Size Classes of Products Synthesized Processively by Two Subassemblies of *Escherichia coli* DNA Polymerase III Holoenzyme*

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Two forms of *Escherichia coli* DNA polymerase III, DNA polymerase III', and DNA polymerase III* have been shown to synthesize DNA products via a processive mechanism with product sizes distinctive for each enzyme form. These forms of DNA polymerase III are intermediate in complexity between the core DNA polymerase III and the DNA polymerase III holoenzyme. In a previous publication (Fay, P. J., Johanson, K. O., McHenry, C. S., and Bambara, R. A. (1981) *J. Biol. Chem.* 256, 976-983), we demonstrated that on a randomly primed 14C DNA template or on an oligo(dT)12 poly(dA) template, the DNA polymerase III holoenzyme adds more than 100 nucleotides before dissociation, whereas the core enzyme adds 10 to 15 nucleotides. Now we show that DNA polymerase III' adds 30 to 40 nucleotides before dissociation. This number can be increased to approximately 60 if spermidine is present, but it is insensitive to the presence of *E. coli* single-stranded DNA-binding protein. DNA polymerase III* adds about 50 nucleotides before dissociation, but this value can be increased to 200 nucleotides in the presence of the binding protein.

Using measurement of product sizes made on an oligo(dT)12 poly(dA) template, reconstitution of holoenzyme activity from DNA polymerase III* and the β subunit was monitored. Finally, it is shown that the products obtained from a purified initiation complex of holoenzyme and oligo(dT)12 poly(dA) derive solely from the holoenzyme.

The DNA polymerase III holoenzyme of *Escherichia coli* is a complex, multisubunit enzyme composed of a DNA polymerase III core and several auxiliary proteins. Although a final determination of the DNA polymerase III holoenzyme structure has not yet been achieved, it is apparent that three auxiliary proteins, β, γ, and δ, are required in addition to a catalytic core form DNA polymerase III for the conversion of single-stranded phage DNAs to the duplex replicative form (McHenry and Kornberg, 1977). The catalytic core of the DNA polymerase III holoenzyme, referred to here as DNA polymerase III, is composed of three subunits, α, ε, and θ (McHenry and Crow, 1979). DNA polymerase III, while able to catalyze limited synthesis on nuclease-activated duplex DNA, is not active by itself on the naturally primed phage DNA templates.

S. Wickner and Hurwitz (1976) have isolated three proteins, Factor I, dnaZ protein, and Factor III which may correspond, respectively, to β, γ, and δ. Furthermore, the β protein probably is copolymerase III* (Wickner et al., 1973). Early studies indicated that the holoenzyme could be resolved by phosphocellulose chromatography into two components, polymerase III* and copolymerase III* (Wickner and Kornberg, 1974; Wickner et al., 1973). DNA polymerase III* was distinguished from DNA polymerase III by its ability to use a primed single-stranded template in the presence of copolymerase III* and by its greater size and lability.

Recently, the polypeptide γ, a seventh component of holoenzyme preparations, has been implicated as a subunit (McHenry, 1980). Purified as a complex with DNA polymerase III, it is termed DNA polymerase III' (McHenry, 1982). The γ polypeptide is not required for reconstitution of holoenzyme activity on single-stranded phage DNA templates, but alters some properties of core polymerase III to make it resemble the holoenzyme.1

The number of nucleotides added by the DNA polymerase III core after a single binding event to the 3' terminus of a DNA primer template (the processivity of polymerization) is about 10 residues, compared to a processivity which exceeds 5000 for the holoenzyme (Fay et al., 1981). This effect must result from the action of one or more of the auxiliary subunits which are associated with the core polymerase in the holoenzyme complex. The present study was conducted using two subassemblies of DNA polymerase III holoenzyme subunits to determine the contributions of particular subunits to synthetic activity, product size, and processivity of DNA polymerization by DNA polymerase III forms.

