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DNA Filter Retention Assay for Exonuclease Activities. Application to the Analysis of Processivity of Phage T5 Induced 5′-Exonuclease

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ABSTRACT: The 5′-exonuclease of phage T5 has been purified nearly to homogeneity by using a simple and fast procedure. The kinetic properties of the purified enzyme have been studied by using a new sensitive assay based upon retention by nitrocellulose filters of DNA with short protruding single-stranded ends. The enzyme is specifically stimulated by KCl. Its K_m is 2.2×10^{-7} M at 30 °C, and its turnover number is 0.33 DNA molecule transformed per minute. The filter retention assay shows that the T5 exonuclease acts by a semiprocessive mechanism, removing from DNA ends about 30 nucleotides on the average per cycle. The degree of enzyme processivity increases with increasing magnesium concentrations.

A 5′-exonuclease, coded for by gene D15 of bacteriophage T5, is required for the transcription of late genes (Chinnadurai & McCorquodale, 1973) and the replication of phage DNA (Frenkel & Richardson, 1971b). It has been suggested that these functions are carried out by a single multienzyme complex which has been isolated from page T5 infected *Escherichia coli* (Ficht & Moyer, 1980). In this complex, phage DNA is found associated with bacterial RNA polymerase and several phage-induced proteins including the 5′-exonuclease and DNA polymerase. The 5′-exonuclease of phage T5 has been purified, and the properties of the purified enzyme have been extensively studied (Paul & Lehman, 1966; Frenckel & Richardson, 1971a). Oligonucleotides of varying lengths (four residues on the average) are released from the 5′ ends of either double-stranded or single-stranded DNA at approximately the same rate. The enzyme can initiate hydrolysis at single-strand breaks in duplex molecules but exhibits no activity on circular single-stranded DNA or covalently closed double-stranded circles and is weakly active on RNA.

New methods of assaying exonucleases may provide meaningful information relevant to the mechanism of action and functions of these enzymes. This prompted us to develop an assay based upon the property of nitrocellulose filters to retain in appropriate conditions double-stranded DNA molecules with short protruding single-stranded ends, whereas intact double-stranded molecules pass through the filter. This specific and sensitive assay has been used to purify nearly to homogeneity the T5-induced 5′-exonuclease and to study the kinetics of hydrolysis of double-stranded DNA by this enzyme. Our results show that the T5-induced exonuclease hydrolyzes DNA through a partially processive mechanism, the number of nucleotides removed between each enzyme–DNA association and dissociation depending on the magnesium concentration.

Experimental Procedures

DNAs. λ DNA was prepared by thermal induction of *E. coli* 159T⁻ (λcI857 S7). ^{14}C-Labeled λ DNA was obtained by addition, after prophage induction, of [2-^{14}C]thymine of specific activity 47.5 mCi/mmol prepared by C.E.A. (France). The [^{14}C]DNA used in this work had a specific activity of 1.31×10^7 cpm/μmol. λ DNA *Sma*I fragments were obtained by two successive 1-h digestions at 25 °C with 30 units/mL restriction endonuclease *Sma*I (Boehringer Mannheim) followed by phenol extraction and dialysis. Before digestion, the DNA was cyclized for 2 h at 50 °C in 2 M NaCl and 10 mM trisodium ethylenediaminetetraacetate (Na_3EDTA) and dialyzed against the incubation medium containing 6 mM $MgCl_2$, 5 mM KCl, and 15 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.5. Cleavage of DNA by *Sma*I was checked by electrophoresis of the fragments on 1% agarose gels. [^{3}H]DNA from phage PM2, kindly provided by Dr. J. Pierre, had a specific activity of 1.07×10^7 cpm/μmol. The covalently closed circular form (I) and open circular form (II) were separated by equilibrium density centrifugation in a CsCl gradient containing 100 μg/mL ethidium bromide. DNA concentrations are expressed in moles of nucleotides per liter.

^{32}P 5′ End Labeling of DNA Fragments. The λ DNA fragments were labeled by the polynucleotide kinase exchange reaction (Berkner & Folk, 1980) using T4 polynucleotide kinase from NEN and [γ-^{32}P]ATP (specific activity 2.9 Ci/μmol from NEN. The excess of [γ-^{32}P]ATP was removed

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Table I: Purification of T5-Induced 5′-Exonuclease

step	volume (mL)	total units[a]	protein (mg/mL)	sp act. (units/mg)	yield (%)
(I) crude extract	57	105 000	5.36	345	(100)
(II) high-speed centrifugation	58	81 200	2.6	538	77
(III) phosphocellulose chromatography	18.5	99 000	0.112	47800	94
(IV) MonoQ chromatography	2	86 600	ND[b]	ND[b]	82
(V) MonoQ chromatography	1	31 000	0.076	4.08×10^5	29

[a] Enzymatic activities were determined by the filter retention assay. The definition of a unit of enzymatic activity is given in the text. [b] Not determined.

by extensive dialysis and gel filtration on Sephadex G-50.

Neurospora crassa Nuclease Digestion. *Neurospora crassa* nuclease (Sigma) was incubated overnight at 0 °C in 0.02 M potassium phosphate buffer, pH 6.5, and 10 mM Na_3EDTA. Digestions were carried out in the absence of Mg^{2+} and in the presence of 0.1 M monovalent ion. In these conditions, nuclease activity on double-stranded DNA is reduced to an undetectable level (Fraser, 1980).

