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Role of the T5 Gene D15 Nuclease in the Generation of Nicked Bacteriophage T5 DNA

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The processing of newly replicated concatameric T5 DNA into both single stranded DNA chains of unit length and single-stranded fragments of sizes comparable to those found in mature T5 virion DNA occurs in the absence of late T5 protein synthesis. The formation of unit-length, single-stranded DNA chains does not require the early T5 gene D15 nuclease; however, the subsequent formation of the single-stranded fragments does require that the D15 nuclease be functional. A reexamination of the properties of the purified D15 nuclease under a variety of conditions showed that, in addition to functioning as a 5′→3′ exonuclease, the enzyme can also introduce endonucleolytic scissions into mature T5 DNA in a reaction that requires duplex T5 DNA and preexisting, single-stranded interruptions.

The location, origin, and function of the specific, single-stranded interruptions (nicks) contained within the genome of bacteriophage T5 DNA have attracted considerable attention for a number of years. The proposed function of the nicks currently centers around their putative role in transcription of late T5 genes. This transcription is known to require the products of both the T5 "early" genes, C2 and D15 (3, 4). Szabo et al. (19) have provided some evidence supporting the proposal of Chinnadurai and McCorquodale (4) that the C2 gene product is a phage "sigma-like" factor, which, as a consequence of binding to *Escherichia coli* RNA polymerase, permits the host enzyme to recognize the late class of T5 DNA promotors. The gene D15 product is known to be a nuclease (6, 9). Enzymatic studies of partially purified D15 nuclease have shown that the protein possesses 5′→3′ exonuclease activity on both single- and double-stranded DNA (6, 14). T5 strains containing amber mutations in gene D15 not only fail to initiate late transcription, but also fail to introduce the single-stranded interruptions into newly replicated phage DNA (7).

There are at least two mechanisms by which the gene D15 product could control the introduction of nicks into replicating T5 DNA. The first, and the most direct, hypothesis would be that the nuclease encoded by gene D15 can also function as a very specific endonuclease and thereby introduce the nicks into replicating DNA. This hypothesis would also be consistent with the proposal that a nicked DNA template is required for late transcription (11). The enzyme must, however, possess very little, if any, general endonucleolytic activity because the protein is unable to cleave either double- or single-stranded circular ϕX174 DNA (6). Alternatively, the introduction of nicks into T5 DNA might be under more indirect control of the D15 gene. For example, gene D15 amber mutants are unable to synthesize late T5 RNA and proteins, and nicking would then be prevented with D15 mutants if either a late protein or complex of late proteins were required for this process. Our results favor the first hypothesis, and we present evidence suggesting that the D15 nuclease plays a direct role in introducing nicks into replicating T5 DNA. Nicking requires the presence of a functional D15 gene product and can occur in the absence of late T5 protein synthesis. A rigorous examination of the in vitro properties of the purified enzyme have shown that, in addition to its 5′→3′ exonucleolytic action on DNA, the enzyme can also cleave mature T5 DNA endonucleolytically.

MATERIALS AND METHODS

Bacterial strains and bacteriophage. *E. coli* K-12 W3110 *thy su*$^-$, a nonpermissive host for amber mutants of T5, was used for these experiments and has been described previously (13). The wild-type, nondeleted strain of bacteriophage T5, referred to as T5$^+$, was used throughout. Single amber mutants of T5$^+$ in either gene C2 (T5C2*am*M142a) or gene D15 (T5D15*am*H50) were provided by D. J. McCorquodale. The protein products produced by phage genes D15 and C2 are thought to be a nuclease (6, 9) and a "σ" factor, which interacts with *E. coli* RNA polymerase (4, 19), respectively.

Media. T-MGM medium modified to permit the growth of *E. coli* thymine auxotrophs has been de-

scribed previously (10). Nutrient broth contained 5 g of NaCl and 8 g of nutrient broth (Difco Laboratories, Detroit, Mich.) per liter. All media were supplemented to 2 mM $CaCl_2$ before use.

Preparation of enzyme substrates. T5 phage that contained ^{32}P-labeled DNA were grown and purified through the polyethylene glycol step as described previously (8). ^{32}P-labeled ColE1 supercoiled DNA, which contains small single-stranded regions of RNA, was prepared as follows. A culture of *E. coli* K-12 W3110 *thy* (ColE1) cells was grown to stationary phase at 37°C in T-MGM medium modified to contain 0.5% glucose, 10 μg of thymine per ml, 0.25% salt-free Casamino Acids (Nutritional Biochemicals Corp., Cleveland, Ohio), and 136 μg of sodium phosphate per ml. A 20-ml portion of these cells was then used to inoculate 1 liter of medium prewarmed to 37°C. The cells were grown with aeration to a density of 3×10^8 to 5×10^8/ml. Chloramphenicol was then added to a final concentration of 150 μg/ml. After 5 min, a 200-ml portion of the cells was transferred to a flask containing 2 mCi of ^{32}P-labeled sodium P_i, and the incubation was continued for both portions of cells for another 18 h at 37°C. At the end of this period, both labeled and unlabeled cells were combined, chilled to 4°C, and harvested by centrifugation. Purification of the labeled plasmid DNA was based on the procedure by Sidikaro and Nomura (17). All operations were carried out at 4°C. Cells from 1 liter of medium were suspended in 15 ml of a 25% sucrose solution prepared in 0.05 M Tris-hydrochloride (pH 8.0) and were incubated for 5 min. A 3-ml portion of a 10-mg/ml solution of egg white lysozyme in 0.05 M Tris-hydrochloride (pH 8.0) was then added to the suspended cells, and the mixture was incubated an additional 10 min. Then, 6 ml of 0.25 M EDTA (pH 8.0) was added, and the mixture was incubated another 10 min. Lysis of the cells was achieved by adding 6.5 ml of 5 M NaCl, followed by 3 ml of 10% sodium lauryl sulfate (SDS). The suspension was then clarified by centrifugation at 35,000 × *g* for 40 min. The supernatant was carefully decanted, and any floating debris was removed by aspiration. The supernatant was dialyzed against 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) containing 0.05% SDS at a final pH of 7.0. A 31.5-ml volume of the dialyzed sample was added to 30 g of CsCl. After solubilization of the CsCl, 0.5 ml of an ethidium bromide solution (12 mg/ml in sterile water) was added. The solution was then centrifuged in a Beckman 50Ti fixed-angle rotor at 40,000 rpm at 15°C for 48 h. Parallel illumination of the tubes with a long-wavelength UV lamp revealed two well-defined bands of DNA. The lower band containing the supercoiled DNA was collected into a sterile syringe by horizontal puncture of the tube. The ethidium bromide present in the DNA was removed by extraction with isopropanol, and the plasmid DNA was then dialyzed against 0.1× SSC. After dialysis, the material was divided into fractions and stored under liquid nitrogen until used. This procedure yielded 600 μg of purified plasmid DNA at an approximate specific activity of 10,000 cpm/μg per liter of cells. At least 85% of the ColE1 DNA prepared in this way contained regions of RNA as judged by susceptibility of the supercoiled molecules to cleavage by alkalai and RNase when compared with a similar preparation of ColE1 DNA isolated from exponentially growing cells.

Ligated T5 DNA, which contained no single-stranded interruptions, was prepared, by the method of Bujard and Hendrickson (1), through the action of T4 DNA ligase (Miles Laboratories, Inc., Elkhart, Ind.) on purified, native $T5^+$ DNA. Native T5 DNA was prepared by phenol extraction of phage that had been extensively purified by banding on CsCl.

A mixture of radiolabeled 28S and 18S ribosomal RNA isolated from baby hamster kidney cells at a specific activity of 10,000 cpm/μg was generously provided by S. A. Moyer (Vanderbilt University, Nashville, Tenn.).

DNA-RNA hybrid molecules were prepared by the action of avian myeloblastosis virus reverse transcriptase on unlabeled avian myeloblastosis virus genome RNA in the presence of oligodeoxythymine and [^3H]TTP. The purified product was a gift of C. Martin Stoltzfus (Vanderbilt University).

Assay of the T5 nuclease encoded by T5 gene D15. The exonucleolytic activity of the T5 D15 nuclease was measured by the method of Paul and Lehman (14). A 50-μl portion of the enzyme solution was added to 0.95 ml of an assay solution that contained 40 μmol of glycine, 8 μmol of $MgCl_2$, 0.8 μmol of EDTA, 52 μg of either heat-denatured salmon sperm or calf thymus DNA, and 500 μg of bovine albumin, at a final pH of 9.3. Samples were incubated for periods up to 30 min at 37°C, and the reaction was terminated by adding 1 ml of 0.5 M perchloric acid. The mixture was chilled to 0°C, and the resulting precipitate was removed by low-speed centrifugation. The optical density of the clarified supernatant was determined at 260 nm against an appropriate blank. If a radioactive substrate was incorporated with the assay, a 0.5-ml sample of the supernatant was added to 5 ml of scintillation fluid (Hydromix, Yorktown Research) and counted. Any corrections for quenching of the radioactivity contained in the supernatant fluid under these conditions was determined by adding known amounts of the radioactive substrate in question to 5 ml of scintillation fluid containing 0.5 ml of acidified assay solution. One unit of enzyme activity is defined as the amount of enzyme that liberates 10 nmol or 3 μg of nucleic acid as acid-soluble material in 30 min as determined by absorption at 260 nm. It should be noted that, although both native and heat-denatured DNA work equally well for the purpose of the assay, we chose to use heat-denatured DNA for most assays. The heat denaturation step inactivates traces of DNase, but not RNase, activity frequently present in most commercial preparations of DNA. For some experiments, assays were carried out at different hydrogen ion concentrations. Where a pH other than 9.3 was chosen for the enzyme assay, the glycine in the assay solution was replaced by 20 μmol of *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) for assays at pH 6.9 or by 20 μmol of Tris-hydrochloride for assays at pH 7.6 and 8.3. All other aspects of the enzyme assay remained unchanged

except where specifically noted in the text.

