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Cloning, Expression, and Purification of Gene 3 Endonuclease from Bacteriophage T7†

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ABSTRACT: The structural gene for the single-stranded endonuclease coded for by gene 3 of bacteriophage T7 has been cloned in pGW7, a derivative of the plasmid pBR322, which contains the λ P_L promoter and the gene for the temperature-sensitive λ repressor, cI857. The complete gene 3 DNA sequence has been placed downstream of the P_L promoter, and the endonuclease is overproduced by temperature induction at mid-log phase of *Escherichia coli* carrying the recombinant plasmid pTP2. Despite the fact that cell growth rapidly declines due to toxic effects of the excess endonuclease, significant amounts of the enzyme can be isolated in nearly homogeneous form from the induced cells. An assay of nuclease activity has been devised using gel electrophoresis of the product DNA fragments from DNA substrates. These assays show the enzyme to have an absolute requirement for Mg(II) (10 mM), a broad pH optimum near pH 7, but significant activity from pH 3 to pH 9, and a 10-100-fold preference for single-stranded DNA (ssDNA). The enzyme is readily inactivated by ethylenediaminetetraacetic acid or high salt. The differential activity in favor of ssDNA can be exploited to map small single-stranded regions in double-stranded DNAs as shown by cleavage of the melted region of an open complex of T7 RNA polymerase and its promoter.

An endonuclease coded for by gene 3 of bacteriophage T7 has been implicated in the degradation of host DNA which occurs after T7 phage infection (Center et al., 1970), in DNA maturation (Paetkau et al., 1977), and in genetic recombination (Powling & Knippers, 1974; Kerr & Sadowski, 1975; Lee et al., 1976; Lee & Sadowski, 1981). The gene 3 enzyme can catalyze an endonucleolytic cleavage of both single- and double-stranded DNA (Center & Richardson, 1970a,b; Sadowski, 1971). The rate of hydrolysis of single-stranded DNA has been reported to be at least 100 times greater than that of duplex DNA (Center et al., 1970). The enzyme introduces

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both nicks and double-strand breaks into duplex DNA. The molecular weight of the T7 endonuclease as calculated from the DNA sequence is 17 171, assuming a single-chain enzyme (Dunn & Studier, 1983).

The preference of the T7 endonuclease for single-stranded DNA has made this enzyme an attractive tool to study the region of the DNA template melted by T7 RNA polymerase (Strothkamp et al., 1980; Osterman & Coleman, 1981), particularly at the well-characterized T7 promoter sequence (Oakley et al., 1979; Oakley & Coleman, 1977). The T7 endonuclease is observed to attack only the noncoding strand, while the coding strand is protected by the bound T7 RNA polymerase in the open complex (Osterman & Coleman, 1977). Therefore, the T7 endonuclease has appeared to be a potentially useful probe for single-stranded regions of DNA.

The drawback has been that the yield of purified T7 endonuclease from bacterial cells infected by T7 phage is extremely poor because the level of expression of the enzyme is very low. To overcome this problem, we have cloned the T7 gene 3 in a plasmid derived from pBR322, placing the gene under the control of the λ P_L promoter also carried by this plasmid. The T7 endonuclease was successfully expressed from the recombinant plasmid which also contained the gene for a temperature-sensitive λ repressor, cI857, so the enzyme could be induced by raising the temperature. The present report describes the purification and properties of the cloned T7 gene 3 endonuclease.

Materials and Methods

Bacteria and Phage Strains. Escherichia coli strain CJ109 (λcI$^+$ lysogen) was used as the host for isolation and characterization of the λP_L promoter-carrying plasmids which were transferred to strain N4830 (λcI857) for the temperature induction experiment (Joyce & Grindley, 1983). Both these strains were provided by N. Grindley (Yale University), who had obtained them from M. Gottesman (Gottesman et al., 1980). Bacteriophage T7 and DNA from wild or the mutant ΔH3 strains were prepared as previously described (Oakley et al., 1975).

Plasmid. pGW7, constructed by G. Wilson in W. Konigsberg's laboratory (Yale University), contains a modified λ-DNA fragment coding for the leftward promoter P_L, the cI857 temperature-sensitive repressor, and the N gene which is inserted between the *Eco*RI and *Bam*HI sites of pBR322.