Filter Retention Assay for Exonuclease Activities. The incubation medium for *E. coli* exonuclease III (obtained from BRL) contained 5 mM $MgCl_2$, 0.3 mM Na_3EDTA, 10 mM 2-mercaptoethanol, 50 mM Tris-HCl, pH 8.0, and 100 μg/mL bovine serum albumin. The standard reaction mixture for the T5-induced enzyme contained 40 mM KCl, 3 mM $MgCl_2$, 1 mM Na_3EDTA, 20 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol, 100 μg/mL bovine serum albumin, λ [^{14}C]DNA fragments, and exonuclease diluted in 1 mM Na_3EDTA, 50 mM Tris-HCl, pH 8.0, 10 mM dithiothreitol, and 500 μg/mL bovine serum albumin. After 15 min of incubation at 30 °C, 100 μL of the incubation mixture was mixed with 4.5 mL of 2 M KCl and 10 mM Na_3EDTA. The solutions were filtered on 25-mm diameter nitrocellulose membranes (0.45 μm, Schleicher & Schuell) under a vacuum of 25 mmHg. The membranes were rinsed with 1 mL of 0.01 N HCl and dried. The amount of labeled DNA retained by the membrane was determined by liquid scintillation counting.

Determination of Acid-Soluble Products. To 0.1 mL of incubation mixture containing either λ [^{14}C]DNA or 5′-^{32}P end-labeled DNA were added 0.2 mL of 0.2 mg/mL calf thymus DNA and 0.4 mL of 10% trichloroacetic acid. After 10 min at 0 °C, the solution was centrifuged at 4 °C for 10 min at 9800*g*. To 0.5 mL of supernatant was added 3.5 mL of Ready-Solv MP (Beckman), and the radioactivity was determined.

Preparation of T5-Infected Bacteria. *E. coli* CR63 was grown at 37.5 °C under forced aeration in a New Brunswick fermentor containing 10 L of medium composed of 8 g of Nutrient Broth Difco, 5 g of NaCl, and 0.78 mL of 1 N NaOH per liter. At a cell density of 3×10^8 cells/mL, the medium was made 1 mM in $CaCl_2$, and T5$^+$ phages were added at a multiplicity of 10. Thirty minutes after infection, the culture was quickly cooled, and the bacteria were harvested by filtration in a Pellicon system manufactured by Millipore, followed by centrifugation at 1750*g* for 20 min at 4 °C. The pellet was stored at −20 °C.

Purification of T5 Exonuclease. All purification steps (summarized in Table I) were carried out at 4 °C. Protein concentrations were determined by the method of Bradford (1976).

T5-infected bacteria (5 g) were thawed and suspended in 58 mL of 1 mM Na_3EDTA, 50 mM Tris-HCl, pH 7.5, 1 mM glutathione, and 25 μg/mL phenylmethanesulfonyl fluoride. The cells were disrupted by using a French press (fraction I). The NaCl concentration was adjusted to 0.2 M, and the suspension was centrifuged for 2 h at 160000*g* in a TFT 65.38 Kontron rotor. The supernatant (fraction II) was diluted 3-fold with 5 mM Na_3EDTA, 10 mM Tris-HCl, pH 7.5, and 1 mM 2-mercaptoethanol and loaded on a phosphocellulose column (Whatman P11). The resin was prepared according to Greene et al. (1978), and the column (2 $cm^2 \times 30$ cm) was equilibrated with 66 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM 2-mercaptoethanol (buffer A). After a wash with one column volume of buffer A, proteins were eluted with a linear salt gradient (200 mL, 0.066–0.46 M NaCl in 10 mM Tris-HCl, pH 7.5, and 1 mM 2-mercaptoethanol). Fractions of 4.8 mL were collected, and enzyme activity was measured by the DNA filter retention assay. The active fractions were pooled (fraction III) and dialyzed overnight against 20 mM Tris-HCl, pH 7.5, 5% glycerol, and 1 mM 2-mercaptoethanol (buffer B). The solution was filtered on a 0.45-μm nucleopore membrane and was applied to a prepacked anion-exchanger Mono Q column (Pharmacia Fine Chemicals) equilibrated with buffer B. The column (0.2 $cm^2 \times 5$ cm) was run under a pressure of ~20 bar at a flow rate of 1 mL/min in conjunction with the Pharmacia Fast Protein Liquid Chromatography system. The column was washed with 4 mL of 0.15 M KCl in buffer B, and proteins were eluted with a linear salt gradient (14 mL, 0.15–0.6 M KCl in buffer B). The active fractions were pooled (fraction IV), and the KCl concentration was adjusted to 0.2 M by dilution with buffer B. The solution was filtered as above and loaded again on a Mono Q column. The column was eluted with a linear salt gradient (14 mL, 0.2–0.5 M KCl in buffer B). The elution profile is shown in Figure 2. Active fractions were made 50% in glycerol and stored at −20 °C.

Polyacrylamide Gel Electrophoresis. Sample denaturation and gel electrophoresis on a 8–16% polyacrylamide gradient containing 0.1% sodium dodecyl sulfate was carried out as described by Laemmli & Favre (1973). After electrophoresis, proteins were stained with 0.25% Coomassie brilliant blue R in 7% acetic acid and 30% methanol. The gel was destained and photographed, and the negative was analyzed with a Joyce-Loebl microdensitometer.