Assay for endonucleolytic activity of the T5 D15 nuclease toward ColE1 plasmid DNA. For assays at pH 9.3, the assay mixture contained 16 μmol of $MgCl_2$, 100 μmol of glycine, 5 U of enzyme, and 2.5 μg of ^{32}P-labeled ColE1 plasmid DNA (10,000 cpm/μg of DNA) in a final volume of 1.0 ml. For assays carried out at pH 6.9, 100 μmol of HEPES was substituted for glycine, and, for those at either pH 7.6 or 8.3, 20 μmol of Tris-hydrochloride was substituted for glycine. Both the polypropylene tubes in which the assays were performed and the necessary solutions were autoclaved before use. Incubations were for periods up to 1 h at 37°C. The reactions were terminated by adding 1.0 ml of 0.5 M perchloric acid. After acidification, 0.2 ml of a solution containing, as coprecipitants, 500 μg of albumin and 100 μg of salmon sperm DNA was added. The solution was clarified by centrifugation. The amount of radioactive plasmid DNA converted to acid-soluble material was determined by adding a portion of the clarified supernatant solution to 5 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) and counting in a liquid scintillation counter.

In vitro assay of T5 nuclease with a T5 DNA substrate. The assay mixture contained 4 μmol of glycine, 0.8 μmol of $MgCl_2$, 8 nmol of EDTA, and 15 μg of T5$^+$ DNA in a final volume of 96 μl at a final pH of 9.3. For assays at pH 7.5, the glycine was replaced by 4 μmol of HEPES. Where specifically noted, the mixture also contained 0.3 μmol of *p*-mercuric benzoic acid (PMB). The sample was incubated at 37°C for 20 min before the addition of 8 U of enzyme contained in 4 μl of buffer. A 10-μl portion of the reaction mixture was immediately added to an equal volume of a solution containing 100 mM EDTA–6 mM PMB and placed at 0°C. This sample, which served as the zero-time sample, was prepared in this way to test for any contaminating nuclease in both the solutions and the DNA substrate. After removal of this zero-time sample, the incubation was continued at 37°C. Additional 10-μl samples were carefully removed at 0.5, 1, 3, 5, 7.5, 10, 20, and 30 min with an Eppendorf pipette, added to equal volumes of the 100 mM EDTA–6 mM PMB solution, and placed at 0°C. Each sample was then treated with a 4-μl volume of 3.5 M NaOH (to achieve strand separation of the DNA) followed by 1 μl of 50% (wt/vol) sucrose. To avoid artifacts generated by shearing of the DNA, some samples were first incubated with enzyme and then treated with 100 mM EDTA–6 mM PMB and alkali before further handling of the DNA. The results of this control experiment were the same as those observed under the routine assay conditions. The entire sample was placed into an individual sample well of a horizontal 0.7% agarose slab gel (11.3 by 13.2 by 0.6 cm) prepared in a buffer containing 0.036 M Tris-hydrochloride, 0.03 M NaH_2PO_4, 1 mM EDTA, and 0.5 μg of ethidium bromide per ml, at a final pH of 7.9. Electrophoresis was carried out in the same buffer at 4°C for 8 h at 30 V. Bands were visualized with long-wavelength UV light and photographed through a Kodak no. 23A Wratten gelatin filter with an MP/4 Polaroid camera that contained P/N 55 film.

Sample preparation and agarose gel analysis of radiolabeled T5 DNA synthesized in infected cells. Bacteria were grown in T-MGM medium and synchronously infected with T5$^+$ by the procedure of Szabo et al. (19) with the following modifications. After growth and collection of the cells by centrifugation, the cellular pellet was suspended, before infection, in T-MGM buffer lacking thymine to increase the amount of [^{3}H]thymidine incorporated into replicating T5 DNA. Newly synthesized phage DNA was then labeled by the addition of [^{3}H]thymidine (10 μCi/ml, 20 Ci/mmol) (New England Nuclear Corp.) and 200 μg of deoxyadenosine per ml to the infected cultures 16 min after the initiation of infection. Labeling was terminated 5 min later (at 21 min after the initiation of viral development) by the addition of unlabeled thymidine to 200 μg/ml. Five-milliliter samples were taken immediately and at later times, as indicated in the text and figures, and immediately added to 15 ml of solution A (0.01 M KCN, 0.15 M NaCl, 0.015 M EDTA, final pH of 7.5) containing 133 μg of chloramphenicol. After chilling to 4°C, the cells were collected by centrifugation at 3,000 × *g*. All subsequent operations were at 4°C. The cellular pellet was suspended in 0.9 ml of solution B (0.1 M Tris-hydrochloride [pH 7.3], 0.01 M KCN, 0.1 M EDTA), and 0.1 ml of egg white lysozyme (10 mg/ml in solution B) was added. The cells were then placed on ice for 30 min. The protoplasts were lysed, and T5 duplex DNA strands were separated by the addition of 0.25 ml of freshly prepared 2 M NaOH. Before electrophoresis, 0.2-ml portions of the lysed samples were combined with 25-μl portions of ^{32}P-labeled T5 phage DNA denatured by the same procedure. The ^{32}P-labeled T5 DNA was added to all samples to provide internal markers for each major single-stranded species of mature T5 DNA. After combination of the lysed cellular extract with the lysed samples of T5 phage DNA, 40 μl of a solution containing 50% (wt/vol) sucrose and 0.1% bromophenol blue dissolved in 1× electrophoresis buffer (0.036 M Tris base, 0.03 M NaH_2PO_4, 1 mM EDTA, 0.04% SDS) was added. The entire 265-μl sample was carefully loaded on top of a 0.6% agarose (Marine Colloids, Inc.) gel (0.6 by 15 cm). Gels were prepared, and electrophoresis was carried out in electrophoresis buffer for 19 h at 45 V and 4°C. After electrophoresis, the gels were extruded and sliced into 0.5-cm disks. Each disk was added to a large test tube containing 0.5 ml of water. The tubes were placed into a boiling-water bath for 15 min. After heating, 10 ml of Hydromix (Yorktown Research Products) was added to each warm sample, followed by transfer of the mixtures to scintillation vials for subsequent counting.

Purification of the T5 gene D15-encoded nuclease. The purification procedure presented here is derived in part from the published procedures of Paul and Lehman (14) as well as that described by Frenkel and Richardson (6). Cells were grown to 2.5 × 10^8/ml in nutrient broth supplemented with 2 mM $CaCl_2$ and were infected with T5 at a multiplicity of 5. They were harvested by centrifugation 25 min after the addition of phage and could be stored as a

frozen paste without appreciable loss of enzyme activity. Each preparation utilized 44 g (wet weight) of infected cells obtained from 50 liters of broth. All operations were at 4°C.

(i) Preparation of crude extract. The 44 g of frozen cells was suspended in 100 ml of buffer A (0.02 M Tris-hydrochloride, 1 mM mercaptoethanol, final pH of 7.5). The cells were disrupted by passage twice through a French press at 7,500 lb/in^2. After cell disruption, the suspension was diluted to a final volume of 1,250 ml in buffer A. Cell debris was removed by centrifugation at 6,000 × *g* for 10 min. The clarified supernatant represents the crude extract.

(ii) Removal of nucleic acid by precipitation with polyethylene glycol 6000. Solid NaCl was added with stirring to the crude extract to a final concentration of 2 M. An equal volume of 30% (wt/vol) polyethylene glycol 6000 (Baker) dissolved in 2 M NaCl was added slowly with stirring. The stirring was continued for an additional 45 min, and the precipitated nucleic acid was then removed by centrifugation at 19,000 × *g* for 15 min. If the protein concentration is maintained at 50 mg/ml or less, virtually no protein is precipitated, although removal of the nucleic acid is nearly complete. The supernatant (3 liters) was dialyzed against two 30-liter changes of buffer B (0.04 M potassium phosphate, 0.01 mM EDTA, 1 mM mercaptoethanol, final pH of 7.4).

(iii) DEAE-cellulose chromatography. The dialyzed extract was passed at 100 ml/h through a 150-ml DEAE-cellulose (Whatman) column that had been equilibrated with buffer B. The enzyme that adsorbed to the column under these conditions was eluted with an 1,800-ml linear salt gradient, which consisted of 900 ml of buffer B and 900 ml of 0.56 M potassium phosphate–0.1 mM EDTA–1 mM mercaptoethanol, final pH of 7.4. The enzyme eluted in a single peak at about 0.4 M potassium phosphate.

(iv) Phosphocellulose chromatography. Fractions that were eluted from the DEAE-cellulose and contained enzyme activity were pooled and dialyzed against two 12-liter batches of buffer C (0.02 M potassium phosphate, 0.1 M NaCl, 1 mM mercaptoethanol, 0.1 mM EDTA, pH 7.4). After dialysis the sample was passed at 25 ml/h through a 40-ml phosphocellulose (Whatman) column that had been equilibrated with buffer C. The enzyme was eluted from the column with a linear salt gradient that contained 900 ml of buffer C and 900 ml of 0.02 M potassium phosphate–0.4 M NaCl–1 mM mercaptoethanol–0.1 mM EDTA, pH 7.4. The enzyme eluted in a single peak centered at about 0.2 M NaCl. With aged preparations, a much smaller second peak of activity, eluting at lower salt, was also seen. The enzyme contained in this second peak was not examined further.