Enzymes and Linkers. Restriction endonucleases (*Bam*HI, *Tha*I, *Hae*II, *Ava*I, and *Hpa*I) and DNA modifying enzymes (T4 ligase, alkaline phosphatase, and T4 kinase) were purchased from Bethesda Research Laboratories, Boehringer Mannheim, and New England BioLabs. The synthetic *Bam*HI linker (G-G-G-A-T-C-C-C) was obtained from Boehringer Mannheim, phosphorylated, and radiolabeled by the kinase using [γ-^{32}P]ATP. The linkers were then ligated to the appropriate DNA fragment.

Cloning. Methods for preparation and cloning of DNA fragments were standard techniques (Wu, 1979a,b; Maniatis et al., 1982). From the T7 nucleotide sequence search, it was determined that digestion of T7 ΔH3 DNA by *Tha*I produces a 1.1-kilobase (kb) DNA fragment which contains the entire coding sequence for T7 endonuclease I as well as a ribosome binding sequence (see Figure 1 below). The *Tha*I digest was electrophoresed on a 2% agarose gel, the section of gel containing the desired fragment cut out, and the DNA electrophoresed out of the gel piece. *Bam*HI linkers were then added to the purified fragment which was then ligated into the *Bam*HI site of pGW7. The ligation mixture was used to transform *E. coli* CJ109 to ampicillin resistance. Plasmids containing gene 3 were detected by in situ hybridization of the bacterial colonies to the ^{32}P-labeled 1.1-kb DNA fragment containing this gene. The orientation of gene 3 with respect to the P_L promoter on the recombinant plasmid was determined by restriction analysis using *Hae*II and *Ava*I (see below).

Expression. The recombinant plasmid containing the T7 endonuclease gene was designated pTP2 and was transferred to the N4830 strain of *E. coli* for expression. The culture was grown at 30 °C in YT broth containing 0.1 mg/mL ampicillin to an optical density of 0.8 at 600 nm. The culture was then rapidly shifted to 42 °C by adding an equal volume of hot (65 °C) broth and incubated at 42 °C for many hours.

Total endonuclease activities of induced cells containing the parent plasmid, pGW7, and the recombinant plasmid, pTP2, were measured with the standard assay using ^{3}H-labeled calf thymus DNA as substrate (see below). This relatively nonspecific endonuclease assay measures all the endonuclease background of the cell, and extracts from cells carrying either no plasmid or pGW7 grown for 3 h at 42 °C showed 750 ± 75 cpm of soluble nucleotide released from the DNA compared to 1100 ± 75 cpm for pTP2-infected cells. Hence, ~30% of the total endonuclease activity in the pTP2-infected cells is represented by the gene 3 product. This increase in endonuclease activity on induction is accompanied by the appearance of a new protein with the molecular weight expected for the gene 3 product as shown below.

Pulse Labeling of Proteins with [^{35}S]Methionine. E. coli N4830 containing either no plasmid, the parent plasmid pGW7, or pTP2 were grown at 30 °C to an optical density of ~0.6 at 600 nm in modified M9 medium which includes, in addition to the standard M9, 2.5 μg/L biotin, 1 μg/L thiamin, and 0.1 mg/mL ampicillin. Cells were collected at room temperature and resuspended in the same volume of modified M9 medium minus methionine. After incubation at room temperature for 30 min, 200-μL aliquots of cell suspensions were transferred to sterile tubes. Cells were induced by adding an equal volume of this same medium prewarmed at 65 °C, followed by incubation at 42 °C. At a selected time after induction, 20 μL of [^{35}S]methionine (New England Nuclear) was added to the mixture which was then incubated at 42 °C for an additional 3 min. The reaction was stopped by adding 2 mM methionine (200 μL), 21% sodium dodecyl sulfate (SDS), and 35% 2-mercaptoethanol (60 μL). After being boiled for 5 min, the tube was placed in an ice–water bath, and these crude cell extracts were subject to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (15% polyacrylamide). Gels were treated with the "Enlightning" rapid autoradiography enhancer (New England Nuclear) before being dried and exposed to Kodak X-omat AR film.

Assay for Endonuclease Activity. Since there are many DNases in the *E. coli* system, both exo- and endonucleases, no assay specific for the T7 endonuclease is possible. Therefore, to detect the T7 endonuclease during the purification process, we have had to rely on the detection of the presence of the newly produced protein of 17 kilodaltons (kDa) by SDS gel electrophoresis. The following assays were used to measure the endonuclease activity of fractions containing the protein.

