EXONUCLEASE VI, A NEW NUCLEASE ACTIVITY ASSOCIATED WITH E. COLI DNA POLYMERASE

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It has been shown[1, 2] that highly purified preparations of *Escherichia coli* DNA polymerase contain a nuclease—exonuclease II—which appears to be inseparable from the polymerase and an integral component of the same protein species. Two important characteristics of this nucleolytic activity are: (1) it behaves as an exonuclease, releasing deoxynucleoside-5′-monophosphates sequentially from the 3′-end of polydeoxynucleotides; (2) it has a distinct preference for d(A-T) copolymer as substrate.

In this paper we describe an additional nuclease activity associated with DNA polymerase. Like exonuclease II it is an exonuclease and releases deoxynucleoside-5′-monophosphates; among the few substrates so far studied, it appears to degrade d(A-T) preferentially. Like exonuclease II, this new activity might reflect a normal polymerizing function of the enzyme. In contrast to exonuclease II, this enzyme catalyzes sequential degradation beginning at the 5′-terminus of polydeoxynucleotides. We propose the designation "exonuclease VI" for this activity.

Materials and Methods.—*E. coli* exonuclease III was prepared by W. Beers according to a modification of a previous procedure;[3] full details will be published elsewhere. Phosphatase and nuclease assays were exactly as described by Richardson and Kornberg.[3, 4] DNA polymerase was prepared by modifications of the procedure of Richardson *et al.*[1] Individual purification steps employed were those of Richardson *et al.*,[1] but the order was changed. Thus, fraction IV was dialyzed to equilibrium against KPO_4 buffer (0.005 *M*, pH 6.4) containing BME (10^{-2} *M*); precipitated material was removed by centrifugation and the supernatant adsorbed and chromatographed on a column of phosphocellulose (30-ml resin bed volume per 100 mg protein, with a ratio of height to cross-sectional area of 5–8). The eluted fractions containing DNA polymerase were then subjected to DEAE-cellulose chromatography.[1] If the specific activity of the pooled fractions at this stage was below 10,000 units/mg, the phosphocellulose step was repeated with column dimensions of height/area = 10. The final procedure was gel filtration through Sephadex G-100 (2.5 cm × 46 cm); DNA polymerase emerges at or immediately behind the void volume. The enzyme obtained in this way was free of endonuclease (no decrease in transforming activity of a sample of pneumococcal DNA was observed following incubation of 4 mμmoles of DNA with 6 units of polymerase at pH 7.0, Mg^{++} 5×10^{-3} *M*, 37°, 13 hr) and of exonuclease III (less than 1 phosphatase unit/650 polymerase units), and migrated as a single, very broad band on electrophoresis in acrylamide gel at pH 8.0. The specific catalytic activity on isolation was 12,000 d(A-T) units[1]/mg protein; on storage during the course of the experiments this decreased to 6000 units/mg. The possibility that contaminating endonuclease might have been introduced during storage was excluded by repeated bacterial transformation tests (we thank Mr. T. Easton for performing these assays). A separate preparation was used for the experiments described in Figure 4.

Polymerase and nuclease activity were measured by the increase or decrease, respectively, of acid-insoluble radioactivity, using a Millipore filter assay. The radioactivity of the filter disks, which were washed with 5% trichloroacetic acid and dried, was measured in a Packard scintillation counter.

Nucleotides were prepared or obtained from commercial sources as previously described.[5] γ-P^{32}-ATP was prepared according to Glynn and Chappell.[6]

Assays: DNA polymerase was assayed as described by Richardson *et al.*;[1] unless otherwise specified, each dNTP was present at 100 mμmoles/ml. The conditions for exonuclease VI assays were: reactions contained, per ml, 30 mμmoles d(A-T)/d($\overline{\text{DAP}}$-T) "hybrid" as substrate [the d(A-T) segments were always radioactive, the d($\overline{\text{DAP}}$-T) only where indicated]; 100 mμmoles each d$\overline{\text{DAP}}$-TP and dTTP, 70 μmoles KPO_4 buffer pH 7, 7 μmoles $MgCl_2$, 1 μmole BME, 125 μg bovine serum albumin (Armour, crystalline); incubation was at 37° for 15 min, usually in a final volume of 0.1 ml. One unit of exonuclease VI is defined as the quantity of enzyme that degrades 10 mμmoles of total polymer nucleotide in 30 min under the conditions described. The assay is linear with respect to enzyme over the range 0.02–0.1 unit/ml.