(v) Hydroxyapatite chromatography and concentration of the purified enzyme. The peak fractions of enzyme eluting from the phosphocellulose column were pooled and rechromatographed by gravity flow onto a column containing 4 g of hydroxyapatite (Bio-Rad Laboratories, Richmond, Calif.) that had been equilibrated with 0.02 M potassium phosphate–1 mM mercaptoethanol–0.1 mM EDTA, pH 7.4. The enzyme was eluted with a linear gradient consisting of 100 ml of 0.1 M potassium phosphate–1 mM mercaptoethanol–0.1 mM EDTA (pH 7.4) and 100 ml of 0.35 M potassium phosphate–1 mM mercaptoethanol–0.1 mM EDTA, pH 7.4. The enzyme eluted midway through the gradient as a single peak. Fractions containing the enzyme were combined, and the final volume (ca. 65 ml) was reduced to about 5 ml by ultrafiltration through an Amicon pressure cell containing a PM10 filter.

RESULTS

Introduction of nicks into replicating T5 DNA in the absence of late T5 protein synthesis. It has been reported that cells infected with T5 strains that contain amber mutations in the early gene, D15, not only fail to nick replicating T5 DNA, but are also incapable of synthesizing late T5 proteins (3). The D15 gene product may participate in the introduction of nicks into replicating DNA in either a direct or indirect fashion. The D15 protein may directly catalyze nicking by introducing single-stranded endonucleolytic cleavages at specific sites within the DNA. Alternatively, nicking could also be controlled indirectly by gene D15. If a late protein or protein complex is responsible for nicking, then an amber mutation in gene D15, which blocks the synthesis of late proteins, could indirectly prevent the introduction of nicks. These two alternatives can be tested experimentally by employing amber mutants of T5 blocked in the early gene C2. T5*am*C2 mutants are another class of early mutants of T5 that, like the T5*am*D15 mutants, fail to synthesize T5 late proteins (4; R. W. Moyer, unpublished data). Cells infected with either D15 or C2 mutants, however, synthesize a normal complement of pre-early proteins and presumably all of the early proteins except for the gene that contains the amber mutation. We would predict that, if the introduction of nicks into T5 DNA requires the presence of late proteins, then cells infected with either a D15 or C2 amber mutant should fail to nick replicating T5 DNA. Conversely, if only early proteins, and in particular the product of gene D15, are required for nick introduction, then the T5 DNA synthesized during T5*am*C2 infection of nonpermissive cells, unlike the DNA derived from T5*am*D15-infected cells, should be nicked normally.

To distinguish between these two possibilities, we labeled newly replicated phage DNA with [^{3}H]thymidine in cells infected with either T5$^+$ (as a control for permissive conditions) or with the two mutants, T5*am*C2 and T5*am*D15 (under nonpermissive conditions), from 16 to 21 min after infection. The labeling period in each

case was terminated by adding excess unlabeled thymidine, and samples were removed immediately and at various times thereafter to follow DNA maturation. After cell lysis and denaturation of the DNA, a portion of each sample was analyzed by agarose gel electrophoresis. The presence of newly generated nicks can be determined by the appearance of discrete, smaller-than-unit-length pieces of DNA comparable in size to nicked fragments found in mature virion DNA. Under our conditions of electrophoresis (see above), unit-length strands and the nicked fragments were readily separated from one another (see, for example, Fig. 1A, 2A, and 3A). The resolution obtained by electrophoresis of the single-stranded DNA chains of T5 is clearly superior compared with that obtained by alkaline sucrose gradients.

The results for T5$^+$-infected cells are shown in Fig. 1. Regardless of the time period after infection, all of the samples showed a considerable amount of labeled T5 DNA that did not enter the gel. We have assumed that this material represents the single-stranded concatameric DNA chains of longer than unit length which has been shown to form and persist during the replicative process (2, 7). This material was not examined further. DNA analyzed immediately after the labeling period was terminated (21 min after infection) showed that the material which enters the gel was heterodisperse and ranged in size from 3×10^6 to 40×10^6 daltons, which is the size of single-stranded unit-length chains (Fig. 1A). By 31 min postinfection, the bulk of the DNA entering the gel migrated as a single sharp band, with an electrophoretic mobility identical to that observed for the marker unit-length T5 DNA (Fig. 1B). At 31 min the presence of discrete, single-stranded fragments of less than unit length, which presumably arose as a result of the nicking process, was also observed. The amount of the single-stranded fragments increased somewhat up to 61 min thereafter (Fig. 1B, C, and D). Unlike what has been observed for DNA derived from purified phage, we consistently noted that the newly labeled in vivo DNA contained a much larger stoichiometric amount of single-stranded DNA of unit length compared with the amount of the shorter fragments.

A similar experiment for T5*am*D15-infected cells under nonpermissive conditions is shown in Fig. 2. As was observed in the wild-type infection, some of the labeled DNA failed to enter the gel irrespective of the time after T5*am*D15 infection that it was examined. At 21 min after infection, the DNA that entered the gel was initially much more heterodisperse compared with the 21-min DNA derived from T5$^+$-infected cells (compare Fig. 1A and 2A). By 31 min, however, the bulk of the labeled DNA that entered the gel migrated at the position of unit-length, single-stranded chains (Fig. 2B). Unlike DNA from T5$^+$-infected cells, there was no subsequent formation of single-stranded fragments (i.e., no renicking of the DNA) in T5*am*D15-infected cells. This finding agrees with the earlier observations of Frenkel and Richardson (7) and Carrington and Lunt (2). We conclude from the results of these experiments that the generation of unit-length, single-stranded T5 DNA can occur before, and by a different mechanism from, the subsequent generation of the single-stranded fragments. Our observation that single-stranded chains of unit length appear in T5*am*D15-infected, nonpermissive cells does not agree with the results reported by Frenkel and Richardson (7). The reason for this discrepancy is not clear.

The processing of T5 DNA during the infection of nonpermissive cells with an T5*am*C2 mutant is presented in Fig. 3. Again, at each time point, a considerable proportion of the labeled T5 DNA was found at the origin. The DNA from the sample at the earliest time (21 min) was also found as heterodisperse species, ranging from small pieces up to genome-length strands (Fig. 3A). It is clear that, during the subsequent chase period, more of the T5 DNA was converted to unit-length DNA strands, and some was also renicked to form the smaller DNA fragments (Fig. 3B to D). Since T5*am*C2 mutants do not synthesize the late class of T5 proteins, the generation of both the single-stranded fragments and the single-stranded DNA of unit length can occur in the absence of late protein synthesis. However, only the formation of the single-stranded fragments would appear to require a functional D15 gene product.

Presence of the gene D15-encoded nuclease in extracts of T5*am*C2-infected cells. Both T5*am*C2- and T5*am*D15-infected nonpermissive cells are defective in late protein synthesis, although each should synthesize a full complement of pre-early proteins and significant levels of all early proteins except for the gene product affected by the amber mutation. If the D15 nuclease itself is required for the nicking of T5 DNA, we would expect this protein to be present in T5*am*C2-infected nonpermissive cells, where nicking of replicated DNA does occur. We showed that this is the case by direct assay of the D15 nuclease in T5*am*C2-infected cells. We also compared the D15 nuclease levels in T5*am*C2-infected cells with those in T5$^+$- and