One assay was designed to detect only endonuclease activity without any interference by exonucleases. In this assay, the substrates for the DNase were circular DNAs, either single-stranded *fd* DNA or a double-stranded plasmid, *pGW7*. This technique is very sensitive because as little as one phosphodiester bond cleavage per molecule (conversion of the circular

DNA to linear DNA) could be detected by gel electrophoresis using ethidium bromide to stain the DNA fragments (see Results and Discussion).

In the second assay, T7 endonuclease activity was determined by the amount of acid-soluble oligonucleotide released from linear, single-stranded DNA. The substrate was denatured DNA (T7 or calf thymus DNA) ^3H labeled by nick translation with the Klenow fragment from DNA polymerase I. The reaction conditions were as previously described, except that the substrate concentration was 2.0 mg/mL single-stranded DNA (1.0×10^6 cpm/mL) and no bovine serum albumin was added. After 1 h at 37 °C 1 volume of calf thymus DNA (0.25 mg/mL) and 2.5 volumes of cold 1 M trichloroacetic acid were added. After incubation on ice for 5 min, the mixtures were centrifuged for 5 min, and 0.1 mL of the supernatant fluid was mixed with the scintillation solution. The radioactivity was measured in a Packard Tri-Carb scintillation counter.

Purification of the T7 Endonuclease: Growth of Cells for Purification. An overnight culture of *E. coli* N4830 containing pTP2 grown at 30 °C (1 L) was added to 10 L of superbroth containing 0.1 mg/mL ampicillin and equilibrated at 30 °C in a Microferm fermentor (New Brunswick Scientific). The culture was maintained at 30 °C to an optical density of 5.0 at 600 nm, then shifted rapidly to 42 °C, and grown for an additional 2 h. Cells were harvested by centrifugation, yielding about 150 g, and subsequently washed with 40 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, before being stored frozen.

Crude Extract. Cells were lysed by a modified procedure described by Seeberg (1978). Frozen cell pellets (80 g) were thawed and resuspended in hypertonic buffer [2.4 M sucrose, 40 mM Tris-HCl, pH 8.0, and 10 mM ethylenediaminetetraacetic acid (EDTA)], 1.5 mL/g of cells at room temperature for 30 min. Soluble proteins inside the cell were released by adding 50 mM Tris-HCl (5 mL/g of cells) containing 1 mM EDTA, 5 mM dithiothreitol (DTT), 0.1 M KCl, pH 8, and 100 μg/mL freshly dissolved lysozyme. After 45 min on ice with intermittent shaking, the mixture was sonicated briefly to decrease the viscosity; cellular debris was removed by a 60-min centrifugation at 25000*g*. The supernatant was the crude extract.

Bovine Pancreas DNase Digestion of DNA in Crude Extracts. The crude extract was adjusted to 10% (v/v) glycerol, 20 mM $MgCl_2$, and 8 mM $CaCl_2$, and solid bovine pancreatic DNase from Sigma was added to 5 μg/mL. The mixture was stirred slowly at room temperature for 4 h, NaCl was added to 0.5 M, and agitation was continued for another 1.5 h. The extracts were centrifuged at 6000*g* for 15 min, and the supernatant was extensively dialyzed at 4 °C against 10 mM Tris-HCl (pH 8.0), 1 mM DTT, 5% glycerol, 20 mM NaCl, and 1 mM EDTA to remove the small oligonucleotides.

Ammonium Sulfate Fractionation. All the following steps were performed at 4 °C on ice. Ammonium sulfate to 25% saturation was added to the extract by the slow addition of solid ammonium sulfate. After 30 min, the suspension was centrifuged and the precipitate discarded. Ammonium sulfate was added slowly to the supernatant to bring it to 60% saturation. The centrifuged pellet was saved, dissolved in a small volume of 10 mM Tris-HCl (pH 7.6), 5% glycerol, and 1 mM DTT, and dialyzed against a large volume of this buffer.