MATERIALS AND METHODS

**Proteins and Enzymes—** *E. coli* DNA polymerase III holoenzyme was prepared by the methods of McHenry and Kornberg (1977). DNA polymerase III* was prepared according to McHenry (1982). DNA polymerase III' was prepared from 1 kg of cells as described for DNA polymerase III through Fraction III (McHenry and Crow, 1979). Fraction III was concentrated by ammonium sulfate precipitation (50% saturation) and redissolved in 1 ml of 50 mM Tris-HCl (pH 7.5), 30% glycerol, 1 mM EDTA, 40 mM NaCl, and 5 mM dithiothreitol. This sample (1.5 × 10⁸ units) was gel filtered through a Sephacryl S-300 column (1.5 × 25 cm) equilibrated in the same buffer resulting in Fraction IV (9.6 × 10⁶ units/mg). The enzyme selected for all experiments reported herein eluted in tube 29 (0.6 ml/tube) and had a specific activity of 1.1 × 10⁶ units/mg. This represented a 9000-fold purification.

1 The minimum subunit assignments for the enzymes are as follows: DNA polymerase III core, α, β, and ε; DNA polymerase III', α, β, ε, and γ; DNA polymerase III*, α, β, ε, γ, and δ; and DNA polymerase III holoenzyme, α, β, ε, γ, δ, and β.

**—**E. coli**—**
tion. Assays were performed in the G4 system in the presence of saturating β subunit.

β and anti-β IgG were prepared according to Johnson and Mc- 
Heffernan (1978). Single-stranded DNA-binding protein from E. coli was prepared by the methods of Meyer et al. (1980) or Weiner et al. (1975). E. coli DNA polymerase I (Fraction VII prepared by method of Jovin et al. (1969)) was kindly provided by Dr. A. Kornberg (Stanford University).

Nucleotides and Polynucleotides—Unlabeled dNTPs were pur-
chased from Boehringer-Mannheim. [3H]dTTP (78 Ci/mmol) and [3H] 
dGTP (10 Ci/mmol) were purchased from New England Nuclear Corp. Poly(dA) (10 μg) was purchased from Miles Laboratories. Oligo(dG)19 and oligo(dT)19 were purchased from P-L Biochemicals. Poly(dA)-oligo(dT)20 (20:1) complex containing adenine and thymine in a molar ratio of 20:1 was prepared by annealing poly(dA) and oligo(dT) at 50°C for 5 min in 0.3 M NaCl and 0.1 M Tris HCl (pH 9). Preparation of randomly primed fd single-stranded DNA containing approximately five primers per fd molecule was previously described (Fay et al., 1981). All concentrations of DNA are given as DNA nucleotide.

Measurement of Processivity of the DNA Polymerase on the 
Primed fd DNA—Processivity was measured for E. coli DNA polym-
erase III and DNA polymerase III* using the kinetic techniques of 
Bambara et al. (1978). A quantitative value for the extent of processive 
DNA synthesis (i.e., the average number of nucleotides added each time the DNA polymerase binds a DNA template) is obtained by this method. The method involves a comparison of the rate of DNA synthesis in the presence of a limited complement (one, two, or three) of dNTPs to the rate when all four dNTPs are present. The reaction with a limited complement of dNTPs indicates the number of 
terminals on which binding and synthesis were attempted, while the reaction with all four dNTPs indicates the total number of nucleotides 
polymerized onto the DNA template. From this information, the 
average number of nucleotides added per binding event is calculated.