Results

Filter Retention Assay of Exonuclease Activities. The hydrogen-bonded circular DNA molecule of phage λ is cleaved by restriction endonuclease *Sma*I into three blunt-ended fragments of 28.0, 12.2, and 8.3 kilobase pairs (kbp) (Roberts, 1981). Upon filtration at high salt concentration through nitrocellulose membranes, 5–8% of these DNA fragments are retained by the membranes. After digestion with *E. coli* exonuclease III, these DNA fragments are converted into forms which are quantitatively retained by the membranes. *E. coli* exonuclease III catalyzes the stepwise release of 5′-mononucleotides from the 3′ ends of double-stranded DNA, generating protruding single-stranded 5′ ends (Richardson et al., 1964). We present below evidence showing that these protruding single-stranded ends induce the retention of the DNA fragments by nitrocellulose membranes.

We have studied the kinetics of transformation of λ DNA fragments into membrane-adsorbable forms upon exonuclease

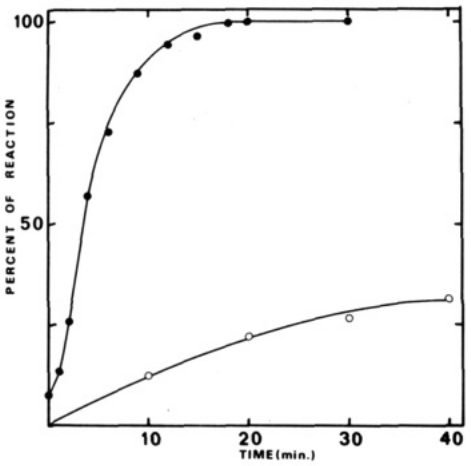

FIGURE 1: Kinetics of digestion of λ [^{14}C]DNA *Sma*I fragments by *E. coli* exonuclease III. λ [^{14}C]DNA concentration was 0.733 μM. After incubation at 30 °C with 0.109 unit/mL *E. coli* exonuclease III, the retention of DNA by nitrocellulose filters was determined as described under Experimental Procedures using 100-μL aliquots withdrawn at the indicated times (●). Acid-soluble products released upon incubation at 30 °C with 7 units/mL exonuclease III were measured by using 150-μL aliquots (○).

III digestion (Figure 1). After a short lag time of about 1 min, the reaction follows apparent first-order kinetics (Figures 1 and 7), and 100% of the DNA is retained after about 20 min of incubation under our conditions. The rate of hydrolysis to acid-soluble products of λ DNA fragments by *E. coli* exonuclease III has been measured in similar conditions, except that the enzyme concentration was raised to 7 units/mL to allow accurate measurements (Figure 1). In these conditions, the rate of the reaction was proportional to the enzyme concentration (data not shown). From the slope of the initial part of the reaction curve, one can calculate that the point of half-transition of the DNA filter retention curve corresponds to the removal of an average of about 18 nucleotides from each DNA fragment. This observation provides the basis for an accurate and sensitive assay of exonuclease activities.

After digestion with the T5-induced exonuclease, the DNA filter retention increases as a function of the incubation time according to first-order kinetics (see below). The rate of transformation of the DNA fragments into forms retained by nitrocellulose membranes is then determined by measuring the fraction of nonretainable DNA at different times, according to the equation:

$$[S]_t/[S]_0 = \exp(-kt) \quad (1)$$

where $[S]_0$ and $[S]_t$ denote the substrate concentrations at the initial time and after t minutes of incubation and k is the rate constant. As shown below for the T5-induced enzyme, k is proportional to the enzyme concentration in the incubation mixture (Figure 8A), and 1 unit of enzyme activity can be defined as the quantity of enzyme which transforms DNA fragments in 1 mL of incubation mixture at a rate constant of $k = 1$ min^{-1}.

Purification and Characterization of the T5-Induced Exonuclease. As previously shown by Frenkel & Richardson (1971a), a 5′-exonuclease appears between 10 and 15 min after infection of *E. coli* with phage T5. We have detected in T5-infected bacteria, within the same time range, the appearance of an enzymatic activity which converts λ DNA fragments into forms retained by nitrocellulose filters. Thirty minutes after infection, this activity reaches a maximum equivalent to a 17-fold increase over the base level measured in uninfected bacteria. We have purified the enzyme from phage-infected bacteria, assaying the activity of the fractions

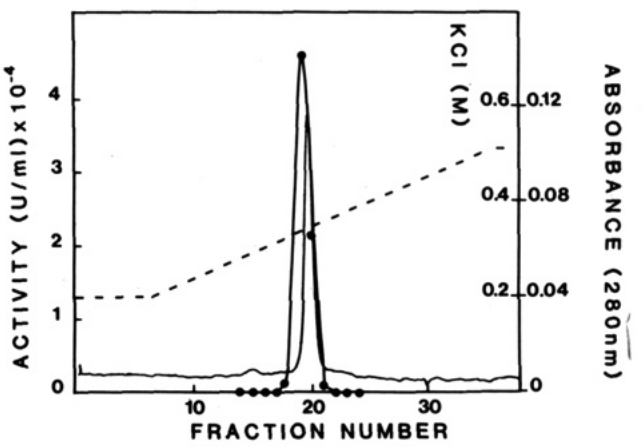

FIGURE 2: Fast liquid chromatography of the phage T5 induced enzyme. Fraction IV was applied to a MonoQ column equilibrated with 0.2 M KCl in buffer B. The column was eluted with a linear KCl gradient (14 mL, 0.2–0.5 M), and fractions of 0.5 mL were assayed by filter retention of λ [^{14}C]DNA fragments. (●) Enzymatic activity; (—) absorbance at 280 nm through a 1-cm light path; (---) KCl concentration.

