double-stranded fragments of host DNA with a molecular weight of approximately 2×10^8 daltons. This mutant failed to induce T4 endonuclease II, an enzyme known to produce single-strand breaks in double-stranded cytosine-containing DNA. The properties of *nd28* give strong support to an earlier suggestion that T4 endonuclease II participates in host DNA degradation. The *nd28* mutation mapped between T4 genes 32 and 63 and was very close to the latter gene. It is, thus, in the region of the T4 map that is occupied by genes for a number of other enzymes, including deoxycytidylate deaminase, thymidylate synthetase, dihydrofolate reductase, and ribonucleotide reductase, that are nonessential to phage production in rich media.

The phenomena of degradation of the *Escherichia coli* deoxyribonucleic acid (DNA) after infection with T-even bacteriophages and the subsequent incorporation of the nucleotides derived from the host into the progeny phage have been known for many years (13, 14, 18-20, 37). Presumably, some of the nucleases which have been observed to be induced after infection with phage play a role in the degradation of the host DNA, but direct evidence on this point has not yet been reported. Enzymes which may be involved in the breakdown of *E. coli* DNA include the four distinct phage-induced nucleases which have been partially purified and characterized. These are endonucleases II and IV, exonuclease A, and an exonuclease activity associated with the phage-induced DNA polymerase. Endonuclease II makes a limited number of single-strand breaks in native cytosine-containing DNA (15, 29, 30), whereas endonuclease IV acts preferen-

tially on single-stranded cytosine-containing DNA (29, 31). Exonuclease A is the most active exonuclease in the crude extract prepared from infected cells and attacks from the 3'-hydroxyl terminus (25, 32, 33). The exonuclease activity associated with the phage-induced DNA polymerase also attacks DNA from the 3'-hydroxyl terminus; this enzyme accounts for only about 10% of the total exonuclease activity in the crude extract (12, 32; Z. J. Lucas, Fed. Proc. **24**:286, 1965). There have also been brief reports or suggestions of other phage-induced nucleases, but these enzymes have not as yet been extensively purified (2; N. R. G. Nossal, Ph.D. Thesis, Univ. of Michigan, Ann Arbor, 1963; S. Bose and N. Nossal, Fed. Proc. **23**:272, 1964).

One way to obtain evidence regarding the role of phage-induced nucleases in the breakdown of *E. coli* DNA is to isolate phage mutants which are unable to degrade this DNA to acid-soluble products. Mutants which may be of this type are the *amber* (*am*) mutants with lesions in genes 46 or 47 (38). Bacteriophages containing mutations in these genes are unable to degrade *E. coli* DNA

¹ This publication is Scientific Paper no. 6848 of the Minnesota Agricultural Experiment Station. A preliminary account of this work has been presented (S. E. Jorgensen, J. F. Koerner, D. P. Snustad, and H. R. Warner, Fed. Proc., **29**:465, 1970).

to acid-soluble products, but the host DNA is broken down to pieces with a molecular weight of about 10^6 or 10^7 daltons (21). The products of genes 46 and 47 have not been characterized to determine whether they are nucleases or whether they stimulate nucleolytic activity in some way. Mutants of these genes have been found to induce normal amounts of exonuclease A, of polymerase-exonuclease (24; E. C. Short, Jr., Ph.D. Thesis, Univ. of Minnesota, Minneapolis, 1968), and of endonucleases II and IV (30, 31). In addition to their inability to induce the complete degradation of *E. coli* DNA, mutants in genes 46 and 47 also have limited ability to make phage DNA and viable phage in the nonpermissive host (38).

The work reported here describes efforts to isolate other mutants which are unable to induce enzymes required for the degradation of host DNA. Since de novo deoxyribonucleotide synthesis occurs in *E. coli* infected with T-even phages, it seems probable that nucleases whose only function is to degrade the host DNA would not be required for the synthesis of viable phage. Mutations in the genes for these enzymes would not be lethal, suggesting that special procedures must be used to screen for such mutants. One possible way to make nuclease deficiency conditionally lethal is to prevent de novo deoxyribonucleotide synthesis. If de novo synthesis does not occur, then phage DNA can be made only from the *E. coli* DNA degradation products, and nuclease mutants might not be able to replicate under these conditions. Previous work has shown that hydroxyurea (HU) effectively inhibits the ribonucleoside diphosphate reductase systems in infected *E. coli*, and that *amb3*, a gene 46 mutant, produces no progeny phage in HU-treated *E. coli* B (35). Thus, it seemed likely that HU could be used to select for mutants with defective nucleases, provided that these nucleases are essential for degradation of *E. coli* DNA to deoxyribonucleotides but are not essential for phage replication in the absence of HU. The procedure described in this paper was used to obtain nuclease-deficient T4 phages.

MATERIALS AND METHODS

Growth of bacteria and phages. For most experiments, the medium described by Fraser and Jerrel was used (11), except that 5 g of Casamino Acids per liter was used rather than 15 g. *E. coli* B was used for all experiments described below unless indicated otherwise, and cultures were grown by gyratory shaking at 30 or 37 C. The *am* mutants (mutants of phage T4 which replicate in *E. coli* CR63 but not in *E. coli* B) used were the generous gifts of R. S. Edgar and W. B. Wood and have been described earlier (7, 9). T-broth was used as the phage dilution medium, and EHA

top and bottom agar, were used for plating, as described by Edgar (6). When cultures were titered, CHCl_3 was always added to the first dilution tube. The plates were incubated overnight at 30 C.