Ultracentrifugation: The conditions used for preparative and analytical ultracentrifugation have been described.[7] For monitoring polymer synthesis, aliquots of reaction mixtures were added to solid NaCl (to yield final concentration of 1 *M*), dialyzed successively against 1 *M* NaCl and 0.01 *M* Tris pH 7.9, and examined by analytical ultracentrifugation. For the isolation of "hybrids," reactions were treated in the same way, and preparative centrifugation performed in CsCl (average density $\rho = 1.70$) in a volume of 3.5 ml under mineral oil, in a Spinco SW39 rotor, 20°, at 33,000 rpm for 72 hr. After puncturing the bottom of the tube, the fractions that corresponded to the regions of "hybrid" density and radioactivity were pooled, dialyzed against 0.01 *M* Tris-HCl, pH 7.9, and recentrifuged under identical conditions in another gradient of CsCl (average density $\rho = 1.45$) in the presence of an excess of actinomycin. The "hybrid" is more dense than d(A-T) in the first gradient, and less dense in the second. All "hybrids" were differentially labeled in the d(A-T) and d($\overline{\text{DAP}}$-T) segments, and purified in two successive gradients. Actinomycin was removed by phenol extraction following dialysis against Tris-HCl 0.01 *M*, pH 7.9.

The thermal denaturation profile of polymers was obtained with a Gilford automatic recording spectrophotometer. We thank Dr. G. Acs for making this equipment available.

Results.—While studying the d(A-T)-directed formation of an alternating deoxynucleotide copolymer consisting of 2,6-diaminopurine and thymine, we have observed that *E. coli* DNA polymerase produces molecules of the form

$$(5')\ p\text{-}[\text{d(A-T)}]_n\text{-}p\text{-}[\text{d}(\overline{\text{DAP}}\text{-T})]_m\text{-OH}\ (3')$$

Thus, the d(A-T) template is, in fact, a primer; the residues of $\overline{\text{DAP}}$ and T are added at the free 3′-OH end few, if any, new chains being formed. This conclusion is supported by the following lines of evidence, full details of which will be published elsewhere:[8] (1) the kinetics of synthesis of d($\overline{\text{DAP}}$-T) are *linear* with time in contrast to those of d(A-T), which are exponential. (2) The rate of synthesis of d($\overline{\text{DAP}}$-T) is a linear function of the amount of primer initially present in the reaction. (3) During the early stages in the synthesis of d($\overline{\text{DAP}}$-T) with d(A-T) primer, the buoyant density of the product in gradients of CsCl is intermediate between that of pure d(A-T) ($\rho = 1.679$) and pure d($\overline{\text{DAP}}$-T) ($\rho = 1.718$). Upon isolation these molecules are found to consist of a region of pure d(A-T) at the 5′-end covalently bonded in tandem, and therefore in linear continuity with pure d($\overline{\text{DAP}}$-T) at the 3′-end, as shown by the following properties: (*a*) the "hybrid" density was not altered following denaturation at elevated temperatures or in dimethyl sulfoxide. (*b*) The release of radioactivity by exonuclease III from "hybrid" molecules differentially labeled in the d(A-T) and d($\overline{\text{DAP}}$-T) segments yields a pattern consistent only with the structure proposed

above. (*c*) The thermal denaturation profile of molecules of "hybrid" density reveals only two transitions corresponding to those of pure d(A-T) and pure d(DAP-T), respectively (Fig. 1). No molecules showing an intermediate "hybrid" *Tm* can be isolated at any stage of the reaction. The existence of "hybrid" molecules is transient; as a function of continuing d(DAP-T) synthesis the "hybrid" increases in density and finally disappears to be replaced progressively by pure d(DAP-T).