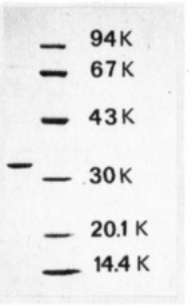

FIGURE 3: Polyacrylamide gel electrophoresis under denaturing conditions of the purified T5 enzyme. About 2 μg of T5 enzyme (fraction V) was loaded on a 8–16% polyacrylamide gel and run in parallel with the molecular weight markers phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 400).

obtained after each step, by measuring the λ [^{14}C]DNA fragment retention by nitrocellulose filters. As shown in Table I, a 1100-fold purification, with a yield of 29%, was achieved in five steps. The enzyme eluted from the phosphocellulose column as a single peak at 0.33 M NaCl with an apparent yield of over 100%. This is presumably due to the presence in the previous fractions of inhibitors removed at this step. Figure 2 displays the elution pattern of the second MonoQ column: a single and symmetrical peak of UV-absorbing material is observed, coinciding with the enzymatic activity, which indicates that a high degree of purification has been attained.

Upon electrophoresis of the purified T5 enzyme (fraction V) on a polyacrylamide gel in the presence of sodium dodecyl sulfate, more than 90% of the material migrates as a polypeptide of M_r 33 000 (Figure 3). In addition, two minor bands corresponding to polypeptides of M_r 42 000 and 71 000 are observed. The exonuclease activity of the purified T5 enzyme was ascertained by incubation with 5′-^{32}P-labeled λ DNA fragments (Figure 4). The conversion of ^{32}P into acid-soluble products follows apparent first-order kinetics with no lag period. No phosphatase activity was detected by incubation of *p*-nitrophenyl phosphate with fraction V in standard buffer containing either 2 or 12 mM $MgCl_2$. No endonucleolytic activity was detected in fraction V after incubation with covalently closed circular DNA of phage PM2 and analysis of the DNA by agarose gel electrophoresis. ^{3}H-Labeled DNA of phage PM2 was incubated in standard buffer for 15 min

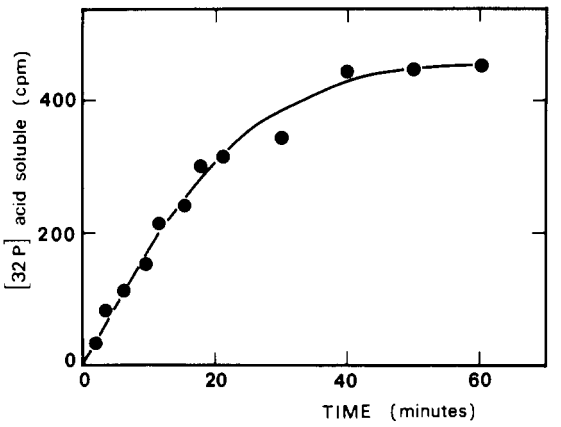

FIGURE 4: Exonuclease activity of the T5-induced enzyme. Incubation was done at 30 °C in standard incubation medium containing 0.45 μM [5′-^{32}P]DNA *Sma*I fragments (specific activity 1.4 × 10^7 cpm/μmol) and 0.198 ng/mL purified T5 enzyme. Aliquots (100 μL) were withdrawn at the indicated times and assayed for ^{32}P, rendered acid soluble as described under Experimental Procedures.

at 30 °C with 0.28 ng/mL T5 enzyme (fraction V). The retention by nitrocellulose filters of covalently closed circular DNA was respectively 6% and 8% before and after incubation. In the same conditions, the retention of the open circular form of PM2 DNA increased from 9% to 67%. All these results show that the purified T5-induced enzyme is endowed with a 5′-exonuclease activity. Since the molecular weight of the main band is very close to the published value of 35 000 (Moyer & Rothe, 1977) for the previously described exonuclease (Frenkel & Richardson, 1971a), it is likely that the two enzymes are identical.

Mechanism of DNA Retention by Nitrocellulose Membranes after Digestion with T5 Exonuclease. Different mechanisms are possibly involved in the retention of DNA fragments by nitrocellulose membranes, after digestion with the T5-induced enzyme. Two experiments were carried out to demonstrate that this retention was in fact the consequence of the exonucleolytic hydrolysis by the T5 enzyme. First, the effect of varying the salt concentration in the filtration medium upon DNA retention was studied. As shown in Figure 5, the retention of intact λ DNA *Sma*I fragments increases only slightly when the KCl concentration increases from 0 to 2.5 M. In contrast, the retention of the DNA fragments digested with the T5 enzyme increases from about 2% to nearly 100% within the same KCl concentration range. This observation indicates that the filter retention of the DNA is not due to the formation of a DNA–protein complex, since in this case, a variation of the salt concentration in the filtration medium is expected to produce an opposite effect. Second, if DNA retention is due to the formation by the T5 enzyme of single-stranded regions at the ends of the DNA fragments, this retention should be abolished after removal of these regions by a single-strand-specific nuclease. Figure 6 shows that the retention of the T5 exonuclease treated DNA fragments is abolished after further digestion with the *Neurospora crassa* nuclease. These results indicate that the T5 exonuclease generates single-stranded regions at the ends of the DNA fragments. These single-stranded ends bind strongly to nitrocellulose filters at high salt concentration and cause the retention by these filters of DNA fragments with such ends.