Chemicals and equipment. HU was obtained from the Sigma Chemical Co. ^{14}C -thymidine and ^3H -thymidine were obtained from the Schwarz BioResearch Inc., and radioactivity was measured with either a Nuclear-Chicago Planchet Counting System (model 4338) or a Packard Tri-Carb liquid scintillation spectrometer (model 3003). The scintillation mixture contained 8.0 g of 2-(4'-*t*-butyl-phenyl)-5-(4'-biphenyl)-1,3,4-oxadiazole and 0.5 g of 2-(4'-biphenyl)-6-phenyl-benzoxole per liter of toluene. All gradients were run in an SW39 rotor in a Spinco ultracentrifuge (model L).

Production and screening of mutants. The procedure described by Tessman (34) was used to obtain phage carrying mutations in nonessential genes. In the control tube, without hydroxylamine, the phage titer decreased only 25% during incubation for 72 hr at 37 C, whereas the titer in the tube with hydroxylamine decreased logarithmically to a survival of about 0.1% during a similar incubation. Survivors included large and small plaque-formers, in addition to the apparently normal plaque-formers. One hundred plaques, including all plaque morphologies observed, were isolated and screened as described below.

Plaques produced by surviving phage were picked with toothpicks which were then placed in 1 ml of growth medium to give phage titers of about 5×10^6 . These phage isolates were replated at a dilution giving 10 to 200 plaques per plate and were repicked in an attempt to eliminate phage with lethal mutations which might be replicated in cells infected with the viable isolates. Only one isolate was lost during this step. This second set of isolates was then used to produce lysates with higher titers by growing in *E. coli* B at 30 C. One more isolate was lost during this step, leaving 98 isolates to be screened as described below.

The lysates described above were screened for presumed nuclease deficiency by comparing phage synthesis in the presence and absence of HU. Lysates were diluted to 10^{10} phage/ml and 0.1 ml of each was added to a 4-ml culture of *E. coli* (4×10^8 cells/ml) containing 0.01 M HU in a 50-ml Erlenmeyer flask. A similar addition was made to a culture without HU, and both were shaken at 37 C for 30 min and were then titered for phage. The value for +HU titer/-HU titer obtained with wild-type phage under these conditions was 0.10 to 0.12. Isolates which had a value of less than 0.09 were then analyzed more carefully to identify those which make no phage in the presence of HU. Lysates were diluted to about 5×10^8 phage/ml, and 0.1 ml of each was added to a 5-ml culture of *E. coli* (5×10^8 cells/ml) in a 50-ml Erlenmeyer flask. These were shaken at 37 C for 1 min, and then 0.1 ml was diluted into 10 ml of fresh medium to minimize reinfection after lysis. After 0.5 min, 5 ml of this solution was transferred to a similar flask containing 4.4 mg of HU (final concentration of 0.012 M). Samples were removed from each flask at various times after infection and titered for phage. Those

phage which replicated in the absence of HU but failed to induce net phage synthesis in the presence of HU were designated nuclease deficient (*nd*).

Purification and mapping. *E. coli* strains B/5, S/6/5, and CR63, obtained from R. S. Edgar, were grown in H-broth (7) for these experiments. For genetic purification, backcrosses were done at 30°C in *E. coli* B/5 by using wild-type/*nd* ratios of 4 as described by Edgar (6), except that antiserum was not used. The *nd* mutants were backcrossed to wild-type six times, and these purified mutants were used for all studies described below, unless otherwise indicated.

Progeny were tested for genotype by plating on S/6/5 indicator, incubating at 30°C overnight, resuspending single plaques in 2 ml of H-broth, and testing the ability of each to grow in the presence and absence of HU. Clonal stocks were then prepared from *nd* isolates for subsequent backcrosses.

One mutant with the *nd* phenotype, designated *nd28*, has been carefully mapped. Mapping this mutant was facilitated by the observation that it produces a considerably smaller plaque than does wild-type T4. Reconstruction experiments indicated that, whereas it was possible to score large plaques unambiguously as *nd*⁺, approximately 5% of the small plaques scored as *nd* were actually found to be *nd*⁺. It is likely, therefore, that the map intervals derived from these data are slightly underestimated. Crosses of *nd28* with *am* mutants were performed at 30°C in *E. coli* strain CR63. The procedure employed was as described by Edgar (6). Superinfection exclusion was prevented by adding KCN; unadsorbed phage were inactivated with antiserum. All adsorption tubes and growth tubes were aerated by slow bubbling. Total progeny were scored on CR63 indicator; wild-type (*nd*⁺*am*⁺) recombinants were scored by enumerating large plaques on S/6/5 indicator. The relative efficiency of plating on CR63 and S/6/5 was determined in each experiment, and the titers determined were corrected accordingly.