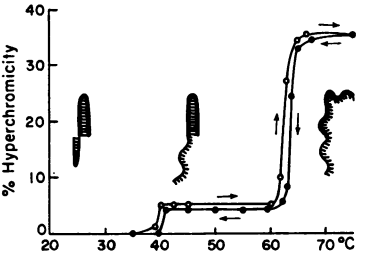


FIG. 1.—Thermal transitions of d(A-T)/d(DAP-T) "hybrid," with the structures proposed to correspond to each temperature range; the light lines in the sketches represent d(A-T), the heavy lines d(DAP-T) segments. Solvent: 0.01 *M* Tris-HCl, pH 7.9; hyperchromicity was followed at 260 mμ.

In view of these facts, it is of interest to note the following additional points of difference between the synthesis of d(A-T) and that of d(DAP-T): (1) the synthesis of d(A-T) reaches a plateau when 45–60 per cent of the precursor triphosphates have been incorporated into polymer,[9] after which the newly formed d(A-T) is slowly degraded, ultimately to completion.[10, 11] In contrast, d(DAP-T) synthesis proceeds until all (>95%) of the dDAP-TP and dTTP are incorporated into polymer and the newly formed d(DAP-T) is stable for many hours. (2) Although the *initial* concentration of d(A-T) primer determines the rate of d(DAP-T) synthesis *throughout the reaction*, and despite the incorporation of d(A-T) into product molecules of "hybrid" density, the d(A-T) primer is totally degraded as the synthesis of d(DAP-T) progresses (Fig. 2). We conclude (*i*) that the polymerase preparations contain a nuclease to which d(A-T) is susceptible and d(DAP-T) is immune, and (*ii*) that this nuclease attacks d(A-T) from the 5′-end.

Exonuclease VI, a DNA-specific nuclease associated with highly purified fractions of DNA polymerase: To demonstrate the presence of this nuclease directly we have isolated d(A-T)/d(DAP-T) "hybrids," differentially labeled in the d(A-T) and d(DAP-T) segments, and have followed the fate of each segment when the "hybrids" serve as primers for the synthesis of additional d(DAP-T). With this primer, as with pure d(A-T) or d(DAP-T), the synthesis of additional d(DAP-T) proceeds linearly to complete exhaustion of precursor triphosphates. As seen in Figure 3, this process is associated with the degradation of the P^{32}-labeled, d(A-T) segments of the "hybrid," while the H^3-d(DAP-T) regions remain intact. The degradation products of the d(A-T) migrate as monophosphates on paper electrophoresis at pH 3.5 and are dephosphorylated by rattlesnake venom. These facts suggest that the nuclease is an exonuclease which catalyzes the stepwise removal of mononucleotides from the 5′-end of polymers.

Several lines of experiments have been performed to test whether exonuclease VI and DNA polymerase might be integral components of the same protein

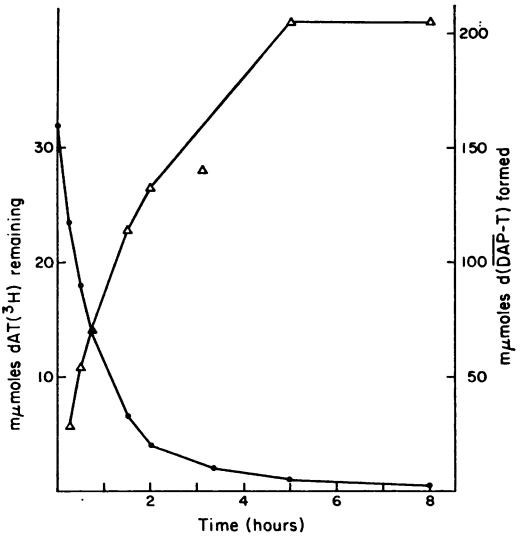


FIG. 2.—Synthesis (Δ—Δ) of d(DAP-T) and degradation (●—●) of the d(A-T-H³) (30 mμmoles/ml), which served as primer. Standard conditions, with 30 units/ml DNA polymerase. The final level of d(DAP-T) synthesis represents complete utilization and incorporation of precursor dDAP-TP + dTTP. One portion of a single reaction mixture was a source of aliquots for following disappearance of primer; a second portion, to which dTTP-H³ of much higher specific activity had been added, was used to follow synthesis. The newly formed d(DAP-T) is not later degraded.