Kinetic Study of the T5-Induced Exonuclease Reaction by the Filter Retention Assay. Upon digestion of λ DNA fragments with T5-induced exonuclease, the retention of the fragments by nitrocellulose filters increases as a function of time up to about 100%. In contrast with the kinetics observed

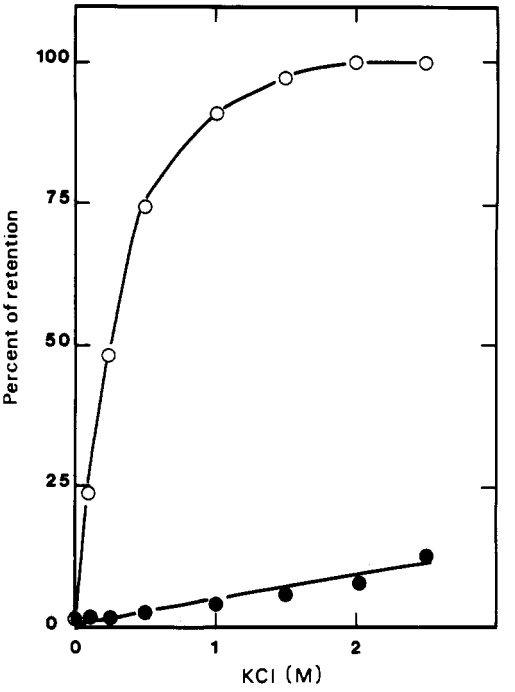

FIGURE 5: Effect of the salt concentration of filtration buffer upon retention by nitrocellulose filters of the *Sma*I fragments of λ [^{14}C]DNA after incubation with T5 enzyme. Digestion was carried out at 30 °C for 15 min in a standard incubation mixture containing 0.78 μM [^{14}C]DNA and 0.69 ng/mL purified T5 enzyme. Aliquots (100 μL) were diluted with 4.5 mL of a solution containing KCl at the indicated concentration and 0.01 M Na_3EDTA and filtered as described under Experimental Procedures. (○) DNA incubated with T5 enzyme; (●) nonincubated DNA.

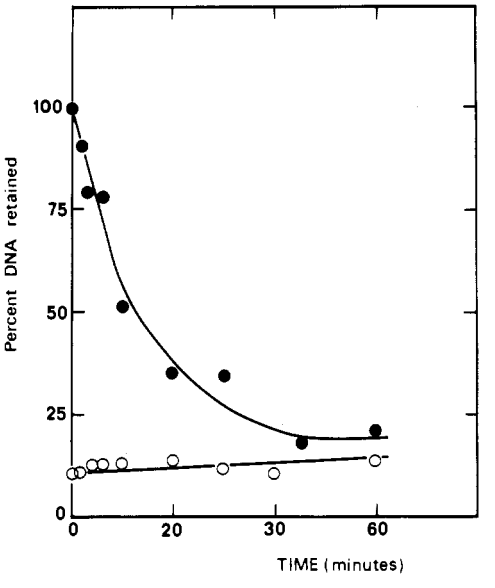

FIGURE 6: Effect of digestion with *Neurospora crassa* nuclease on the retention of λ DNA transformed by T5 enzyme. *Sma*I fragments of λ [^{14}C]DNA at a concentration of 0.82 μM were incubated with 0.278 ng/mL purified T5 enzyme at 30 °C in standard buffer. After 15 min, Na_3EDTA was added to complex Mg^{2+} ions, the salt concentration was adjusted to 37 mM KCl and 70 mM NaCl, and *Neurospora crassa* nuclease was added to a final concentration of 4.4 units/mL. Digestion was carried out at 37 °C, and aliquots of 110 μL taken at the indicated times were diluted with 4.5 mL of 2 M KCl, 0.01 M Na_3EDTA and filtered as described under Experimental Procedures. (●) DNA incubated with T5 enzyme; (○) nonincubated DNA.

with *E. coli* exonuclease III, there is no lag time in the transformation of the fragments into filter-adsorbable forms. Figure 7 shows that the reaction follows apparent first-order