Double mutants (*nd am*) were also identified among the progeny on the CR63 plates from the above crosses by screening for the *am* mutation on *E. coli* B and then testing these *am* mutants for the presence of *nd* as indicated above. All double mutants obtained in this way were tested for complementation with *am* mutations in genes 30, 42-46, and 56 as described by Wiberg (38). These double mutants were then used in the experiments described below.

Degradation of host DNA after phage infection. For labeling of host DNA with ¹⁴C, *E. coli* B was grown to a concentration of about 10⁸ cells/ml at 37°C, and then 0.5 mg of uridine was added per ml of culture. Four minutes later ¹⁴C-thymidine was added (specific activity = 47.9 µCi/µmole; final concentration = 0.04 µCi/ml), and the culture was shaken for 80 min at 37°C. Cells were sedimented by centrifugation for 5 min at 10,000 × *g* and resuspended in warmed, non-radioactive medium.

To measure acid solubilization of the *E. coli* DNA, 0.5-ml samples were removed from infected cultures at various times and added to 0.5 ml of 10% trichloroacetic acid. The precipitates were sedimented by centrifugation for 10 min at 2,500 × *g*, washed with 2

ml of cold 5% trichloroacetic acid, and dissolved in 2 ml of 1 M NH₄OH. Samples were plated on planchets and counted.

To characterize further the *E. coli* DNA after infection with *nd* mutants, lysed cultures of thymidine-labeled cells were also analyzed on sucrose gradients. For these experiments, the ¹⁴C-thymidine concentration was 0.1 µCi/ml, and 0.8 µmole of unlabeled thymidine was included per ml in the medium used to resuspend the cells. Lysates were prepared at various times after infection by treating the phage-infected cells with lysozyme and sodium lauryl sarcosinate as described by Frankel (10). The lysates were stored at 4°C.

Sucrose gradients were prepared by a modification of the procedure described by Kutter and Wiberg (21). The 5 to 20% gradients were generated by carefully layering 0.9-ml samples of 20, 16.25, 12.5, 8.75, and 5% sucrose solutions, respectively, in Spinco SW39 tubes. All of the solutions for the neutral gradients also contained 0.1 M NaCl, 0.05 M ethylenediaminetetraacetic (EDTA; pH 8.5), and 0.01% NaN₃. The gradients were stored at 4°C for 2 to 16 hr prior to use. After this equilibration time, 0.1 ml of the lysate sample and 0.05 ml of the appropriate marker DNA were layered onto the top of the gradient with a sawed-off 1-ml pipette. The tubes were then centrifuged at 35,000 rev/min for 3 hr. The fractions were collected by removing successive 0.1-ml samples from the meniscus by using a 0.1-ml Biopipette (Schwarz BioResearch). The 0.1-ml fractions were applied to 2-cm square pieces of Whatman 31ET chromatography paper. The papers were washed twice with cold 5% trichloroacetic acid, twice with 95% ethanol, and once with ether (5 ml of all wash solutions per paper). They were then dried, placed in scintillation vials containing 3 ml of scintillation fluid, and counted.

The ³H-thymidine-labeled T4 DNA used as a marker was made by infecting a 10-ml culture of ³H-thymidine-labeled *E. coli* with wild-type T4 and preparing a lysate 2 hr after infection. The cell debris was removed by centrifugation at 7,000 × *g* for 10 min. The supernatant was then centrifuged at 50,000 × *g* for 20 min to sediment the phage. The phage pellet was resuspended in 1 ml of medium containing 0.1 M NaCl, 0.001 M MgSO₄, 0.02 M tris(hydroxymethyl)aminomethane (Tris)-acetate buffer (pH 7.5), and 100 µg of gelatin per ml, and the T4 DNA was released from the phage by adding sodium dodecyl sulfate to a final concentration of 0.1% and incubating the mixture at 65°C for 10 min (21). The ³H-labeled ϕ29 DNA used as a marker was a gift from Charles Schachte. The molecular weight of this DNA is 11 × 10⁶ (1).

Transfer of thymidine from host DNA to phage DNA. To measure the amount of ¹⁴C-thymidine from *E. coli* DNA which was incorporated into phage DNA after infection, 5-ml samples of infected cultures were removed at various times and lysed with CHCl₃. After standing for at least 30 min in the presence of CHCl₃, the lysates were centrifuged for 10 min at 5,000 × *g* to sediment bacterial debris. Samples (4 ml) of these supernatants were transferred without mixing to Spinco SW39 centrifuge tubes containing 4 g of CsCl. The tubes were centrifuged for 20 hr at 30,000 rev/

min. The gradient is established during this period by partial dissolution of the CsCl. Samples (0.15 ml) were removed from the top of the gradient with a Biopipette (Schwarz BioResearch). These fractions were diluted to 3.0 ml with Fraser and Jerrel medium and assayed for acid-insoluble ^{14}C and viable phage as described above.