DEAE-cellulose Fraction. The dialyzed ammonium sulfate fraction was applied to a 2.5 × 50 cm DEAE-cellulose (DE52, Whatman) column equilibrated with 10 mM Tris-HCl (pH 7.6), 5% glycerol, 10 mM NaCl, and 1 mM DTT. The column was washed with 1 column volume of buffer and then eluted with a linear gradient of 0.01–0.5 M NaCl in the above buffer. The majority of the T7 endonuclease did not bind to the DEAE-cellulose column at pH 7.6 and came out in the void volume to form the DEAE-cellulose fraction.

Hydroxylapatite Fraction. A column (1.5 × 23 cm) of hydroxylapatite (Bio-Gel HTP, Bio-Rad) was equilibrated with 10 mM potassium phosphate (pH 7.6), 5% glycerol, and 1 mM DTT. The DEAE-cellulose-excluded fraction after dialysis against this buffer was loaded onto the column. The column was washed with the same buffer, and the T7 endonuclease was eluted by a gradient of 0.01–0.1 M potassium phosphate (pH 7.6) containing glycerol and DTT. Fractions containing the 17-kDa protein were determined by SDS–PAGE and pooled to form the hydroxylapatite fraction.

PBE94 Fractionation. Isoelectric separation of the nuclease using a PBE94 (Pharmacia) chromatofocusing column separated the enzyme from a number of other contaminants at this stage, but the extensive dialysis required to remove the amphoteric polybuffer resulted in large losses of activity. On the other hand, the use of the PBE94 resin for simple ion-exchange chromatography of the protein is a useful step, and the PBE94 column was routinely used in the ion-exchange mode. A column (1 × 13 cm) of anion-exchanger PBE94 was equilibrated with 10 mM Tris-HCl (pH 8.5), 5% glycerol, 5 mM NaCl, and 1 mM DTT. The hydroxylapatite fraction was dialyzed against this buffer and then applied to the column. After 1 column volume of buffer wash, the T7 endonuclease was eluted with a linear gradient of 0.01–0.2 M NaCl in the same buffer. Tubes containing T7 endonuclease appeared to be close to 95% purified, with only one minor contaminant protein left as determined by SDS–PAGE. These tubes were pooled to form the PBE94 fraction.

Acrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) employed the gel and buffer system according to the Laemmli formulation (Laemmli, 1970).

Results and Discussion

Construction of the Plasmid pTP2 Containing T7 Gene 3. A search of the nucleotide sequence of T7 bacteriophage revealed restriction sites for *Tha*I on both sides of gene 3. T7 DNA was digested by *Tha*I and then separated on a 2% agarose gel. This gave a convenient fragment of 1.1 kilobases (kb) containing all of the gene 3 coding sequence and the ribosome binding site (Figure 1). *Bam*HI linkers were attached to this 1.1-kb blunt-ended DNA fragment containing gene 3, and after *Bam*HI digestion, the purified fragment was ligated into the *Bam*HI site of pGW7 (8.0 kb) so that the gene 3 sequence is positioned downstream from the P_L promoter (Figure 1). For isolation and characterization of the recombinant plasmids, these P_L-bearing plasmids were transferred to strain CJ109 (a λcI^+ lysogen) in order to repress expression of gene 3 at this stage. Out of thousands of transformed CJ109 colonies screened by the hybridization technique, two colonies were found that contained the gene 3 sequence. The plasmids were isolated, and the orientation of the initial codon of gene 3 with respect to the P_L promoter was determined by restriction with *Hae*I and *Ava*I, since the fragment contains sites for these enzymes in asymmetrical positions. In both cases, the T7 endonuclease gene was in the correct orientation for expression from the P_L promoter.

Expression of the Endonuclease from the Recombinant pTP2. Plasmid containing gene 3, named pTP2, was transferred to *E. coli* strain N4830 which also carries the temperature-sensitive cI857 repressor. The detection of the newly