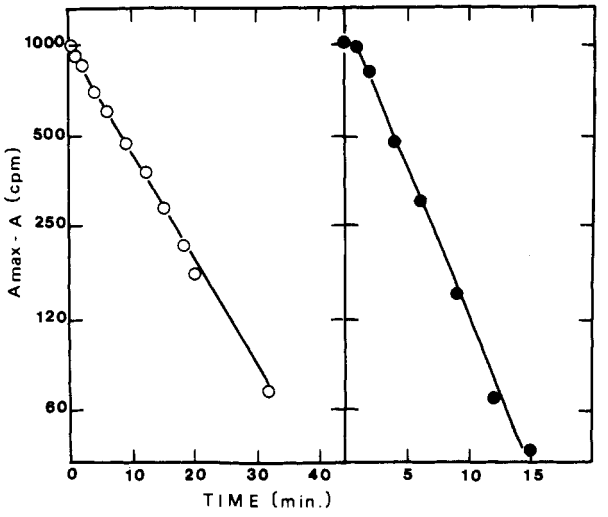

FIGURE 7: Retention by nitrocellulose membranes of the *Sma*I fragments of λ [^{14}C]DNA after exonuclease digestion. Incubation was carried out at 30 °C in the standard reaction medium containing 0.73 μM [^{14}C]DNA fragments and 0.164 ng/mL T5-induced exonuclease (fraction V) (○). The amount of DNA retained by the filters was determined on 100-μL samples taken at the specified times, and the concentration of nontransformed substrate was expressed as $A_{max} - A$ where A is the amount of ^{14}C retained by the filter at the indicated times and A_{max} = 1078 cpm is the amount of ^{14}C retained by the filter after 60 min of incubation. For the reaction with *E. coli* exonuclease III, the data taken from Figure 1 were replotted in the same fashion (●).

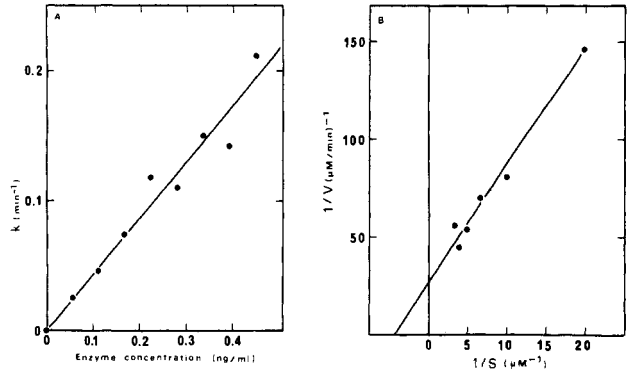

FIGURE 8: Retention of λ [^{14}C]DNA fragments after T5 exonuclease digestion: effect of enzyme and substrate concentration upon the rate of the reaction. (A) Incubation was carried out at 30 °C in 100 μL of standard reaction mixture containing 0.75 μM λ [^{14}C]DNA and purified T5 exonuclease at the indicated concentrations. After 15 min of incubation, the quantity of DNA retained by the filter was determined, and the rate constant of the reaction as defined by eq 1 was calculated (the value of A_{max} obtained by incubation with 2.5 ng/mL T5 exonuclease was used for this calculation). (B) Incubation was carried out at 30 °C in standard incubation mixture (0.1–1 mL) containing 0.113 ng/mL purified T5 exonuclease and various concentrations of λ DNA fragments. The rate constant of the reaction was determined after 15 min of incubation, and the reciprocal of the initial rate, expressed in micromolar nucleotides transformed per minute, was calculated.

kinetics, adequately described by eq 1, and the rate constant is proportional to the enzyme concentration (Figure 8A). In addition, the initial rate of the reaction was determined at constant enzyme concentration as a function of the DNA concentration. From the data plotted in Figure 8B, the Michaelis constant of the T5 exonuclease is found to be 2.2×10^{-7} M at 30 °C (substrate concentration expressed in nucleotides), and the maximum velocity corresponds to a turnover number of 0.33 fragment transformed per minute per enzyme molecule. The temperature dependence of the reaction rate was studied between 10 and 37 °C by the DNA filter retention

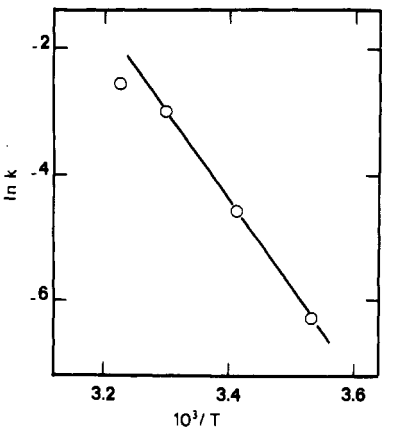

FIGURE 9: Temperature dependence of the rate of the reaction catalyzed by T5 exonuclease. λ [^{14}C]DNA *Sma*I fragments at a concentration of 1.1 μM were incubated at various temperatures with purified T5 exonuclease in standard incubation buffer containing 20 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.5, instead of Tris-HCl. The rate of the reaction was determined by the filter retention assay after 15 min of incubation with T5 exonuclease at a concentration of 0.128 ng/mL (assays at 30 and 37 °C), 0.643 ng/mL (assay at 20 °C), and 6.43 ng/mL (assay at 10 °C). The rate constants figured were calculated for an enzyme concentration of 0.128 ng/mL.

assay. A value of 28 kcal/mol for the activation energy of the reaction was obtained from a plot of the data according to the Arrhenius equation (Figure 9). One can hypothesize that this high value might reflect the mechanism of action of this enzyme: to remove oligonucleotides, the enzyme would first open the DNA ends prior to phosphodiester bond hydrolysis.