Preparation of extracts: enzyme assays. Extracts were prepared from cells grown at 37°C and infected with phage. Twelve minutes after infection, 400 ml of infected cells was harvested by centrifugation for 10 min at $10,000 \times g$. These cells were resuspended in 15 ml of solutions containing 0.01 M Tris-acetate buffer (pH 8.0), 0.01 M magnesium acetate, and were broken by sonically treating the suspension 10 times for 20-sec intervals with a Bronwill Biosonik II. These extracts were then centrifuged for 10 min at $10,000 \times g$, and the precipitate was discarded. They were assayed for deoxycytidinetriphosphatase (27), dihydrofolate reductase (36), deoxycytidine monophosphate (dCMP) deaminase (36), and exonuclease (25).

Chromatography of extracts on diethylaminoethyl (DEAE) cellulose. The cell-free extracts were treated with pancreatic deoxyribonuclease and ribonuclease as described by Short and Koerner (32). The protein was precipitated by the addition of ammonium sulfate to attain 80% saturation. A solution of the 80% ammonium sulfate precipitate containing approximately 30 mg of protein was desalted with Sephadex G-25 and applied to a DEAE-cellulose column (1 cm by 10 cm). The protein was eluted with a concave gradient generated by three identical vessels connected in series (26). The vessels contained equal volumes of 0.007 M potassium phosphate buffer (pH 7.5) and 0.005 M EDTA. The two vessels closest to the column also contained 0.003 M NaCl, and the third vessel contained 0.8 M NaCl. The extract from uninfected cells was eluted with a total gradient volume of 240 ml, and fractions of 6 ml were collected. The extracts from infected cells were eluted with a total gradient volume of 120 ml, and fractions of 3 ml were collected. The fractions were assayed for deoxyribonuclease activity with partially degraded DNA as described by Oleson and Koerner (25).

RESULTS

When 100 isolates from the heavily mutagenized phage stock were tested for their ability to replicate in the presence of HU, four isolates clearly lacked this ability but could replicate in its absence. These isolates were purified as described above and have been designated *nd* for their presumed nuclease deficiency. Figure 1 shows a typical growth curve for wild-type T4 and one of these *nd* mutants, *nd28*. Whereas *nd28* replicates as well as T4 in *E. coli* B in the absence of HU, there is no net phage production in cells infected with *nd28* for up to 40 min after infection in the presence of HU. The mutation is not an *am* mutation since the same effect occurs when *E. coli* CR63 is used as the host. Double mutants were isolated from crosses of *nd28* with various *am*

mutants and Fig. 1 shows that these double mutants also fail to replicate in the presence of HU.

To test whether the host DNA is degraded after infection with *nd28*, the amount of *E. coli* DNA rendered acid-soluble was measured at various times after infection. Because phage DNA synthesis obscures the degradation pattern of host DNA in cells infected with wild-type T4, these studies were conducted with the double mutant *nd28* $\times$ *amN82* in addition to *nd28*, T4, and *amN82* alone. *AmN82* is a gene 44 mutant which induces little or no phage DNA synthesis in *E. coli* B (9). Figure 2 shows the patterns of degradation of host DNA to acid-soluble fragments within 1 hr after infection. Whereas infection with *amN82* caused degradation of more than 80% of the host DNA to acid-soluble form, less than 25% solubilization occurred after infection with *nd28* $\times$ *amN82*, indicating that the *nd28* mutation retards the normal breakdown of the host DNA. Similar results were obtained with *nd28* $\times$ *amB22* (gene 43), *nd28* $\times$ *amE10* (gene 45), and *nd28* $\times$ *amE56* (gene 56).

Another test of the ability of *nd28* to prevent degradation of host DNA is to determine the amount of ^{14}C -thymidine from host DNA which can be incorporated into phage DNA. Since transfer of the bases from the DNA of the host to that of the phage progeny is presumed to occur through degradation of host DNA to mononucleotides and subsequent reincorporation into the nascent-phage DNA strands (8), failure of *nd28*

FIG. 1. Phage production in the presence and absence of hydroxyurea. Cells were infected with phage at a multiplicity of infection of about 0.02 and incubated in the absence of HU (●) or in the presence of 0.01 M HU (○) at 37°C. Phage production is expressed as log (progeny/parent); thus, the horizontal line at a value of zero on the ordinate represents the input phage titer.

to degrade host DNA should preclude transfer of label from the host DNA to the newly synthesized phage. To test this corollary, cells were labeled with ^{14}C -thymidine, phage were added, and samples were taken at various times. The infected cultures were lysed with chloroform and centrifuged at $5,000 \times g$ for 10 min, and the supernatants were analyzed by CsCl gradient centrifugation as described above. Three radioactive bands were obtained as shown in Fig. 3:

FIG. 2. Degradation of host DNA to acid-soluble form after phage infection. The *E. coli* B DNA was labeled with ^{14}C -thymidine before infection. The multiplicity of infection was 5. The net conversion of this DNA to acid-soluble form was determined after infection with T4 (●), nd28 (○), amN82 (■), and nd28 $\times$ amN82 (□). The final phage titers after 60 min for T4, nd28, amN82, and nd28 $\times$ amN82 in this experiment were 8.1×10^{10} , 9.1×10^{10} , 1.4×10^8 , and $<5 \times 10^6$ plaque-forming units/ml.