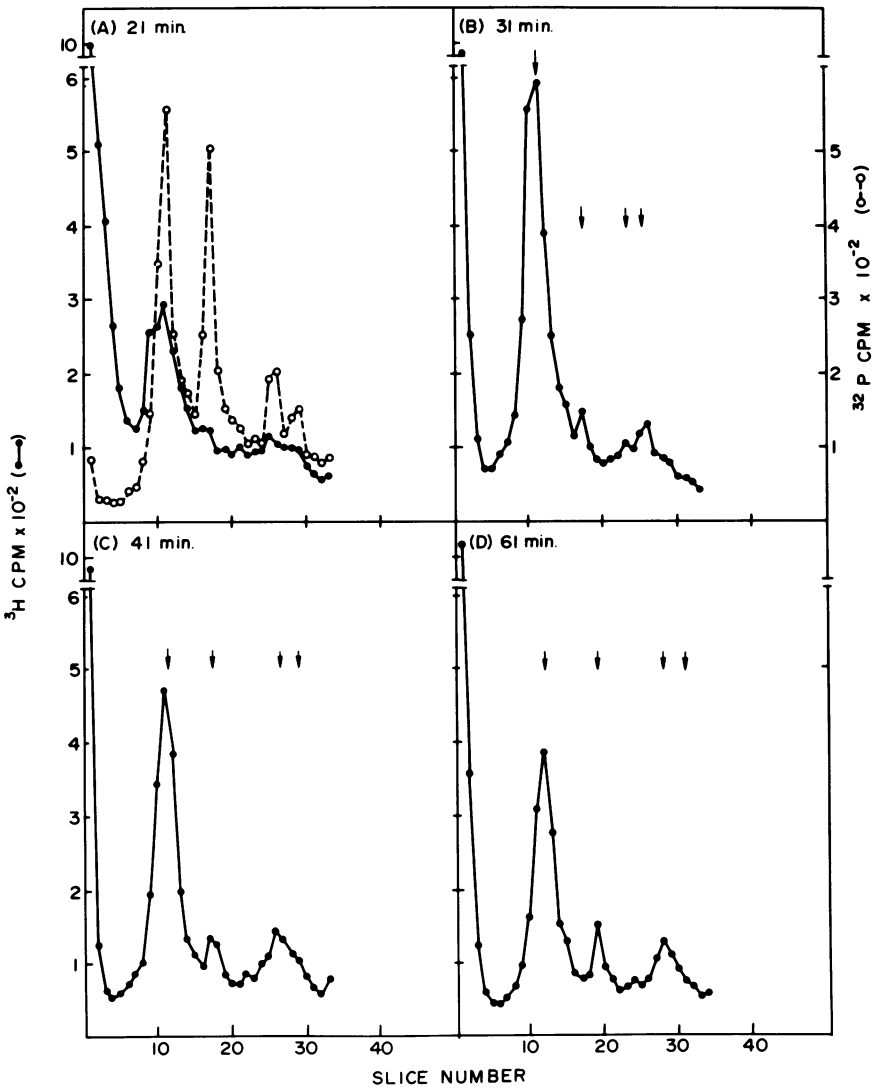

FIG. 1. *Analysis, by agarose gel electrophoresis, of alkaline-denatured, newly labeled T5 DNA derived from T5$^+$-infected E. coli. Newly synthesized phage DNA was labeled from 16 to 21 min after infection as described in Materials and Methods. At 21 min after infection, the labeling was terminated by adding excess unlabeled thymidine. Samples were removed immediately (21 min) and at later times as indicated. After treatment of the cells with alkali, portions of the lysed samples containing the denatured DNA strands were subjected to electrophoresis, for 19 h at 45 V and 4°C, together with approximately 10,000 cpm of ^{32}P-labeled denatured T5 DNA derived from purified virions. (A) Electrophoretic pattern of ^{32}P-labeled marker DNA (○); electrophoretic pattern of ^{3}H-labeled DNA from the experimental sample (●). The direction of electrophoresis is from left to right. The molecular weights of the four peak fractions of the marked T5 DNA from left to right are: 40 × 10^6; an unresolved mixture of 14.5 × 10^6 and 13.9 × 10^6 fragments; 6 × 10^6; and a mixture of 3.7 × 10^6 and 3.1 × 10^6 fragments. For panels (B) to (D), the mobilities of the major species of the marker T5 DNA are indicated by arrows (↓) for simplicity.*

T5*am*D15-infected cells (Fig. 4). In T5$^+$-infected cells, the nuclease appeared at 10 min after infection, reached a maximum at about 25 min, and remained relatively constant throughout the remainder of the infectious cycle. In T5*am*D15-infected cells, as expected, there was no significant increase in the nuclease level over background for up to 60 min after infection. From this control we may also conclude that our assay does indeed provide a measure of

the nuclease encoded by gene D15. T5*am*C2-infected cells also synthesized the D15-encoded nuclease beginning at 10 min after infection, as observed in the wild-type infection. However, between 15 and 20 min after infection, the synthesis of the enzyme stopped, resulting in a somewhat diminished final specific activity compared with that of T5$^+$-infected cells. Nevertheless, appreciable levels of the nuclease were formed. A somewhat reduced final level of the early D15 nuclease was not unexpected since another property of T5*am*C2-infected cells is the premature shutoff of all early protein synthesis. The T5*am*C2-mediated shutoff of early protein synthesis is not thought to be a property pertinent to these studies, since even the somewhat reduced level of the D15 nuclease is sufficient to generate the nicked, single-stranded fragments in replicating T5 DNA.

Purification of gene D15-encoded T5 nuclease. The partially purified nuclease encoded by gene D15 has been studied by both Paul and Lehman (14) and Frenkel and Richardson (6), who concluded that the protein possesses 5'→3' exonuclease activity toward both single- and double-stranded DNA. Frenkel and Richardson (7) and later Chinnadurai and McCorquodale (3) also speculated that this enzyme might pos-

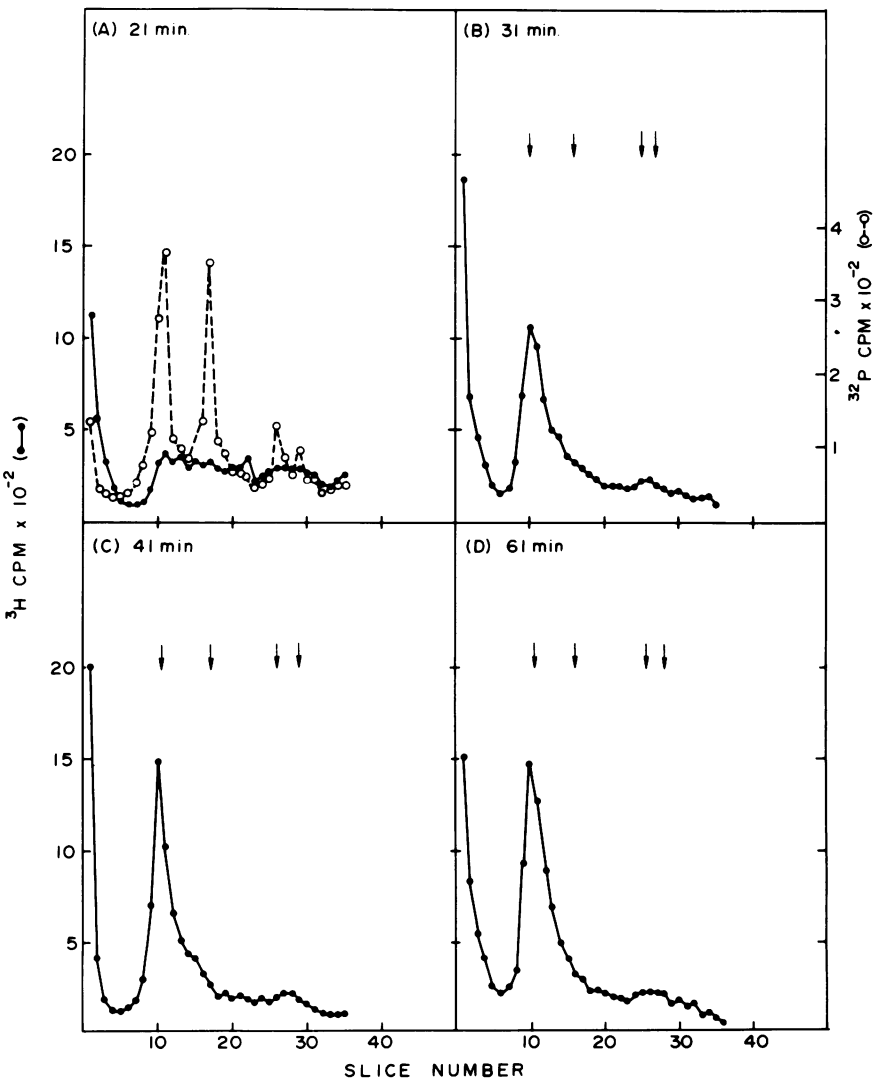

FIG. 2. *Analysis, by agarose gel electrophoresis, of alkaline-denatured, newly labeled T5 DNA derived from T5amD15-infected, nonpermissive E. coli strains. The experimental design for the analysis of DNA from T5amD15-infected cells and the presentation of the data are as described in the legend to Fig. 1.*

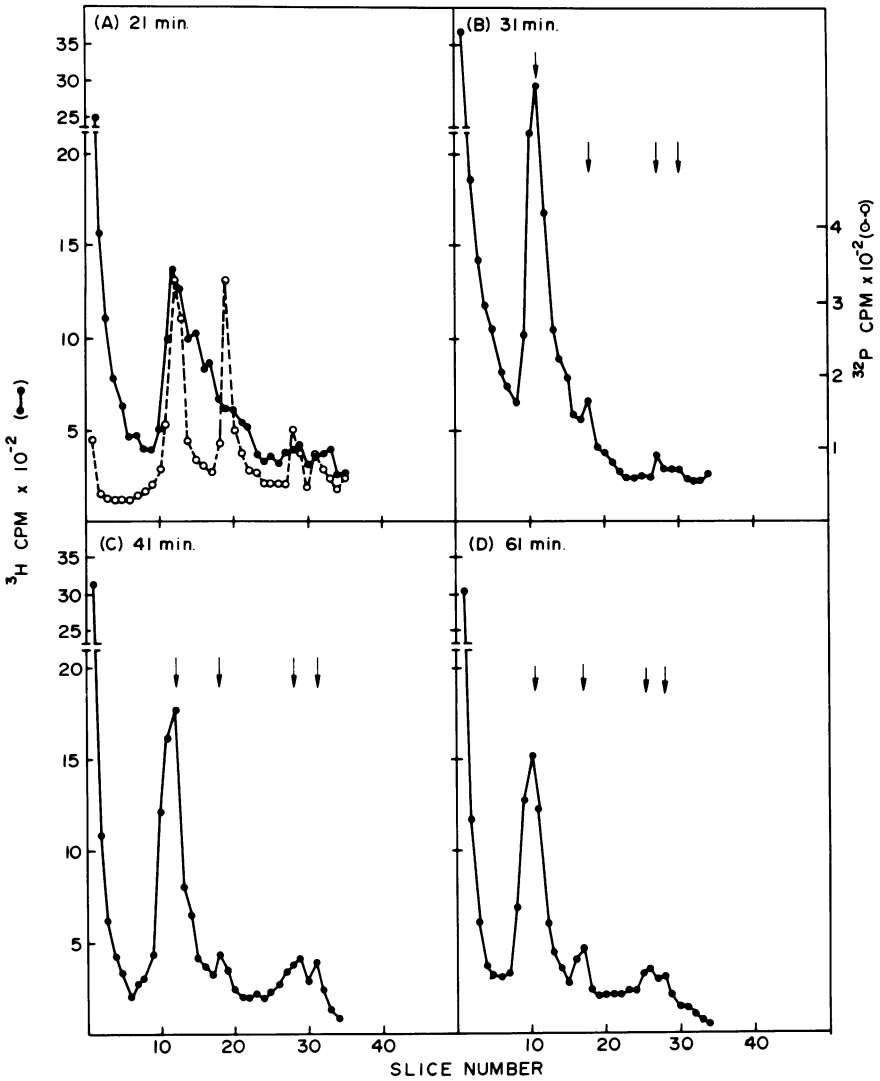

FIG. 3. *Analysis, by agarose gel electrophoresis, of alkaline-denatured, newly labeled T5 DNA derived from T5amC2-infected, nonpermissive E. coli strains. The experimental design for the analysis of DNA from T5amC2-infected cells and the presentation of the data are as described in the legend to Fig. 1.*

sess rather specific endonucleolytic activity toward T5 DNA (even though attempts to cleave closed circular ϕX174 DNA endonucleolytically were unsuccessful [6]). Since the results from the in vivo experiments presented above strongly suggest that the D15 nuclease participated directly in the generation of nicked T5 DNA, we felt that a more thorough reexamination of the catalytic properties of the protein was warranted.