The absence of lag in the kinetics indicates that the T5 exonuclease does not dissociate from its substrate before it has created a single-stranded end of a sufficient length to retain DNA fragments on the filters. However, the turnover number determined above shows that the DNA–enzyme complex dissociates before complete hydrolysis of the DNA fragment. In other words, the T5 exonuclease does not dissociate after each hydrolytic event but stays bound to the DNA through several hydrolytic events. Our results allow us to determine the average number of nucleotides removed per enzyme–DNA association, which is a measure of the enzyme processivity. Assuming that enzyme molecules bind randomly to DNA fragments, the probability that a fragment remains intact after an average of n enzyme–DNA associations per fragment is $P = e^{-n}$. Hence, the rate constant in eq 1 is equal to n/t. In the experiment shown in Figure 7, $n/t = 8.3 \times 10^{-2}$ min^{-1}, indicating that each enzyme molecule binds to a DNA fragment 0.38 time per minute. The rate of release of acid-soluble products from λ DNA fragments by T5 exonuclease was measured at the same DNA concentration as in Figure 7: for an enzyme concentration of 28 ng/mL, 1.29% of the DNA is made acid soluble per minute, indicating that each enzyme molecule removes an average of 11.1 nucleotides per minute. In this range, the rate of hydrolysis is proportional to the enzyme concentration (data not shown). The average number of nucleotides removed per enzyme–DNA association is then 30 nucleotides. The above results have also allowed us to calculate that 1 unit of activity catalyzes the conversion of 0.66 pmol of nucleotides per minute to acid-soluble products in standard conditions.

Effect of Monovalent Ions on the T5 Exonuclease Activity. The effect of monovalent ion concentration upon the activity of the T5-induced exonuclease has been studied. As shown in Figure 10, the activity as measured by the DNA filter

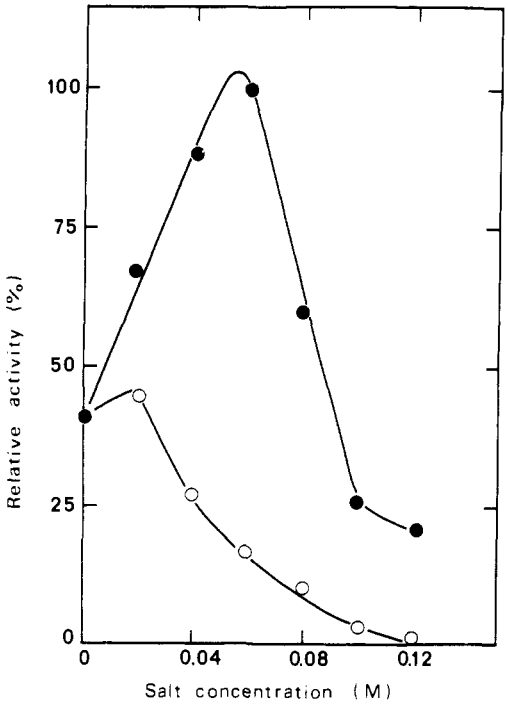

FIGURE 10: Effect of monovalent ions on the T5 exonuclease activity. λ [^{14}C]DNA at a concentration of 0.79 μM was incubated for 15 min at 30 °C with 0.088 ng/mL T5 exonuclease in the standard reaction mixture containing NaCl (○) or KCl (●) at the indicated concentrations. The activity measured by the DNA filter retention assay in the presence of 60 mM KCl is taken as 100%.

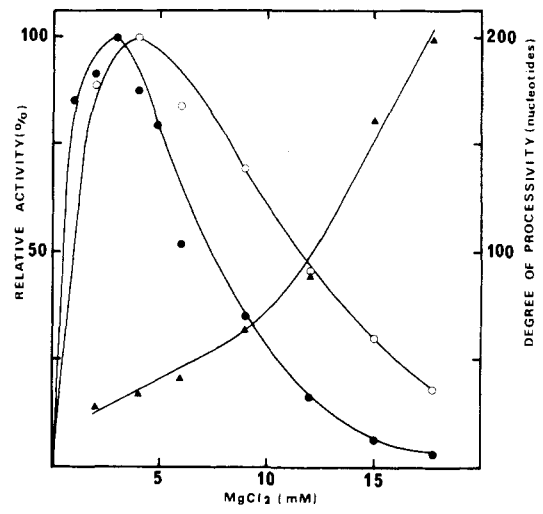

FIGURE 11: Effect of $MgCl_2$ concentration on T5 exonuclease activity and processivity. λ [^{14}C]DNA fragments at a concentration of 1.02 μM were incubated for 15 min at 30 °C with T5 exonuclease (0.113 ng/mL up to 5 mM $MgCl_2$; 0.282 ng/mL above 5 mM $MgCl_2$) in the standard incubation mixture containing $MgCl_2$ at the indicated concentrations (Mg^{2+} complexed with EDTA excluded). The enzyme activity measured by the DNA filter retention assay is expressed as a percent of the activity determined in the presence of 3 mM $MgCl_2$ (●). The rate of hydrolysis to acid-soluble products was measured after 10 and 20 min of incubation in the presence of 28 ng/mL T5 exonuclease. The activity is expressed as the percent of the activity determined in the presence of 4 mM $MgCl_2$ (○). The number of nucleotides removed per enzyme–DNA association was derived from the experimental data as explained in the text (▲).

retention assay is markedly inhibited by NaCl. In contrast, a 2.5-fold stimulation of the activity is observed in the presence of 60 mM KCl, followed by an inhibition at higher concentration.