FIG. 3. Transfer of ^{14}C -thymidine from host DNA to phage. Cells were labeled with ^{14}C -thymidine, infected with either T4 or nd28 phage at a multiplicity of infection of 5, lysed with CHCl_3 after 60 min, and analyzed by CsCl gradient centrifugation. The results are plotted as counts/min (●) and plaque-forming units (○) per fraction. The density increases from left to right.

band A contains debris, band B contains viable phage, and band C contains DNA (both phage and host). The specific activity of the T4 phage produced during the 60-min incubation is about six times that of the nd28 phage, confirming that nd28 cannot efficiently use the host DNA as a precursor for phage DNA synthesis. Similar results were obtained when the lysates were analyzed 36 min after infection.

It is important to note that the reduced amount of ^{14}C -thymidine in the phage peak is not accompanied by an increased amount in the DNA peak. Instead, the radioactivity missing from the gradient was found in the precipitate obtained from the $5,000 \times g$ centrifugation. Apparently, the failure to degrade the host DNA causes it to remain in some sort of very large complex. Preliminary experiments with the technique described by Earhart et al. (5) suggests that a sizable fraction of this DNA may still be membrane-bound.

To determine the size of the host DNA which remains acid-insoluble after infection with nd28, lysates were prepared from ^{14}C -thymidine labeled cultures of *E. coli* at various times after infection with T4 and nd28 phage. Analysis of these lysates by sucrose gradient centrifugation showed that after infection with T4, the host DNA was reduced to fragments sedimenting with a velocity corresponding to a molecular weight of about 10^7 daltons by 10 min after infection (Fig. 4). In contrast, the DNA from the cells infected with nd28 remained a constant size from 6 to 30 min after infection. Comparison of the sedimentation rates of a T4 marker (4, 21) and the host DNA after infection with nd28 shows that the latter has an average molecular weight of approximately 2×10^8 daltons. A sample of the nd28 lysate withdrawn 10 min after infection was analyzed on an alkaline sucrose gradient to determine whether single strand breaks were present. The ^{14}C -labeled host DNA sedimented slightly faster than the T4 marker, at a rate corresponding to a molecular weight of 10^8 daltons. Thus, the molecular weight of the single strands is one half that of the duplex molecules, suggesting there were no single-strand breaks present at 10 min after infection.

The accumulation of high-molecular-weight fragments of host DNA without single-strand nicks suggested that nd28 failed to induce T4 endonuclease II, an enzyme recently described by Sadowski and Hurwitz (30). This hypothesis was verified when Sadowski assayed extracts of *E. coli* infected with our nd28 phage and found that endonuclease II activity was absent (P. D. Sadowski, personal communication).

FIG. 4. Sedimentation analyses of ^{14}C -labeled host DNA after phage infection. Cells were labeled with ^{14}C -thymidine, infected with either T4 (dashed line) or nd28 (solid line) phage at a multiplicity of infection of 3, lysed at various times after infection, and applied to 5 to 20% sucrose gradients as described in the text. ^3H -labeled T4 DNA (molecular weight = 130×10^6) and $\phi 29$ DNA (molecular weight = 11×10^6) (I) were added as markers. The right ordinate represents the meniscus of each solution.

In attempting to assess the overall enzymatic capabilities of these mutants, other phage-induced enzymes were also measured. It was found that normal amounts of deoxycytidinetriphosphatase, dCMP hydroxymethylase, dCMP deaminase, and dihydrofolate reductase were present in extracts from cells infected with nd28 (Table 1). The data also indicate that the total exonuclease activity was normal in the crude extract. In an effort to determine whether the distribution of the known exonucleases was similar to that of wild-type T4, the extract was subjected to chromatography on DEAE-cellulose. This analysis

(Fig. 5) showed that there was little or no exonuclease activity in the nd28 chromatogram in the fractions corresponding to the T4 exonuclease A region of the wild-type T4 chromatogram (peak A, Fig. 5). Since T4 exonuclease A is responsible for the major portion of the exonuclease activity

TABLE 1. Phage-induced enzyme activities in crude extracts

Extract	Relative specific enzyme activity ^a				
	dCTPase	dCMP hydroxymethylase	dCMP deaminase	Dihydrofolate reductase	Exonuclease
Uninfected...	0.00	0.00	0.00	0.14	0.28
nd28 ^b	1.09	0.99	0.92	0.98	0.99
T4.....	1.00	1.00	1.00	1.00	1.00

^a Abbreviations: dCTPase, deoxycytidinetriphosphatase; dCMP, deoxycytidine monophosphate.

^b Unpurified isolate was used to prepare this extract.

FIG. 5. DEAE-cellulose chromatography of protein fractions from uninfected *E. coli* B, and *E. coli* infected with T4 and nd28 phage. The A_{280} of the effluent is indicated by the dashed line and the exonuclease activity is indicated by the solid line. Peaks IVa, IVb, and I correspond to *E. coli* exonucleases IVa, IVb, and I, respectively (16). Peaks B and A correspond to phage-induced DNA polymerase-exonuclease and exonuclease A, respectively (32). The multiplicity of infection was 2.

in extracts of infected cells, these results suggest that T4 exonuclease A is induced by *nd28* but that the protein induced by this mutant is altered and is not stable during chromatography on DEAE-cellulose.