The D15 nuclease purification procedure that we adopted is summarized in Materials and Methods. The pertinent data on specific steps of the purification procedure are presented in Table 1. Since our procedure was derived in part from the methods of Paul and Lehman (14) and Frenkel and Richardson (4), only the relevant points about particular steps of the procedure are mentioned, as follows.

(i) Hydroxyapatite chromatography. Although little in the way of overall purification was achieved by hydroxyapatite chromatography, traces of RNase activity that frequently copurify with the T5 nuclease up to this step are removed by this procedure.

(ii) Ultrafiltration. The enzyme activity of the final preparations was found to be unstable in dilute solutions. This problem was overcome

when the protein was somewhat more concentrated.

(iii) Stability of the purified, concentrated enzyme. At a protein concentration of 100 μg/ml, the enzyme is stable when stored in a solution of 0.05 M potassium phosphate buffer (pH 7.4) and 50% glycerol under liquid nitrogen. All of the reactions and assays using purified enzyme were performed in plastic tubes, since the enzyme has a tendency to adsorb to glass.

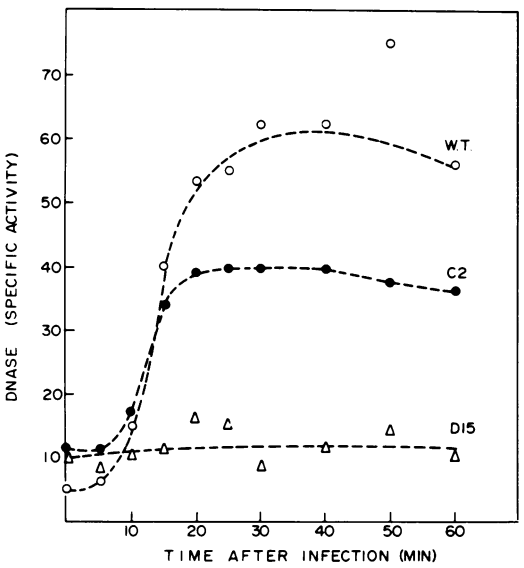

FIG. 4. *Formation of the gene D15-encoded nuclease in productively infected cells and in nonpermissive cells infected with either mutant T5amC2 or mutant T5amD15. Cells were grown and synchronously infected at a multiplicity of infection of 10 by the method of Szabo et al. (19). The final concentration of infected cells was 5 × 10⁸/ml. For each time point indicated, 25 ml of infected cells was removed and added to 10 g of crushed ice containing 3.75 mg of chloramphenicol. All further operations were at 4°C. The cells were pelleted by centrifugation at 12,000 × g for 10 min. The pellet was suspended in 5 ml of a 0.05 M glycylglycine–1 mM mercaptoethanol solution at a final pH of 9.3. The cells were disrupted by sonic treatment, and the resulting cellular debris was removed by centrifugation at 12,000 × g for 10 min. Enzyme assays were performed on the clarified supernatant at pH 9.3, as described in Materials and Methods. The specific activity is given in units of enzyme per milligram of protein present in the clarified extracts. W.T., Wild type.*

Purity and molecular properties of the gene D15-encoded nuclease. Samples of the purified enzyme were subjected to electrophoresis on polyacrylamide gels under several different sets of conditions as a test of its homogeneity. The native enzyme was first subjected to electrophoresis under nondissociating conditions and was found to migrate as two distinct bands (Fig. 5A). Although both bands were invariably present, the relative amount of protein in each band depended on the age of the preparation. The faster-moving band of protein was the predominant species in fresh preparations of the enzyme. This band was apparently converted to the material exhibiting a slower mobility on storage at 0°C.

When the purified D15 nuclease was treated with, and subsequently subjected to, electrophoresis in the presence of either urea or SDS (Fig. 5B and C, respectively), only a single band of protein resulted in both cases. A single subunit was observed regardless of the relative amount of material present in each of the two bands observed when a sample of the enzyme was subjected to electrophoresis on native gels. Therefore, we believe that the faster-moving species of protein observed during electrophoresis under nondenaturing conditions represents a monomeric form of the D15 nuclease, whereas the slower-moving species represents a dimeric form of the enzyme.

By comparing the electrophoretic mobility on SDS gels of the dissociated enzyme with proteins of known molecular weight (Fig. 5C) we were able to assign a tentative molecular weight of 35,000 to the monomeric form of the D15 nuclease. To estimate the molecular weight of the active form of the enzyme, a portion of the purified nuclease was chromatographed in the presence of proteins of known molecular weight on a Sephadex G-100 column. A peak of enzyme activity eluted in a position just after ovalbumin (Fig. 6). This elution position of the enzyme, when compared with that of

TABLE 1. *Summary of the D15 nuclease purification*

Fraction	Total vol (ml)	Total units	Total protein (mg)	Sp act (units/mg)	Yield (%)
Crude extract	1,200	490,000	5,000	98	100
DEAE eluate	187	207,000	46.8	4,400	42
Phosphocellulose eluate	200	119,000	1.49	80,000	25
Hydroxyapatite eluate	63	80,000	ND[a]	ND	16
Ultrafiltration	5.5	73,000	1.14	64,000	15

[a] ND, Not done.

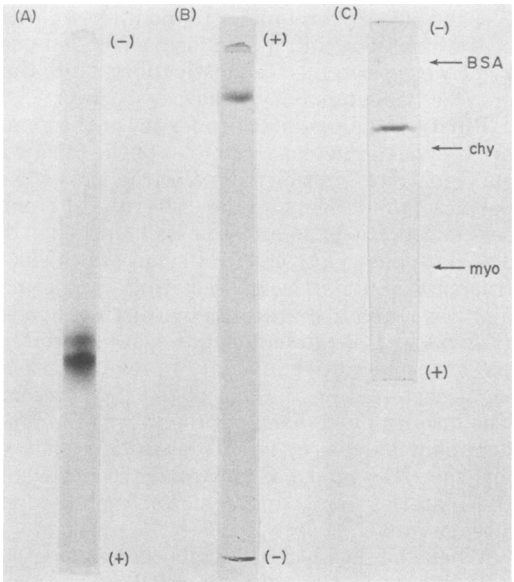

FIG. 5. *Electrophoretic analysis, on polyacrylamide gels, of the gene D15-encoded nuclease under native and denaturing conditions. Samples of the enzyme were electrophoretically analyzed under nondenaturing conditions (A), after treatment with 8 M urea (B), or after denaturation with SDS (C). Electrophoresis under nondenaturing conditions was carried out on 5 μg of enzyme at pH 8.3, with 10-cm 10% polyacrylamide tube gels, by the method of Davis (5). Electrophoresis in the presence of 8 M urea at pH 4.5 was performed on a 10-μg sample of enzyme, with 10-cm 10% polyacrylamide tube gels, as described by Traub et al. (20). Electrophoresis of a sample denatured with SDS was performed, on 10% polyacrylamide gel slabs as described by Laemmli (12), on a 10-μg sample of the purified enzyme. After electrophoresis of nondenatured or urea-denatured samples, the proteins were visualized with amido black staining. (B) The faint band migrating near the end of the gel represents the tracking dye. Coomassie brilliant blue staining was used to visualize the protein bands on the SDS gels. (C) Electrophoretic mobilities of bovine serum albumin (BSA) (67,000), chymotrypsinogen (chy) (25,000), and myoglobin (myo) (17,000) were run simultaneously in adjacent wells of the SDS slab gels.*

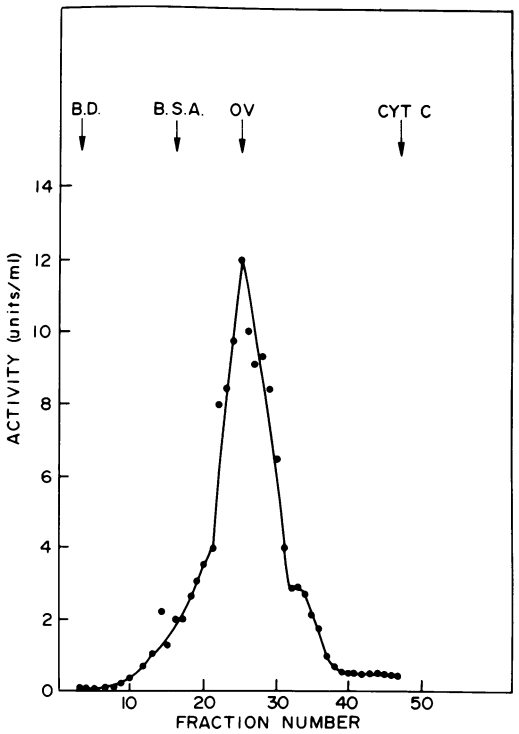

FIG. 6. *Chromatography of the T5 gene D15-encoded nuclease on Sephadex G-100. A portion of the purified enzyme (200 U), together with marker proteins, was chromatographed on a column (1.5 by 90 cm) of Sephadex G-100. All procedures were carried out at 4°C in 0.02 M potassium phosphate buffer (pH 7.4) containing 0.25 M NaCl and 0.1 M EDTA. The void volume of the column was determined with blue dextran (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) of molecular weight 2 × 10^6. The enzyme was chromatographed either separately or together with blue dextran (B.D.), bovine serum albumin (B.S.A.), ovalbumin (OV), or cytochrome c (CYT C). The column fractions were assayed for the D15 nuclease at pH 9.3 as described in Materials and Methods.*

both ovalbumin and the other standard proteins, is consistent with an enzymatically active monomeric form of the enzyme with a molecular weight of approximately 35,000.