Effect of $MgCl_2$ on the T5 Exonuclease Processivity. The T5-induced exonuclease shows an absolute requirement for a divalent cation (Paul & Lehman, 1966). The Mg^{2+} ion concentration dependence of the T5 exonuclease activity was examined both by the DNA filter retention assay and by the determination of the release of acid-soluble products. Figure 11 shows that both curves do not superimpose: the relative activity measured by the DNA filter retention assay displays a lower optimal Mg^{2+} concentration and decreases faster than the activity measured by the release of acid-soluble products. Using the same treatment of the data as described earlier, it was also possible to determine the enzyme processivity as a function of Mg^{2+} concentration. As shown in Figure 11, the T5 exonuclease processivity is markedly dependent on the Mg^{2+} concentration: it increases from about 30 nucleotides in 2 mM Mg^{2+} (standard assay conditions) up to about 200 nucleotides in 18 mM Mg^{2+}.

Discussion

A simple purification procedure for the phage T5 induced 5′-exonuclease is described in this paper. By this procedure, based upon fast protein liquid chromatography, the enzyme is obtained at a purity of ~90%. Appropriate modifications of the elution conditions from the last column should eliminate the two contaminating polypeptides remaining in the preparation.

Exonucleases are generally assayed by measuring the rate of release of acid-soluble products from DNA ends. The hydrolysis of uniformly labeled substrates can be monitored until completion of the reaction, whereas only the first cleavage of ^{32}P end-labeled substrates is detected. We have designed a new assay which detects short single-stranded regions created by exonucleases at DNA ends and therefore allows one to study the early stages of the reaction. This assay is based on the retention of double-stranded DNA molecules with short single-stranded protruding ends by nitrocellulose filters at high salt concentration. A comparable assay was previously used to measure the *rec A* catalyzed assimilation of single strands into duplex DNA (Mc Entee et al., 1979). However, the most striking feature of our assay is that a very short single-stranded region is able to provoke the filter retention of a double-stranded DNA molecule. In our experimental conditions, the removal of about 30 nucleotides by the T5 exonuclease induces the quantitative retention of the λ *Sma*I DNA fragments. This represents about 0.1% of the average length of these fragments. This estimate reflects the sensitivity of the assay which is close to that of other methods measuring the hydrolysis of a ^{32}P-labeled terminal nucleotide. By contrast to these latter methods, the DNA filter retention assay does not distinguish enzymes of opposite polarities. However, this assay is insensitive to the presence of contaminating phosphatases. Two kinds of proteins, nucleases and DNA binding proteins, may possibly interfere in this assay. The possible interference of noncovalently bound proteins is ruled out by the high salt concentration in the filtration medium. A protease treatment, prior to filtration, would allow one to detect the interference of any protein able to covalently bind to DNA. With the exception of pancreatic DNase I, the mechanism of action of known endonucleases is unlikely to generate single-stranded regions of sufficient length to provoke the filter retention of the substrate. During the purification of the T5 exonuclease, even at the early stages, the only activity which was detected at a significant level by the DNA filter retention assay was the 5′-exonuclease. Therefore, this assay appears to be rather specific for the detection of exonucleases active on double-stranded DNA.

An important question regarding the mechanism of action of the exonucleases is whether the removal of nucleotides

proceeds in a processive or nonprocessive manner. Several years ago, Thomas & Oliveira (1978) developed a method to investigate the processivity of DNA exonucleases and observed, among the different enzymes studied, a great variability in the degree of processivity. As shown in this paper, the presence or the absence of a lag period in the filter retention kinetics allows one to readily distinguish between rather processive or rather distributive enzymes. Mononucleotides are removed by *E. coli* exonuclease III according to a rather distributive mechanism (Thomas & Oliveira, 1978), and the lag observed before the first DNA fragments are retained by filters corresponds to the time required to remove the appropriate number of nucleotides from the ends of the fragments. The absence of an initial lag in the retention kinetics, observed with the T5 exonuclease, shows that this enzyme displays a higher degree of processivity. Combining the data obtained from both the retention and acid-soluble product release kinetics allows quantification of this processivity. It is then possible, with this simple technique, to precisely analyze the differences of processivity previously observed by Thomas & Oliveira (1978) with a variety of other exonucleases (i.e., T7 exonuclease, λ-exonuclease, etc.). Finally, we also observed that the T5 exonuclease processivity strongly depends on the Mg^{2+} concentration in the assay. This suggests that the processivity of exonucleases may be modulated by environmental conditions.

We have observed a specific stimulation of the activity of the T5 exonuclease by KCl and determined the Michaelis constant and the turnover number of the enzyme in the presence of this salt. The average number of nucleotides removed per minute per enzyme molecule is equal to 11. This low efficiency of hydrolysis in our in vitro conditions suggests that these conditions are not optimal and that in vivo the enzyme functions more efficiently, possibly exhibiting a higher degree of processivity, because of its integration in a multienzyme complex (Ficht & Moyer, 1980). The role of the T5-induced exonuclease in the DNA metabolism of the phage has been a matter of speculation. The enzyme is not involved in host DNA degradation but is required for the maturation of phage DNA (Frenkel & Richardson, 1971b). By creating gaps in double-stranded DNA, the enzyme may allow DNA decatenation by a type I topoisomerase according to a mechanism recently described (Low et al., 1984). These gaps, maintained by the exonuclease throughout DNA replication and maturation, could possibly be filled just before DNA packaging, leaving on one DNA strand interruptions which are a distinctive feature of the T5 DNA molecule in the phage (Everett, 1981).

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