molecules: all results obtained so far point to the physical unity of the two catalytic entities. Thus, (1) the elution profiles of nuclease and polymerase are superimposable, and the ratio of the two activities are constant throughout chromatography on anion- and cation-exchange resins and on gel filtration through G-200 Sephadex (Figs. 4*a–c*). (2) Neither DNA polymerase nor exonuclease VI is inhibited when PCMB is present in the reaction mixtures. However, we have found that the rate of polymerase inactivation is markedly accelerated when the enzyme is preincubated with PCMB at 37°. Therefore it is of interest that under these conditions the rates of disappearance of both nuclease and polymerase are identical.

Finally, nuclease assays have been performed using "hybrid" molecule and calf thymus DNA preparations in which the 5′-phosphoryl termini had been labeled with P^{32} by means of polynucleotide kinase.[12] These termini are released almost instantaneously by the enzyme and appear as 5′-monophosphates. Nuclease assays performed in the absence of dNTP also reveal that the enzyme has an absolute requirement for Mg^{++}. The enzyme does not require a 5′-phosphoryl group, and attacks molecules bearing 5′-OH termini with equal efficiency. The enzyme is active throughout the pH range 6.5–9.2, and the ratio of polymerase/exonuclease VI activity is approximately 2 at pH 7. Exonuclease II and VI are differentiated by the fact (*i*) that d(DAP-T) is very susceptible to the former at pH 9.2 and resistant to the latter at pH 7, whereas d(A-T) is degraded

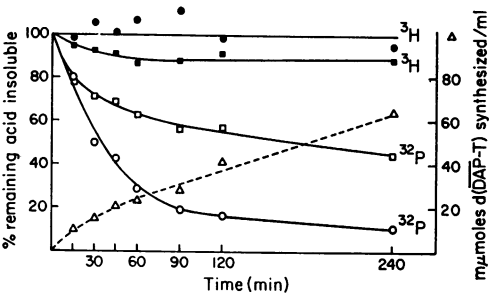


FIG. 3.—Action of DNA polymerase on d(A-T-P^{32})/d(DAP-T-H^3) hybrids in the presence (○, ●) and absence (□, ■) of dDAP-TP and dTTP. The addition of dNTP accelerates the degradation of the d(A-T) segments and protects the d(DAP-T) segments. The synthesis of d(DAP-T) (△—△) was followed in a separate aliquot of the reaction mixture by measuring the incorporation of dTTP-H^3 of specific radioactivity much higher than that of the primer. The concentration of polymerase was 30 units/ml.

by both; and (*ii*) that the ratio of the two activities at pH 9.2 and pH 7 is very different. We have no experimental findings as yet that bear on any possible identity in the active sites of polymerase, exonuclease II, and exonuclease VI.

Discussion.—The results described in this paper reveal that highly purified preparations of DNA polymerase contain an exonuclease that cannot be separated from the polymerase by a variety of physical procedures. The nuclease attacks DNA molecules at the 5′-terminus, and differs in this respect from another polymerase-associated nuclease, exonuclease II. These observations may be relevant for two questions pertaining to DNA synthesis, namely, chain initiation and simultaneous unidirectional replication of the two strands of a helix.

On the basis of the evidence cited above, we conclude that the qualitative kinetic differences in the synthesis of d(A-T) and d(DAP-T) can best be understood by assuming that the initiation of new chains proceeds easily in the former case and very poorly in the latter. In the presence of enzyme excess, the rapid formation of new chains satisfactorily accounts for the observed exponential kinetics with d(A-T), whereas failure to initiate new chains at a significant rate would explain the persisting linear pattern of synthesis of d(DAP-T). It is important to note that dDAP-TP is not simply inert with respect to chain initiation, but in fact acts to inhibit this process. When polymer synthesis is conducted with mixtures of dDAP-TP and dATP, the base composition of the product faithfully reflects the input ratio of precursor triphosphates. At a ratio dDAP-TP/dATP = 0.2, the kinetics of synthesis change from exponential to linear, although the initial rates of polymerization at ratios of 0.1 (exponential pattern) and 0.25 (linear) differ only by a factor of 2. Thus, increasing proportions of dDAP-TP in the reaction mixture appear to inhibit chain initiation to a greater extent than chain propagation. It follows that the two processes, each with its own substrate specificity, may reflect qualitatively distinct catalytic functions of DNA polymerase; however, we cannot as yet exclude other possible alternatives.