Other mutants lacking T4 endonuclease II have recently been isolated (K. Hercules et al., Fed. Proc. 29:465 1970). We prepared extracts from three mutants, kindly furnished by J. S. Wiberg, and subjected them to chromatography on DEAE-cellulose. In two of these mutants, the amount of the T4 exonuclease A obtained by DEAE-chromatography was somewhat less than the level induced by wild-type T4, and in the third mutant the level of this enzyme was similar to that of T4.

Preliminary crosses of *nd28* with various *am* mutants in genes distributed throughout the map of phage T4 indicated that it is linked only to mutants in the region of gene 63. Two-factor cross data (Fig. 6) showed that *nd28* mapped close to *amM69* (gene 63) and between *amA453* (gene 32) and *amN54* (gene 31) (7). The order shown in Fig. 6 was deduced from these data and appropriate three-point crosses. When *nd28* was crossed with *amM69 amA453*, the per cent recombination was 1.18 and 1.24 in two experiments; crosses between *nd28* and *amM69 amN54* gave values of 2.70 and 2.61. These results indicate that *nd28* is located between *amA453* and *amM69*.

DISCUSSION

The results clearly establish that the *nd28* genome carries a nonlethal mutation which blocks its ability to induce degradation of the bacterial DNA of the host and thereby prevents incorporation of the host DNA into progeny phage. Isolation of this nuclease-deficient mutant was facilitated by comparing its ability to grow in the presence and absence of HU, since inability to use host deoxyribonucleotides becomes conditionally lethal if the pathway for de novo deoxyribonucleotide synthesis is also blocked.

FIG. 6. Location of the *nd28* mutation on the T4 chromosome (7, 39). The values indicate the average per cent recombination observed for each interval; the superscripts indicate the number of times each cross was performed. For the crosses shown, the average deviation from the mean was 11%. The standard deviation for the *nd28*—*amM69* interval was 0.16.

The *nd28* mutant does not induce synthesis of T4 endonuclease II. Correlation of the absence of T4 endonuclease II activity with the inability to degrade host DNA strongly suggests that this enzyme is required in the degradative pathway. The specificity of endonuclease II for cytosine-containing DNA and its failure to degrade T4 DNA also implicate this enzyme in the role of host DNA degradation.

Although the data presented in this paper constitute the only direct evidence implicating a specific enzyme in the pathway for the degradation of host DNA, a more detailed tentative model for the complete degradative sequence can be deduced from a number of other observations. The pathway outlined in Fig. 7 suggests that at least five distinct enzymatic steps may be involved. Several observations imply that there may be at least one degradative step prior to hydrolysis by T4 endonuclease II. The host DNA fragments which accumulate in the absence of T4 endonuclease II have a molecular weight of about 2×10^8 daltons which is only one-tenth of the size of the original *E. coli* genome. The relative constancy of the size of these fragments throughout the first 30 min suggests that degradation occurs at specific sites and then stops. This pattern is more consistent with one of an enzymatic hydrolysis rather than one of random breakage. In addition, the data of Bose and Warren (3) suggest that there is an initial discrete state in the degradation of host DNA which yields a product with a molecular weight of 10^8 to 2×10^8 which is about the same size as the fragments that accumulate after infection with *nd28*. This initial step may account for the observations, made by light and electron microscopy (17, 22, 23), which indicate that by 2 min after infection with T2 there is a dispersal of the bacterial nucleus, a process that is blocked by the addition of chloramphenicol (17). However, the techniques for ultracentrifugal analysis of these large DNA molecules are still poorly developed, and artifactual breakage of DNA strands, or formation and disruption of

FIG. 7. Hypothetical scheme for degradation of host DNA after infection with T4 phage. The stage at which degradation is blocked in cells infected with T4*nd28* is designated. Also shown is the step or steps at which degradation is interrupted in cells infected with phage carrying mutations in genes 46 or 47.

complexes between DNA and other cellular material, may make this interpretation invalid.

The second step in the pathway of host DNA degradation outlined in Fig. 7 is the introduction of nicks in one of the strands of the DNA duplex, catalyzed by T4 endonuclease II. This enzyme is not induced by *nd28*, and, after infection with this mutant, the host DNA accumulates as a high-molecular-weight duplex without any single-strand nicks. According to the scheme envisioned by Sadowski and Hurwitz (31), in the next step of host DNA degradation, mononucleotides are released from the 3'-hydroxyl side of the nicked strand by an exonuclease. Enzymes that may participate in this step include T4 exonuclease A, T4 polymerase-exonuclease, the known exonucleases of the host (28), or other enzymes which have not yet been characterized. Once the single-strand gaps have been widened by an exonuclease, the complementary strand can be cleaved by T4 endonuclease IV (31), giving shorter duplex molecules with molecular weights of 10^6 to 10^7 daltons. Duplex fragments of this size accumulate in cells infected with phage bearing mutations in genes 46 and 47 (21), suggesting that the products of these genes are involved in the one or more final steps in which these fragments are hydrolyzed to mononucleotides.