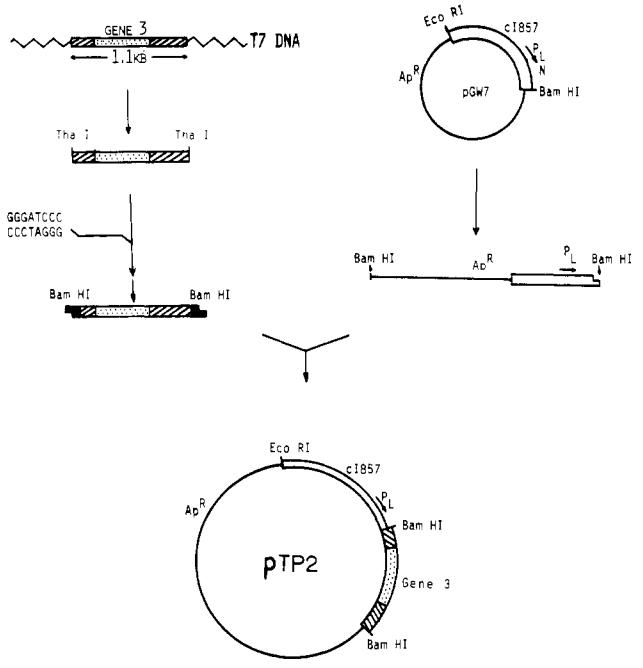

FIGURE 1: Construction of the plasmid pTP2 containing T7 gene 3 (the endonuclease gene) under the control of the P_L promoter. *Bam*HI linkers were attached to a *Tha*I fragment from T7 DNA containing the complete gene 3 structural gene and the ribosome binding site 5′ to the gene. The fragment of 1073 bp in length from T7 9943 to 11 069 bp. The structural gene 3 extends from T7 10 257–10 704 bp. After treatment with *Bam*HI, the linked fragment was ligated into the *Bam*HI site of the plasmid pGW7 which carries λP_L and a short piece of the λN gene ending at a *Bam*HI site. In both recombinant plasmids which produce gene 3, the proximal *Bam*HI site (nearest P_L) has been either mutated or deleted such that only one *Bam*HI site is present in the recombinant. Within the precision of gel calibration, the length is as expected; hence, the change is very small and clearly does not affect gene 3.

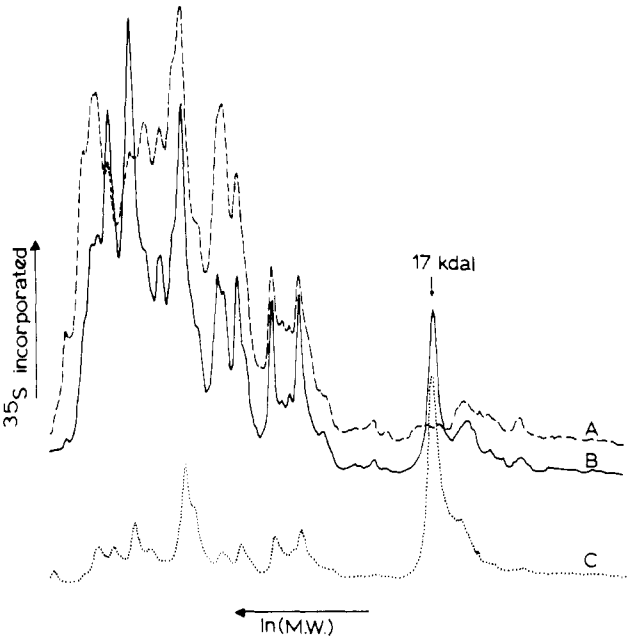

FIGURE 2: Densitometer traces of SDS–15% polyacrylamide gels of [^{35}S]methionine-labeled proteins. (A) (---) Proteins produced by *E. coli* containing pGW7, incubated at 42 °C for 20 min; (B) (—) proteins produced by *E. coli* containing pTP2, incubated at 42 °C for 20 min; (C) (···) proteins produced by *E. coli* containing pTP2, incubated at 42 °C for 40 min. The amount of 17-kDa protein produced during a 3-min period after 40 min at 42 °C is about 40% of all the proteins produced at this stage.