The unusual substrate properties of dDAP-TP permit the synthesis of a polymer with helical regions of different base composition and stability at the two ends of a single, continuous polynucleotide chain. With each region suitably labeled, such molecules are ideal substrates for characterizing the degradative polarity of exonucleases. The use of these substrates has revealed the presence of a new

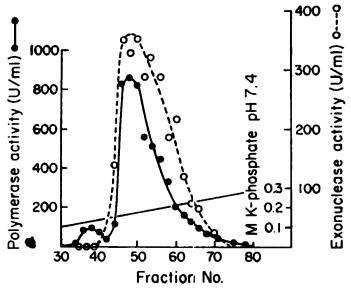
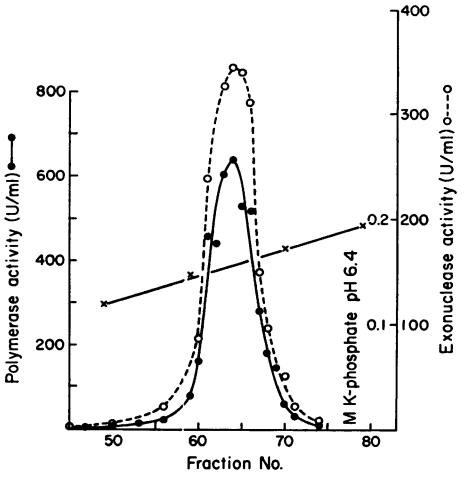
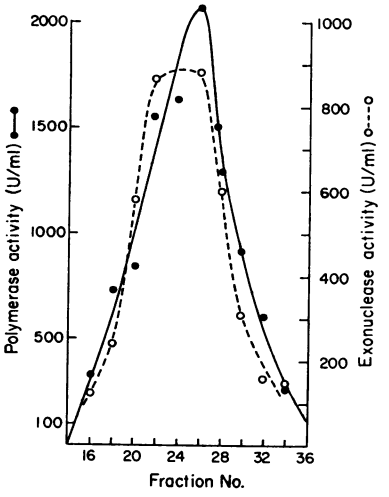


FIG. 4.—A separate batch of DNA polymerase was isolated as described in *Methods;* the specific activity was 16,000 units/mg. Aliquots (approximately 25,000 units) were subjected to three analytical procedures: (*Left*) (*a*) DEAE-cellulose chromatography: 0.55 × 9.5-cm column equilibrated and sample applied in KPO_4 buffer (0.01 *M*, pH 7.4 + BME 10^{-2} *M*). Enzyme eluted with linear gradient (60 ml) 0.01 *M*–0.3 *M* KPO_4 (pH 7.4) + BME 10^{-2} *M;* fractions were 0.6 ml.



(*b*) Phosphocellulose chromatography: 0.9 cm × 15.5-cm column equilibrated and sample applied in 0.005 *M* KPO_4 buffer pH 6.4 + BME 10^{-2} *M*. The column was washed with 20 ml 0.005 *M* KPO_4, 10 ml 0.1 KPO_4, and eluted with linear gradient (120 ml) 0.1 *M*–0.3 *M* KPO_4 (pH 6.4). All buffers contained BME 10^{-2} *M*. Fractions were 3 ml.



(*c*) Gel filtration through Sephadex G-200. The column (1.2 × 42 cm) was equilibrated and developed with 0.05 *M* KPO_4, pH 7.4, containing BME (10^{-2} *M*). Fractions were 1 ml.

exonuclease activity in the four highly purified preparations of DNA polymerase examined to date. This nuclease and the polymerase are not merely inseparable, but behave as though they were identical during a variety of physical procedures. The presence of this nuclease may account for several phenomena associated with the *in vitro* action of DNA polymerase. These include (*a*) failure to observe 5′-triphosphate termini associated with the formation of new chains, (*b*) the requirement of boiled extracts (presumably containing oligodeoxynucleotides that promote efficient chain initiation) for optimal utilization of single-stranded DNA templates, and (*c*) multiple 5′-termini in the products formed with such templates.[13, 14]