Substrate specificity of the purified T5 gene D15-encoded nuclease. The nucleolytic activity of the D15 nuclease toward various forms of nucleic acid was investigated (Table 2). As was first reported by Paul and Lehman (14), the nuclease activity toward single- or double-stranded DNA from a given source is quite similar. Eucaryotic RNA, when added to an assay mixture that also contains DNA, was almost completely resistant to nucleolytic attack. In addition, we observed that the undenatured avian myeloblastosis virus DNA-RNA hybrid product synthesized by reverse transcriptase action on avian myeloblastosis virus RNA was considerably more resistant to digestion by the nuclease than duplex or single-stranded DNA. If the hybrid product was heated, the DNA was much more rapidly attacked by the nuclease. Therefore, it would appear that a significant degree of protection to degradation by the T5 nuclease is conferred on DNA when that DNA is present as part of a DNA-RNA hybrid duplex molecule.

Attempts to demonstrate general endonu-

TABLE 2. *Relative substrate specificity of the T5 gene D15-encoded nuclease*[a]

Substrate	Relative activity (%)
Salmon sperm DNA	
Native	100
Denatured	95
rRNA[b]	0.06
DNA-RNA hybrid[c]	
Minus heat	2.5
Plus heat	30

[a] The nucleolytic activity was determined at pH 9.3 on salmon sperm DNA as described in Materials and Methods.

[b] The susceptibility of rRNA to nucleolytic attack was determined by including 50,000 cpm of ^{3}H-labeled rRNA derived from baby hamster kidney cells (10,000 cpm/μg) in the standard assay mix together with salmon sperm DNA.

[c] Hybrid molecules containing 2,500 cpm derived from [^{3}H]thymidine were included, together with salmon sperm DNA, in the assay mixture at pH 9.3. Denaturation was accomplished before addition of the enzyme by heating the hybrids to 100°C for 2 min followed by quick cooling.

cleolytic activity of the T5 gene D15-encoded nuclease. In earlier experiments, Frenkel and Richardson (6) tested the rate of endonucleolytic cleavage of ϕX174 circular DNA by the D15 nuclease. They concluded that the endonucleolytic activity of the enzyme toward both single- and double-stranded circular DNA was virtually absent. Since our in vivo experiments suggested a direct role for the gene D15-encoded nuclease in nicking replicating T5 DNA, we reinvestigated the general levels of endonuclease associated with the purified enzyme. The substrate we chose for our studies was ColE1 supercoiled DNA isolated from colicinogenic cells that had been exposed to chloramphenicol before isolation of the plasmid DNA. This DNA was chosen as a substrate for two reasons: (i) supercoiled DNA has no free 5′ termini to serve as substrate for the active exonucleolytic activity of the enzyme; and (ii) a high percentage of the ColE1 plasmid DNA isolated from cells exposed to chloramphenicol contains a small region of RNA within one DNA strand (18). Hence, we could test for any potential endonucleolytic cleavage of this supercoiled substrate due either to cleavage of DNA or to endoribonuclease H activity.

The total endonuclease activity was measured by two methods. The first method was to simply expose the ColE1 supercoiled DNA to the purified D15 nuclease for different time periods at different pH values. The treated DNA was then subjected to electrophoresis on agarose gels in the presence of ethidium bromide to separate supercoiled DNA from the potential endonucleolytic and/or exonucleolytic reaction products. The gels were then exposed to UV light, and the loss of fluorescent material from the supercoiled DNA band was estimated visually. No loss of supercoiled material was observed over a pH range of 6.9 to 9.3 in either the presence or the absence of ATP, indicating that the level of endonuclease possessed by the D15 nuclease is quite low (data not shown).

A second, more quantitative, measure of endonuclease activity is an indirect assay based on the premise that the endonucleolytic rate of strand scission catalyzed by the enzyme is very slow compared with the rate of exonucleolytic degradation of a suitable substrate. The introduction of a single nick into a supercoiled DNA molecule should yield a free 5′ terminus. A nick would render the newly cleaved strand susceptible to the much more rapid exonucleolytic activity of the enzyme, resulting in a complete conversion of the strand to acid-soluble material over a time interval that is short compared with that required for the production of a single endonucleolytic cleavage. Therefore, we assume that the rate of release of acid-soluble material from the supercoiled ColE1 plasmid molecules will reflect the slower rate of single-stranded nick introduction. We assayed for endonucleolytic cleavage over a pH range of 6.9 to 9.3 and compared it to the rate of exonucleolytic cleavage over the same pH range (Table 3). The maximum exonuclease activity occurred at pH 9.3. The enzyme activity decreased gradually as the pH was lowered, although considerable activity remained even at pH 6.9. Throughout the pH range measured, virtually no endonuclease activity was observed. In additional experiments, not shown here, we found these results to be unaffected by the addition of ATP to the

TABLE 3. *Relative exonucleolytic and endonucleolytic activities of the T5 Gene D15-encoded nuclease*

pH	Exonuclease[a] (%)	Endonuclease[b] ($\times 10^{-5}$) (%)
9.3	100	1.1
8.3	49	3.1
7.3	20	2.5
6.9	12	8.6

[a] The exonuclease activity was determined on heat-denatured calf thymus DNA as described in Materials and Methods. The maximum exonucleolytic activity observed at pH 9.3 is taken as 100%.

[b] The percent endonuclease activity is calculated relative to the exonucleolytic activity at the same pH.

reaction mixture or by replacing the RNA-containing plasmid by ColE1 supercoiled DNA molecules that lack any regions of RNA.

Action of the T5 gene D15 nuclease toward various forms of T5 DNA. Thus far, our in vitro studies on the substrate specificity of the T5 nuclease with heterologous DNAs agree with those of others (6)—that the primary action of the enzyme is exonucleolytic, with no evidence of endonucleolytic cleavage. Nevertheless, we decided to examine the mode of action of the purified enzyme on its natural substrate, T5 DNA. The results of the digestion of native T5 virion DNA by the D15 nuclease at pH 9.3 and 7.5 is shown in Fig. 7A and B, respectively. After treatment of the native DNA with the enzyme for different lengths of time, the samples were alkali denatured to achieve strand separation of any remaining double-stranded material before the reaction products were analyzed by agarose gel electrophoresis. Within 30 s after addition of the en-

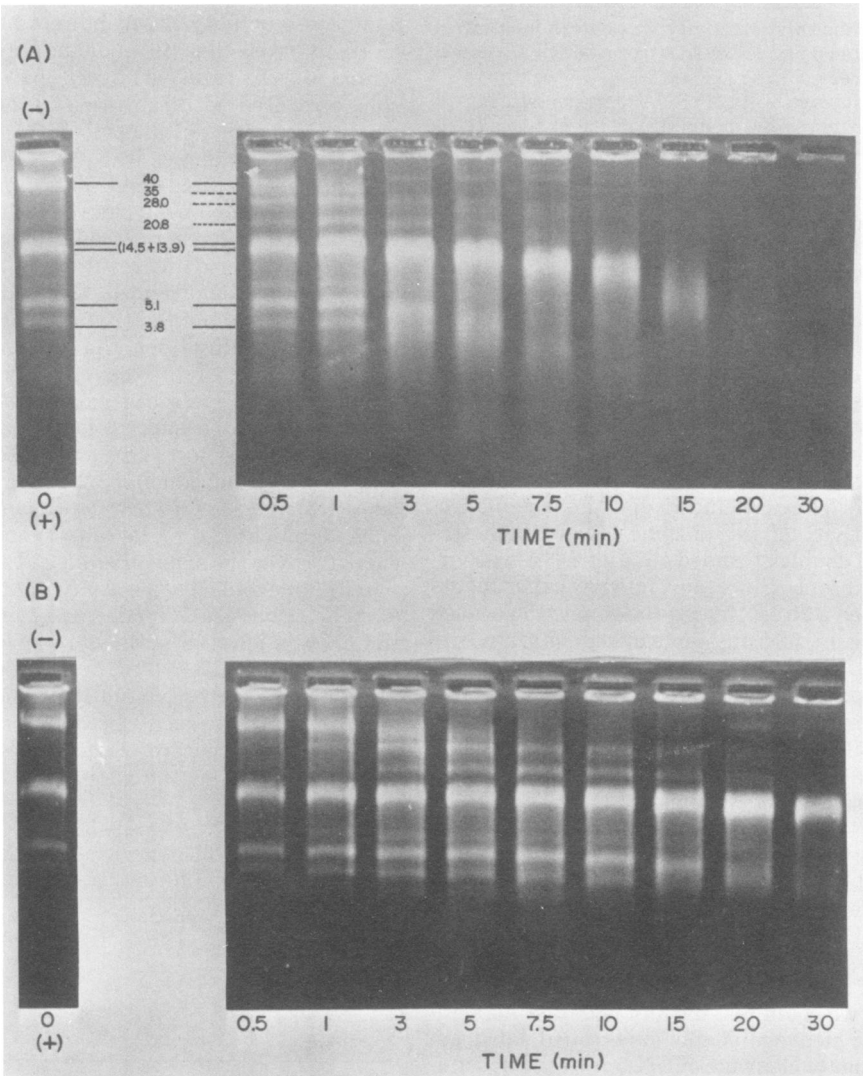

FIG. 7. *Kinetics of digestion of native T5 duplex DNA by the purified T5 gene D15-encoded nuclease at pH 9.3 or 7.5. Native duplex T5 DNA was digested by the D15 nuclease for up to 30 min, and the products were analyzed by agarose gel electrophoresis after strand separation as described in Materials and Methods. The digestion was carried out at either pH 9.3 (A) or pH 7.5 (B). (——) Molecular weights (10^6) of the preexisting major species of single-stranded DNA that arose from strand separation of purified phage T5 DNA; (----) those that appear as the results of nuclease action.*

zyme at pH 9.3, the amount of unit-length DNA strands diminished noticeably, and two discrete single-stranded fragments of 28 × 10^6 and 21 × 10^6 daltons appeared. After a 1-min exposure to the enzyme, the intact strand was almost totally degraded, allowing a third discrete band with a molecular weight of 36 × 10^6 to be resolved. After 3 min of digestion, the fluorescent background of the gel increased throughout, and there was a "broadening" and increase of the 17 × 10^6- and (14.5 + 13.9) × 10^6-dalton bands. After 5 min of digestion, a noticeable decrease in all of the remaining material larger than 17 × 10^6 daltons was observed, accompanied by a loss of resolution and a broadening of the remaining DNA bands toward regions of lower molecular weight. Only significant amounts of material of approximately 17 × 10^6 daltons remained at 7.5 min of digestion, although the area of the peak had shifted to a somewhat lower molecular weight by this time. The average molecular weight of the DNA in this region decreased steadily thereafter between 10 and 20 min of digestion, and by 30 min the DNA was virtually completely digested to material too small to be retained within the gel.