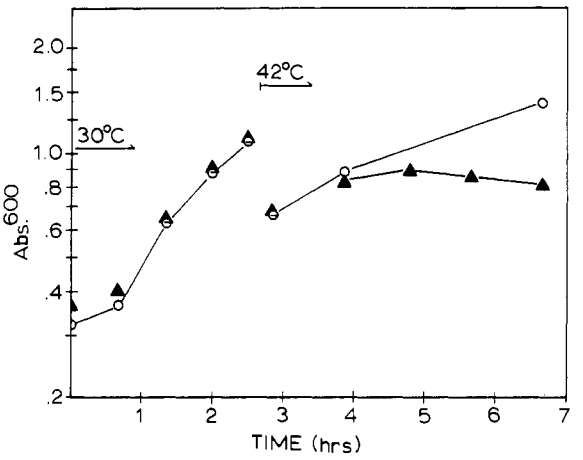

FIGURE 3: Growth curves of *E. coli* containing plasmid pGW7 or pTP2. Overnight cultures of *E. coli* containing pGW7 or pTP2 were diluted 25-fold in LB broth including 100 μg/mL of ampicillin and grown at 30 °C with vigorous shaking. Cell densities were monitored at 600 nm. Cells grown to approximately 1 OD U were induced by adding an equal volume of broth at 60 °C followed by incubation at 42 °C. (○) *E. coli* containing pGW7; (▲) *E. coli* containing pTP2.

synthesized proteins and their level of expression after temperature induction were determined by pulse labeling of proteins with [^{35}S]methionine. After the temperature shift to 42 °C, only pTP2-bearing strains have a newly produced protein of 17 kDa, as determined by denaturing SDS gel electrophoresis (Figure 2). The pTP2-containing *E. coli* did not show this 17-kDa protein unless they were shifted to 42 °C (Figure 2). Therefore, the newly produced protein is the product of the cloned gene 3 expressed from the P_L promoter on pTP2. The densitometer traces of the gel in Figure 2 suggest that over 40% of all the protein produced in the 3-min interval after 40 min of induction at 42 °C was the 17-kDa T7 endonuclease (Figure 2). The standard calf thymus DNA assay shows endonuclease activity of the cells to rise rapidly over this 40-min period. The percentage is even higher after longer induction time (60 min).

Other than the initiating *N*-formylmethionine residue, the endonuclease contains no methionine. Hence, the initial very rapid labeling with ^{35}S when plasmid transcription is temperature induced (Figure 2) represents the rapid buildup of the unprocessed gene product which still contains the *N*-formylmethionine. This conclusion was also supported by the observation that the standard Edman reaction conditions did not give any phenylthiohydantoin (PTH)-amino acids with T7 endonuclease.

The presence of significant quantities of the T7 endonuclease appears to be lethal to the host cell containing pTP2. As shown in Figure 3, the growth curve of pTP2-bearing *E. coli* indicated that cell density peaked at about 2 h after the jump to 42 °C and then decreased slowly. In contrast, the cell density of *E. coli* containing pGW7 was much higher and remained so for many hours at 42 °C (Figure 3). We examined the level of T7 endonuclease expression as a function of time after the temperature shift for the pTP2 carrying strains. Whole cell lysates of samples of similar cell density were analyzed by SDS–PAGE. The synthesis of T7 endonuclease from the P_L promoter peaked at about 2 h after induction as compared to all other proteins. At later times, the relative amount of T7 endonuclease present actually decreases.

Despite its rapid production initially as shown by ^{35}S labeling (Figure 2), the amount of T7 endonuclease produced is never close to that of the major proteins of the cell lysate, apparently because it rapidly begins to inhibit cell growth. In fact, its