It is also of interest that the *nd28* mutant appears to induce an unstable T4 exonuclease A. In view of the lengthy mutagenic treatment preceding the isolation of these mutants, one would expect to find many additional nonlethal mutations in the *nd* isolates. When three other suspected *nd* isolates were assayed for the enzymes listed in Table 1, two of the mutants induced defective dCMP deaminase, one induced a defective dCMP hydroxymethylase, and all three induced defective T4 exonuclease A. No dihydrofolate reductase or deoxycytidinetriphosphatase mutants were identified. Although extraneous mutations are apparently prevalent, the high frequency of T4 exonuclease A deficiency in the HU-sensitive isolates would seem to indicate that such mutations may be more than fortuitous. This conclusion is supported by the finding that two of the three T4 endonuclease II-deficient mutants isolated by Wiberg, by a procedure which did not involve extensive mutagenesis, were found by us to induce less than normal amounts of T4 exonuclease A. The fact that T4 exonuclease A deficiency in *nd28* was not eliminated by backcrossing with wild-type T4 six times suggests that the gene for this nuclease may be closely linked to the *nd28* gene.

The location of the *nd28* mutation on the map of the phage chromosome (7, 39) is of considerable interest. It is completely unlinked to the

other two genes implicated in host DNA degradation, genes 46 and 47 (21, 38). These two genes are essential for normal phage replication. In contrast, *nd28* replicates normally, and the *nd28* mutation is located close to genes for the non-essential enzymes dihydrofolate reductase, dCMP deaminase, thymidylate synthetase, and ribonucleotide reductase (39).

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grants AI-07898 to Huber R. Warner, AI-07946 to D. Peter Snustad, and AI-04479 to James F. Koerner from the National Institute of Allergy and Infectious Diseases.

We thank Carol Lien, Suzanne Rodell, and Joanne Smith for technical assistance.

LITERATURE CITED

- Anderson, D. L., D. D. Hickman, and B. E. Reilly. 1966. Structure of *Bacillus subtilis* bacteriophage $\phi 29$ and the length of $\phi 29$ deoxyribonucleic acid. *J. Bacteriol.* 91:2081-2089.
- Ando, T., J. Takagi, T. Kosawa, and Y. Ikeda. 1969. Isolation and characterization of enzymes with nicking action from phage T4-infected *Escherichia coli*. *J. Biochem. (Japan)* 66:1-10.
- Bose, S., and R. J. Warren. 1969. Bacteriophage-induced inhibition of host functions. II. Evidence for multiple, sequential bacteriophage-induced deoxyribonucleases responsible for degradation of cellular deoxyribonucleic acid. *J. Virol.* 3:549-556.
- Burgi, E., and A. D. Hershey. 1963. Sedimentation rate as a measure of molecular weight of DNA. *Biophys. J.* 3:309-321.
- Earhart, C. F., G. Y. Tremblay, M. J. Daniels, and M. Schaechter. 1968. DNA replication studied by a new method for the isolation of cell membrane-DNA complexes. *Cold Spring Harbor Symp. Quant. Biol.* 33:707-710.
- Edgar, R. S. 1963. Some technical considerations concerning experiments on phage recombination, p. 19-36. *In* W. J. Burdette (ed.), *Methodology in basic genetics*. Holden-Day Inc., San Francisco.
- Edgar, R. S., and W. B. Wood. 1966. Morphogenesis of bacteriophage T4 in extracts of mutant-infected cells. *Proc. Nat. Acad. Sci. U.S.A.* 55:498-505.
- Elam, J. S., and J. F. Koerner. 1970. Sources of the nucleotide pools in *Escherichia coli* after infection with bacteriophage T2. *J. Biol. Chem.* 245:1012-1019.
- Epstein, R. H., A. Bolle, C. M. Steinberg, E. Kellenberger, E. Boy de la Tour, R. Chevalley, R. S. Edgar, M. Susman, G. H. Denhardt, and A. Lielausis. 1963. Physiological studies of conditional lethal mutants of bacteriophage T4D. *Cold Spring Harbor Symp. Quant. Biol.* 28:375-392.
- Frankel, F. R. 1968. Evidence for long DNA strands in the replicating pool after T4 infection. *Proc. Nat. Acad. Sci. U.S.A.* 59:131-138.
- Fraser, D., and E. A. Jerrel. 1953. The amino acid composition of T3 bacteriophage. *J. Biol. Chem.* 205:291-295.
- Goulian, M., Z. J. Lucas, and A. Kornberg. 1968. Enzymatic synthesis of deoxyribonucleic acid. XXV. Purification and properties of deoxyribonucleic acid polymerase induced by infection with phage T4. *J. Biol. Chem.* 243:627-638.
- Hershey, A. D., J. Dixon, and M. Chase. 1953. Nucleic acid economy in bacteria infected with bacteriophage T2. I. Purine and pyrimidine composition. *J. Gen. Physiol.* 36:777-789.
- Hershey, A. D., and N. E. Melechen. 1957. Synthesis of phage-precursor nucleic acid in the presence of chloramphenicol. *Virology* 3:207-236.