At pH 7.5 the same overall pattern of digestion of T5 DNA seen at pH 9.3 was observed, although the rate was significantly slower. This result was expected because the D15 nuclease is only 20% as active at pH 7.5 as compared with pH 9.3 (Table 3). We believe that the rapid formation of new, discrete DNA bands of 35 × 10^6, 28 × 10^6, and 21 × 10^6 daltons at both pH 9.3 and 7.5 is best explained by a specific endonucleolytic activity of the enzyme toward T5 DNA. However, the eventual broadening and slow decrease in the average molecular weight of each DNA band observed later in the digestion are best explained by the expected exonucleolytic action of the enzyme. These results suggest that the D15 nuclease can function (in vitro) as a specialized endonuclease in addition to its known function as an exonuclease.

We next examined the digestion pattern of ligated T5 DNA by the D15 nuclease at pH 9.3 (Fig. 8). If the nicks contained within the T5 DNA were repaired by the action of T4 DNA ligase before treatment with the D15 nuclease, no new, discrete single-stranded DNA bands (35 × 10^6, 28 × 10^6, and 21 × 10^6 daltons) were observed (cf. Fig. 7 and 8). After 1 min of digestion, the single band of DNA representing both strands of the ligated T5 DNA began to decrease in intensity and broaden. By 20 min of digestion, however, a gradual decrease in the average molecular weight of the remaining DNA was readily apparent. This digestion pattern of ligated DNA is most readily explained by the exclusive exonucleolytic action of the enzyme. It appears, therefore, that the presence of the single-stranded interruptions normally found in native phage DNA is essential for the D15 nuclease to act endonucleolytically. Furthermore, the molecular weights observed for the transiently generated, single-stranded DNA bands would be consistent with their formation by an endonucleolytic cleavage of the intact strand of the duplex T5 DNA across from the preexisting nicks.

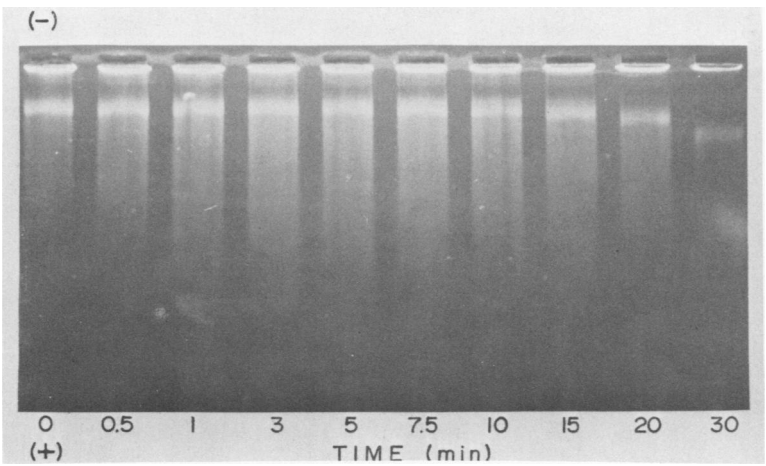

FIG. 8. *Kinetics digestion of ligated T5 duplex DNA by the purified T5 gene D15-encoded nuclease at pH 9.3. Purified T5 DNA was ligated and repurified before digestion by the D15 nuclease. The digestion was carried out for up to 30 min, and the products were analyzed as described in Materials and Methods. As expected, only a single band of single-stranded DNA of approximate molecular weight 40 × 10^6 was initially present owing to repair of the nicked strand by action of the T4 ligase.*

We also determined that the T5 DNA must be double-stranded to allow nicking with the D15 nuclease. If denatured T5 DNA was used as a substrate for the nuclease, no new, discrete single-stranded DNA fragments were observed (Fig. 9). The pattern of DNA digestion is consistent only with the exonucleolytic degradation of the DNA. It is also apparent here that the fragments of T5 DNA with molecular weights of 14.5×10^6 and 13.9×10^6 are relatively refractile to degradation compared with the rest of the DNA. Similar results were also observed to a lesser extent in the other experiments (Fig. 7). The reason for this is not known.

Rogers and Rhoades (15) purified from T5-infected cells several enzyme activities that were capable of introducing single-stranded nicks into a variety of duplex DNA molecules, including ligated T5 DNA. To facilitate detection of these nicking enzymes, the authors treated cell extracts with 3 mM PMB, which completely inhibits the gene D15-encoded nuclease but has little effect on the nicking enzymes they describe. To eliminate the possibility that our nuclease preparations were contaminated with small quantities of the enzymes described by Rogers and Rhoades, we added 3 mM PMB to a reaction mixture containing native T5 DNA. The results (Fig. 10) show that PMB totally inhibited all nuclease activity, including the generation of the single-stranded fragments described in Fig. 7. No evidence of any digestion of the DNA was observed even after 30 min of incubation. These results strongly suggest that both the exonucleolytic and the apparent endonucleolytic activities observed with native T5 DNA as the substrate were catalyzed by the gene D15-encoded nuclease alone.

DISCUSSION

We followed the maturation of newly replicated T5 DNA formed both during the wild-type infection and in nonpermissive cells infected with T5 amber mutants in either early gene C2 or D15. The genes affected are thought to be a phage-encoded "σ" factor and a nuclease, respectively. Both of these amber phages, as a consequence of their individual mutations, fail to synthesize any appreciable quantities of late T5 RNA and protein in nonpermissive hosts (3, 4). The results of these studies show that single-stranded cutting of the newly replicated T5 DNA occurs in at least two distinct steps.

The DNA synthesized in T5*am*D15-infected cells has been shown by sedimentation in neutral sucrose to remain concatameric (2, 7) However, we showed that when this DNA is denatured it consists in part of unit-length, single-stranded chains. Therefore, the first step of cutting appears to be the introduction of single-stranded interruptions or gaps into the concatameric DNA at genome length distances, which would yield single-stranded, unit-length chains after denaturation. In T5*am*D15-infected cells, these single-stranded chains of unit length are the smallest cleavage products generated. A second step of cutting can be observed in both the wild-type and T5*am*C2-infected cells. The end product of this subsequent cutting step is the creation of T5 DNA molecules, which after

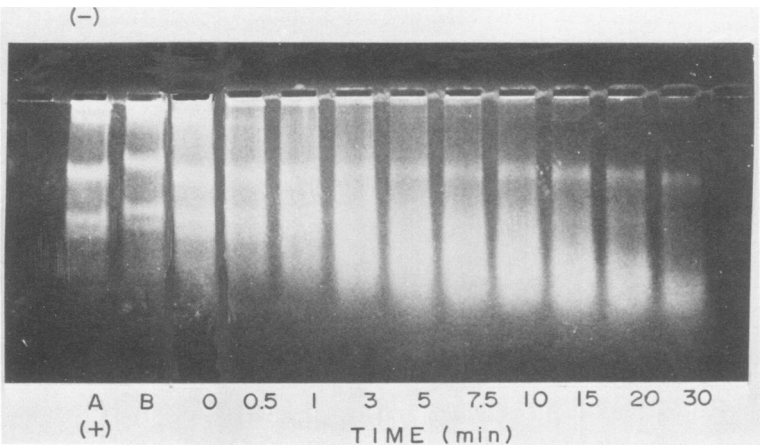

FIG. 9. *Kinetics of digestion of single-stranded T5 DNA by the purified T5 D15-encoded nuclease at pH 9.3. The conditions of digestion were as described for Fig. 7A, except that single-stranded T5 DNA prepared by alkaline denaturation was used as the substrate. In addition, a sample of the denatured DNA before (A) and after (B) a 20-min preincubation at 37°C without enzyme is included.*

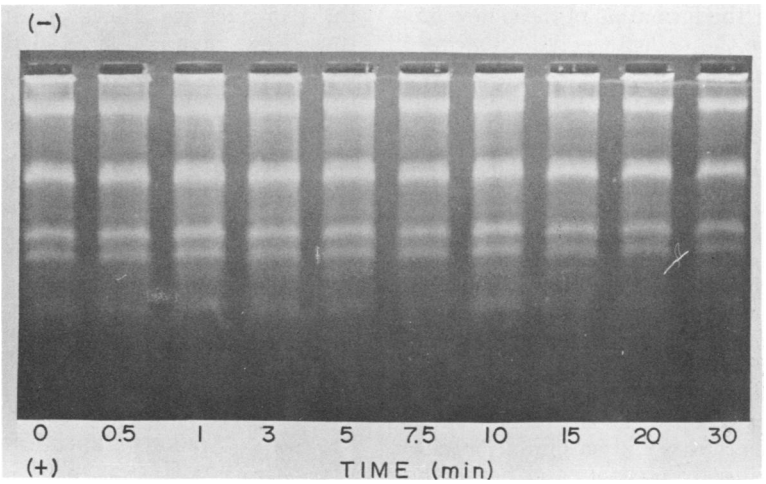

FIG. 10. *Inhibition by 3 mM PMB of the hydrolysis of native T5 DNA by the D15 nuclease at pH 9.3. The reaction conditions were exactly as described in the legend to Fig. 7A, except that the reaction mixture also included 0.03 μmol of PMB.*

denaturation yield not only single-stranded chains of unit length, but also chains of smaller sizes, which are comparable to the less-than-unit-length fragments found in T5 virion DNA (Fig. 1 and 3). These findings directly implicate the D15 nuclease in the second step of DNA cutting, which is the formation of the single-stranded chains of smaller than unit length.