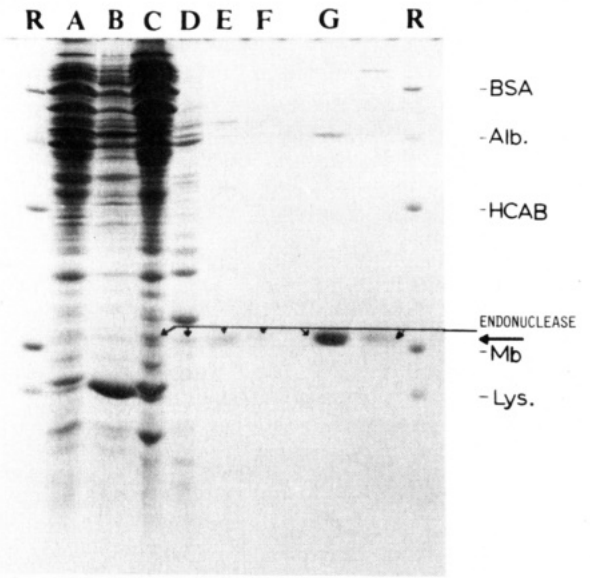

FIGURE 4: Gels of fractions isolated during the purification of gene 3 endonuclease from induced *E. coli* containing pTP2. Samples from different stages of the purification procedure were examined on a SDS–18% polyacrylamide gel. Lane A, crude extract; lane B, crude extract of *E. coli* containing pGW7; lane C, precipitate from 60% saturated ammonium sulfate; lane D, fraction excluded from DEAE column; lane E, pooled fractions from HAP column; lane F, phosphocellulose fraction (phosphocellulose chromatography can be used to obtain a relatively pure enzyme eluting at ~0.2 M salt, but this step was not routinely used); lane G, chromatofocusing PBE94 fraction; lane R, protein standards: BSA, egg albumin, human carbonic anhydrase B, myoglobin, and lysozyme.

level of expression may be less than 1% of all the proteins produced. This level is, however, much greater than that observed in T7-infected cells where no band can be observed on SDS-PAGE. It is not surprising that a protein which is involved in the degradation of host DNA becomes deleterious at relatively low concentrations. The same result has been observed when pTP2 was transferred to other *E. coli* strains, B and HB101.

Purification of the T7 Endonuclease from Induced Cells. Cultures of *E. coli* N4830 bearing pTP2 grown in rich superbroth medium and induced for 2 h were used to purify T7 endonuclease. Details of the purification are given under Materials and Methods. The enzyme can be purified more than 3-fold by ammonium sulfate fractionation. The T7 endonuclease seems to bind tightly to some other larger proteins of *E. coli*. This interaction cannot be separated completely by high salt (0.3 M NaCl) or mild detergent (0.1% deoxycholate), since the endonuclease still comes out in the excluded volume on a Sephadex G-100 column.

Gene 3 endonuclease contains 21 Lys and 6 Arg residues (Dunn & Studier, 1983) which account for its relatively high p*I*, about 8.3. The high positive charge density of the protein may account for its binding to other proteins and its failure to bind to DEAE-cellulose. Not unexpectedly, it binds so tightly to CM-cellulose that we have not been able to develop a convenient elution technique.

The protein compositions at different stages of the purification procedure (see Materials and Methods) are illustrated by SDS–PAGE (Figure 4). There are only two protein bands in the PBE94 fraction as judged by the overloaded sample on the gel (lane G, Figure 4). The T7 endonuclease represents at least 95% of the proteins in this fraction; the other contaminating protein has a molecular weight of about 46 000. We have used this PBE94 fraction to characterize the T7 endonuclease purified from our clones.

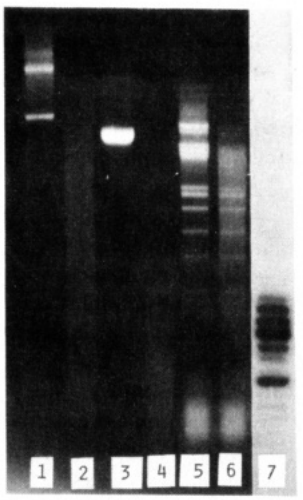

FIGURE 5: Cleavage of several forms of DNA by the T7 gene 3 endonuclease assayed by electrophoresis of the substrates and cleavage products on a 1.3% agarose gel. Each reaction mix was 10 μL containing 0.15 μg of enzyme, 0.05 M Tris-HCl, 7 mM $MgCl_2$, and 5 mM β-mercaptoethanol, pH 8, 37 °C. Reactions were halted with EDTA at 45 min. (1) Circular dsDNA (pGW7 DNA); (2) pGW7 DNA plus endonuclease; (3) circular ssDNA (fd DNA); (4) fd DNA plus endonuclease; (5) *Hpa*I fragments of T7 DNA; (6) *Hpa*I fragments of T7 DNA plus endonuclease; (7) specific cleavages induced by gene 3 endonuclease in a promoter-containing *Hpa*II fragment (L3) of T7 DNA in an open complex with T7 RNA polymerase according to the conditions of Osterman & Coleman (1981). The 5′ end of the noncoding strand was labeled with ^{32}P. The radioautograph of an 8% acrylamide gel is shown.

Characterization of the T7 Endonuclease Purified from the Clone. A convenient assay for the cleavage of both double- and single-stranded DNA has been devised by electrophoresing the digests on agarose gels and then staining with ethidium bromide. Qualitative interpretation can be made easily, while quantitative comparisons can be estimated by taking into account the less efficient staining of single-stranded DNA by ethidium bromide.