15. Hurwitz, J., A. Becker, M. L. Gefter, and M. Gold. 1967. Enzymatic reactions at termini of DNA. *J. Cell. Physiol.* 70 (Suppl. 1):181-199.
16. Jorgensen, S. E., and J. F. Koerner. 1966. Separation and characterization of deoxyribonucleases of *Escherichia coli* B. I. Chromatographic separation and properties of two deoxyribo-oligonucleotidases. *J. Biol. Chem.* 241:3090-3096.
17. Kellenberger, E., J. Séchaud, and A. Ryter. 1959. Electron microscopical studies of phage multiplication. IV. The establishment of the DNA pool of vegetative phage and the maturation of phage particles. *Virology* 8:478-498.
18. Koch, A. L., F. W. Putnam, and E. A. Evans, Jr. 1952. Biochemical studies of virus reproduction. VIII. Purine metabolism. *J. Biol. Chem.* 197:113-120.
19. Kozloff, L. M. 1953. Origin and fate of bacteriophage material. Cold Spring Harbor Symp. Quant. Biol. 18:209-220.
20. Kozloff, L. M., and F. W. Putnam. 1950. Biochemical studies of virus reproduction. III. The origin of virus phosphorus in the *Escherichia coli* T6 bacteriophage system. *J. Biol. Chem.* 182:229-242.
21. Kutter, E. M., and J. S. Wiberg. 1968. Degradation of cytosine-containing bacterial and bacteriophage DNA after infection of *Escherichia coli* B with bacteriophage T4D wild type and with mutants defective in genes 46, 47, and 56. *J. Mol. Biol.* 38:395-411.
22. Luria, S. E., and M. L. Human. 1950. Chromatin staining of bacteria during bacteriophage infection. *J. Bacteriol.* 59: 551-560.
23. Murray, R. G. E., D. H. Gillen, and F. C. Heagy. 1950. Cytological changes in *Escherichia coli* produced by infection with phage T2. *J. Bacteriol.* 59:603-615.
24. Nossal, N. G. 1969. A T4 bacteriophage mutant which lacks deoxyribonucleic acid polymerase but retains the polymerase-associated nuclease. *J. Biol. Chem.* 244:218-220.
25. Oleson, A. E., and J. F. Koerner. 1964. A deoxyribonuclease induced by infection with bacteriophage T2. *J. Biol. Chem.* 239:2935-2943.
26. Peterson, E. A., and H. A. Sober. 1959. Variable gradient device for chromatography. *Anal. Chem.* 31:857-862.
27. Price, A. R., and H. R. Warner. 1968. A structural gene for bacteriophage T4-induced deoxycytidine triphosphate-deoxyuridine triphosphate nucleotidohydrolase. *Virology* 36:523-526.
28. Richardson, C. C. 1969. Enzymes in DNA metabolism. *Annu. Rev. Biochem.* 38:795-840.
29. Sadowski, P., B. Ginsberg, A. Yudelevich, L. Feiner, and J. Hurwitz. 1968. Enzymatic mechanisms of the repair and breakage of DNA. Cold Spring Harbor Symp. Quant. Biol. 33:165-177.
30. Sadowski, P. D., and J. Hurwitz. 1969. Enzymatic breakage of deoxyribonucleic acid. I. Purification and properties of endonuclease II from T4 phage-infected *Escherichia coli*. *J. Biol. Chem.* 244:6182-6191.
31. Sadowski, P. D., and J. Hurwitz. 1969. Enzymatic breakage of deoxyribonucleic acid. II. Purification and properties of endonuclease IV from T4 phage-infected *Escherichia coli*. *J. Biol. Chem.* 244:6192-6198.
32. Short, E. C., Jr., and J. F. Koerner. 1965. Separation of deoxyribooligonucleotidases induced by infection with bacteriophage T2. *Proc. Nat. Acad. Sci. U. S. A.* 54:595-600.
33. Short, E. C., Jr., and J. F. Koerner. 1969. Separation and characterization of deoxyribonucleases of *Escherichia coli* B. II. Further purification and properties of an exonuclease induced by infection with bacteriophage T2. *J. Biol. Chem.* 244:1487-1496.
34. Tessman, I. 1968. Mutagenic treatment of double- and single-stranded phages T4 and S13 with hydroxylamine. *Virology* 35:330-333.
35. Warner, H. R., and M. D. Hobbs. 1969. Effect of hydroxyurea on replication of bacteriophage T4 in *Escherichia coli*. *J. Virol.* 3:331-336.
36. Warner, H. R., and N. Lewis. 1966. The synthesis of deoxycytidylate deaminase and dihydrofolate reductase and its control in *Escherichia coli* infected with bacteriophage T4 and T4 amber mutants. *Virology* 29:172-175.
37. Weed, L. L., and S. S. Cohen. 1951. The utilization of host pyrimidines in the synthesis of bacterial viruses. *J. Biol. Chem.* 192:693-700.
38. Wiberg, J. S. 1966. Mutants of bacteriophage T4 unable to cause breakdown of host DNA. *Proc. Nat. Acad. Sci. U. S. A.* 55:614-621.
39. Yeh, Y. C., E. J. Dubovi, and I. Tessman. 1969. Control of pyrimidine biosynthesis by phage T4: mutants unable to catalyze the reduction of cytidine diphosphate. *Virology* 37:615-623.