Based on the results of these experiments, we propose that the cutting of replicating T5 DNA to yield the unit-length chains can occur before the second cleavage step and does not require either the C2 or D15 gene products. Whether this order is an obligatory one for phage DNA processing is not yet known. At the present time we do not know which DNA sequences are represented in each single-stranded DNA of unit or less than unit length. It is possible that the intracellular DNA sequences contained within a given single-stranded piece of DNA in vivo are different from the sequences of the pieces of comparable size found in virion phage DNA. In addition, it has been shown that replicating T5 DNA is extensively gapped (2). We do not know whether either or both of the cutting steps we have discussed are accompanied by the simultaneous gapping of the DNA or whether gapping is a separate event. These questions are currently under study in our laboratory.

We have previously shown that the nicks found in input parental T5 DNA are repaired very early in the infectious process (10). We now believe that the subsequent regeneration of nicked T5 DNA requires only early proteins, since nicking occurs in T5*am*C2-infected non-permissive cells (Fig. 3), in which no late RNA or protein synthesis occurs. Our observations are consistent with, but do not prove, the fact that a fully "nicked" T5 DNA template must be recreated from the replicated concatameric DNA to allow late transcription to occur. The protein products that arise from late RNA are the structural proteins of the virus, which implies that both cutting steps that we have described occur before assembly of completed phage particles. These last conclusions are dependent upon the "non-leakiness" of the T5*am*C2 mutant employed for this study. Although it is clear from our unpublished data and the published work of others (4) that little, if any, late proteins can be detected in cells infected with this mutant, we cannot completely rule out the formation of a small amount of a late protein(s), which might constitute a "nicking" enzyme or complex of enzymes.

Our data from studies of the in vivo maturation of T5 DNA suggest a direct role for the D15 nuclease in the generation of the fully nicked T5 DNA. We have purified and reinvestigated the in vitro properties of the D15 nuclease for specific nicking activity toward T5 DNA. Although earlier studies showed that the partially purified enzyme acts only as a 5′→3′ exonuclease (6, 14), we have shown that the enzyme can also act in vitro on native, nicked duplex T5 DNA to generate a product that, after strand separation, yields several new species of single-stranded DNA of defined length (Fig. 7). For the enzyme to generate these unique-sized fragments, a T5 DNA substrate that is both nicked and double-stranded is required. We feel that the mechanism most likely

to account for the formation of these new products would be the endonucleolytic cleavage of the normally intact strand of the T5 duplex DNA across from the preexisting nicks. Such a mechanism would explain our lack of success in showing a similar distribution of products when either ligated T5 DNA or denatured T5 DNA is used as substrate for the enzyme. This proposed mechanism is analogous to the action of S1 nuclease on T5 DNA (16). Endonucleolytic cleavage of native T5 DNA would, however, also lead us to expect that many new bands of DNA should be present, since a number of nicks are contained in native T5 DNA. Examination of the T5 DNA map shows that we would fail to observe many of the other expected bands because they would be similar or identical to ones already present, and hence their detection would be difficult.

The question that served to initiate these studies remains unanswered. We still do not know the enzymatic mechanism by which the single-stranded nicks are introduced into T5 DNA. It is clear that when the early T5 gene D15 is defective there is no nicking of T5 DNA to less-than-unit-length fragments. This finding clearly implies a direct role of the D15 nuclease in the nicking of T5 DNA. Other enzyme activities, however, that must be considered in this process are those characterized by Rogers and Rhoades (15). These workers described three enzyme activities induced upon T5 infection that also appear to be "early" enzymes but, unlike the D15 nuclease, are capable of introducing single-stranded interruptions in vitro into ligated T5 DNA. None of these enzymes or "nickases," however, generates the nicking pattern found in mature T5 DNA extracted from the phage. The "nickases" are distinct from the D15 nuclease since they are resistant to the inhibitor PMB under conditions in which the D15 nuclease is completely inhibited (15). Our experiments do show that these "nickases" cannot be solely responsible for the generation of the mature nicked T5 DNA. In T5*am*D15-infected cells, where the amber mutation is known to reside in the D15-encoded nuclease, no nicking of the T5 DNA occurs in vivo, although one would expect the normal synthesis and expression of the three "nickases."

Our data are compatible with the idea that the D15 nuclease has a direct role in the generation of the single-stranded nicks of mature T5 DNA in vivo. Likewise, the results of our in vitro studies have shown that single-stranded DNA fragments appear to be generated by endonucleolytic cleavage of the native T5 DNA by the D15 nuclease. However, at least some of these new, uniquely sized chains are of sizes different from those of chains of T5 DNA extracted from the virion and must have arisen from the cleavage of the intact strand of T5 DNA. The reason for the failure to reproduce the nick pattern found in native T5 DNA is not clear but may indicate that the "correct" pattern of nicking of T5 DNA in vivo is produced by a protein complex, of which the D15 nuclease is only one component. The specificity of nucleolytic action of the D15 nuclease may thus be modulated by the presence of other proteins within an enzyme complex. This hypothesis is currently under investigation.

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LITERATURE CITED

1. **Bujard, H., and H. E. Hendrickson.** 1973. Structure and function of the Genome of coli phage T5. I. The physical structure of the chromosome of T5+. Eur. J. Biochem. 33:517-528.
2. **Carrington, J. M., and M. R. Lunt.** 1973. Studies on the replication of bacteriophage T5. J. Gen. Virol. 18:91-109.
3. **Chinnadurai, G., and D. J. McCorquodale.** 1973. Requirement of a phage-induced 5'-exonuclease for the expression of late genes of bacteriophage T5. Proc. Natl. Acad. Sci. U.S.A. 70:3502-3505.
4. **Chinnadurai, G., and D. J. McCorquodale.** 1974. Regulation of expression of late genes of bacteriophage T5. J. Virol. 13:85-93.
5. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Anal. N.Y. Acad. Sci. 121:404-427.
6. **Frenkel, G. D., and C. C. Richardson.** 1971. The deoxynuclease induced after infection of *Escherichia coli* by bacteriophage T5. I. Characterization of the enzyme as a 5'-exonuclease. J. Biol. Chem. 246:4839-4847.
7. **Frenkel, G. D., and C. C. Richardson.** 1971. The deoxynuclease induced after infection of *Escherichia coli* by bacteriophage T5. II. Role of the enzyme in replication of phage deoxynucleic acid. J. Biol. Chem. 246:4848-4852.
8. **Hayward, A. S., and M. G. Smith.** 1972. The chromosome of bacteriophage T5. I. Analysis of the single-stranded DNA fragments by agarose gel electrophoresis. J. Mol. Biol. 63:383-395.
9. **Hendrickson, H. E., and D. J. McCorquodale.** 1972. Genetic and physiological studies of bacteriophage T5. J. Virol. 9:981-989.
10. **Herman, R. C., and R. W. Moyer.** 1974. *In vivo* repair of the single-stranded interruptions contained in bacteriophage T5 DNA. Proc. Natl. Acad. Sci. U.S.A. 71:680-684.
11. **Herman, R. C., and R. W. Moyer.** 1975. *In vivo* repair of bacteriophage T5 DNA: an assay for viral growth control. Virology 66:393-407.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins

during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.
13. **Moyer, R. W., A. S. Fu, and C. Szabo.** 1972. Regulation of bacteriophage T5 development of Col II factors. J. Virol. 9:804-812.
14. **Paul, A. V., and I. R. Lehman.** 1966. The deoxynucleases of *Escherichia coli*. VII. A deoxynuclease induced by infection with phage T5. J. Biol. Chem. 214:3441-3451.
15. **Rogers, S. G., and M. Rhoades.** 1976. Bacteriophage T5 induced endonucleases that introduce site-specific single chain interruptions in duplex DNA. Proc. Natl. Acad. Sci. U.S.A. 73:1576-1580.
16. **Shishido, K., and T. Ando.** 1975. Site specific fragmentation of bacteriophage T5 DNA by single-strand specific S1 endonuclease. Biochim. Biophys. Acta 390:125-132.
17. **Sidikaro, J., and M. Nomura.** 1975. *In vitro* synthesis of the ColE3 immunity protein directed by ColE3 plasmid DNA. J. Biol. Chem. 250:1123-1131.
18. **Sugino, Y., J.-I. Tomizawa, and T. Kakefuda.** 1975. Location of non-DNA components of closed circular colicin E1 plasmid DNA. Nature (London) 253:652-654.
19. **Szabo, C., B. Dharmgrongartama, and R. Moyer.** 1975. The regulation of transcription in bacteriophage T5-infected *Escherichia coli*. Biochemistry 14:989-997.
20. **Traub, P., S. Miroshima, C. V. Lowry, and M. Nomura.** 1971. Reconstitution of ribosomes from subribosomal components, p. 391-407. *In* K. Moldane and L. Grossman (ed.), Methods in enzymology, vol. 20. Academic Press Inc., New York.