The purified gene 3 endonuclease can degrade both single-stranded (fd DNA) and double-stranded (pGW7) circular DNA (Figure 5). In the assays shown by the gel in Figure 5, there was at least twice as much fd DNA present as double-stranded plasmid DNA, yet the enzyme degraded the fd DNA to smaller pieces over the same time period. Rough calculations based on the amounts of pGW7 and fd DNA present in the assay mix suggest a 10–100-fold more rapid digestion of the fd DNA. To further test this, a mixture of double-stranded fragments of T7 DNA varying in length from 5956 to 640 base pairs (bp), provided by the *Hpa*I digest of T7 DNA, was used as substrate. While this mixture is attacked by the enzyme, these fragments are relatively resistant, and the *Hpa*I fragments are not completely hydrolyzed over the same time period by the same amount of enzyme used for the first two assays, again showing the greater activity against single-stranded DNA (Figure 5). The specific cleavage by the gene 3 endonuclease of the eight-base single-stranded region at a T7-specific promoter in its open complex with T7 RNA polymerase is illustrated in lane 7 of Figure 5.

Conditions for Optimal Activity: Inhibitors. By use of single-stranded fd DNA as the substrate, the gel assay system can be used to accurately determine optimal reaction conditions (Figure 6). The enzyme requires Mg(II). Very little if any activity is observed in its absence, and maximal activity is observed at ~10 mM Mg(II) (Figure 6, top). Mg(II) above 30 mM begins to inhibit the activity. In view of the Mg(II) requirement, it is not surprising that 5 mM EDTA inhibits

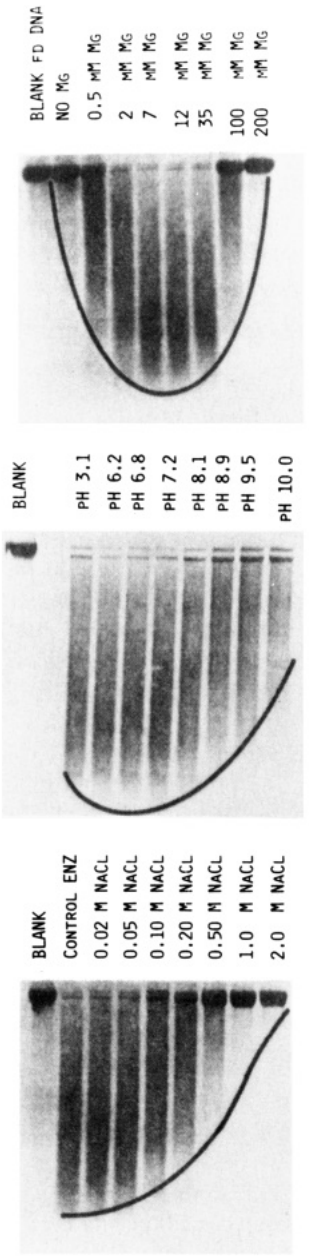

FIGURE 6: Effects of Mg(II), pH, and salt (NaCl) on the activity of the T7 gene 3 endonuclease as assayed by the hydrolysis of fd DNA according to the conditions in Figure 5.

the enzyme almost completely.

The enzyme has a rather broad pH optimum (Figure 6, middle). While a plateau of maximal activity is observed from pH 6 to pH 7, there is not much decrease on the acid side until pH 3 and below and no significant decrease in activity at alkaline pH until pH 9 (Figure 6, middle); hence, it should be useful under a variety of reaction conditions.

The enzyme is sensitive to very high salt but will maintain significant activity in 0.2 M NaCl (Figure 6, bottom). Salt concentrations of 1 or 2 M can be used to almost totally inactivate the enzyme, although EDTA would appear to be the most effective agent. The relative preference of this enzyme for the diester bonds of single-stranded regions of DNA (cleavage producing a free 3′-hydroxyl) has been graphically demonstrated by using it in low concentration to cleave the 8-bp sequence melted by T7 RNA polymerase at the promoters for this enzyme on T7 DNA (Figure 5) (Strothkamp et al., 1980; Osterman & Coleman, 1981). This activity should be useful as a probe for regions of single-stranded DNA produced under a variety of conditions.

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