It is of interest to consider the possible implications of this nuclease activity for DNA replication *in vivo*. Genetic[15] and autoradiographic[16] evidence suggest that DNA replication in bacteria is unidirectional along the length of the chromosome

and that both strands of the helix are replicated simultaneously. Neither of these techniques can resolve molecular events at the level (single or even hundreds of nucleotides) required for precise insight into enzymatic mechanisms. Even so, the catalytic properties *in vitro* of DNA polymerase, hitherto restricted to the 3′-end of polynucleotides, are not comfortably reconciled with observations *in vivo*.[17]

The activity of polymerase at the 3′-OH termini of DNA strands possesses two facets, namely, stepwise addition (polymerase) and/or removal of single nucleotides (exonuclease II). The degradative action may be viewed as a perverted reversal of polymerization, in which the enzyme preferentially selects OH^-, rather than PP_i, to displace the terminal nucleotide from its diester bond, yielding deoxynucleoside-5′-monophosphates. Exonuclease VI also catalyzes the stepwise hydrolysis of polymers, and liberates deoxynucleoside-5′-monophosphate from the 5′-terminus. Therefore, it is tempting to infer that this activity, like its counterpart at the 3′-terminus, may also reflect a normal addition of nucleotides.

One plausible scheme, illustrated in Figure 5, forms the basis for current experi-

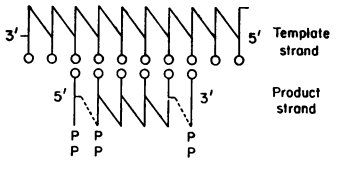


FIG. 5.—A possible scheme for DNA polymerase function. Given the release of 5′-monophosphates from the 5′-end, the postulated corresponding addition of nucleotides would require the maintenance of terminal 5′-triphosphates at the growing 5′-end, with PP_i being displaced by the 3′-OH of the incoming triphosphate. This is in contrast to the established action of polymerase at the 3′-end where the 3′-OH of the polymer terminus displaces PP_i from the incoming triphosphate.

mental attempts to detect pyrophosphorolysis and chain propagation, if they occur, at the 5′-end of appropriately protected and labeled oligonucleotide-polynucleotide complexes. An attractive aspect of this postulate derives from the implied similarities in steric and mechanistic requirements for polymerization at both termini: in both cases (1) 3′-OH acts to displace PP_i from 5′-triphosphates, (2) the helical relationship between template base, primer base, and incoming nucleotide are preserved. Thus a single catalytic site, suitably constructed to accommodate optionally the extra pyrophosphate moiety needed at the 5′-end could function interchangeably to add (or remove) nucleotides at either end of polynucleotide chains. The failure to observe synthesis at 5′-termini of DNA molecules *in vitro* could be due to a variety of causes which cannot be considered here.

The present results provide some concrete experimental basis for considering that a single enzyme—DNA polymerase—may possess the capacity to replicate both strands of a DNA helix in a single direction by the simultaneous stepwise addition of nucleotides at the 3′-terminus of one growing strand and the 5′-terminus of the other. Recent evidence suggests that the enzymatic synthesis of bacteriophage RNA may be catalyzed by an enzyme which can promote chain elongation at both 3′- and 5′-termini.[18]

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Abbreviations: BME, β-mercaptoethanol; dNTP, deoxynucleoside-5'-triphosphate; dATP, dTTP, dDAP-TP, deoxynucleoside 5'-triphosphate of adenine, thymine, and 2,6-diaminopurine, respectively; d(A-T), d(DAP-T), alternating deoxynucleotide copolymers of adenine and thymine, 2,6-diaminopurine and thymine, respectively; PCMB, *p*-chloromercuribenzoic acid; d(A-T)/d(DAP-T), density "hybrids" with pure d(A-T) segments at the 5' and pure d(DAP-T) at the 3' regions; DEAE-cellulose, *O*-(diethylaminoethyl) cellulose; Tris-HCl, tris(hydroxymethyl) aminomethane-HCl; PP_i, inorganic pyrophosphate.

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