Because the period required for the polymerase molecules to cycle 
from one template to the next in the limited versus the four dNTP 
reactions may differ, a correction factor for this difference must be 
included in the analysis. A template analogue inhibitor is added to two 
additional reactions, one with a limited complement of dNTPs and the 
other with all four dNTPs. The extent to which the inhibitor reversibly 
binds the polymerase as it moves from one template to the next. A 
comparison of the extent of inhibition in the limited reactions to the 
inhibition in the unlimited reactions yields the "cycling time" correction factor. The cycling times in the presence of three dNTPs with respect to those in the presence of four dNTPs range from 0.2 to 0.4 for DNA polymerase III and 0.15 to 0.4 for DNA polymerase III*, depending on reaction conditions. The template analogue inhibitor used in these studies is a poly(dA)- 
oligo(dT)19 template. [3H]dTTP is the labeled dNTP in all reactions 
such that all radioactivity incorporated into acid-insoluble product is 
the result of synthesis on the primed fd DNA template. As a result, 
the syntheses at [3H]dTTP functions as an inhibitor DNA synthesis. It also fulfills the criteria for inhibitor DNA (Bambara et al., 1978) since it reversibly binds to the DNA polymerase, and the 
DNA and dTTP interact with the DNA polymerase in the same manner in all reactions.

For processivity determinations of DNA polymerase III and DNA 
polymerase III*, reaction mixtures (50 μl) contained the following 
buffer components: 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 200 μg/ml of bovine serum albumin, 20% glycerol (v/v), and 15 mM diethio-
treitol. Reaction mixtures used for DNA polymerase III contained 100 μM 
primed fd DNA and 8 to 24 units of polymerase. Limited reactions contained [3H]dTTP (10 Ci/mmol) plus dATP and dTTP at 100 μM 
each. Unlimited reactions contained [3H]dTTP and all four 
dNTPs at 100 μM each. In inhibited reactions, the mixtures 
were altered to contain 25 μM primed fd DNA and 75 μM poly(dA)- 
oligo(dT)19 (20:1). All assays were performed at 30 °C. Samples of 

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2 The abbreviations used are: dNTP, deoxynucleoside triphosphate; SSB, single-stranded DNA-binding protein.
3 One unit of polymerase III activity is the amount of enzyme catalyzing the incorporation of 1 pmol of (total) deoxyribonucleotide/men on an activated salmon sperm DNA template.
4 Processivity determination reactions are performed at approxi-
nately 0.85 Vₛ, with respect to DNA concentration so that reaction rates are essentially independent of the concentration of this sub-
strate. DNA concentration is adjusted in inhibited reactions for this

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5 One unit of DNA polymerase III activity is defined as 1 pmol of (total) deoxynucleotide incorporated/min on a G4 DNA template with priming of dnaG primase in situ when supplied with saturating levels of the β auxiliary subunit.
6 Processivity determination reactions are performed at greater 
than the 0.85 Vₛ, with respect to dNTP concentration so that reaction 
rate is essentially independent of the dNTP concentration. Indepen-
dence of rate with dNTP concentration is achieved at a lower 
dNTP concentration with DNA polymerase III* than with DNA polymerase III.

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7 One unit of holoenzyme activity is defined as 1 pmol of (total) deoxynucleotide incorporated/min on a G4 DNA template with priming by dnaG primase in situ.
column (0.9 x 17 cm) equilibrated in enzyme dilution buffer at room temperature. The initiation complex eluted in the excluded column volume and was detected by the incorporation of [3H]dATP (9000 cpm/pmol). The total activity of the preincubation mixture prior to gel filtration was 710 pmol of dTMP incorporation, while the total activity of the initiation complex recovered from the column was about 20 pmol of incorporation.

An aliquot (60 μl) of the preincubation mixture (not gel filtered) and an aliquot of the isolated initiation complex (800 μl) were reacted with 50 μM [3H]dATP (9000 cpm/pmol) for 10 min at 30 °C. The initiation complex reaction mixture contained anti-β IgG (3.1 μg) to prevent reinitiation of the holoenzyme. The lengths of the extended primers were determined as described above.

RESULTS

Effects of Spermidine and SSB on Synthetic Activity of Forms of DNA Polymerase III—We have previously shown that spermidine or SSB is inhibitory to the synthetic activity of the DNA polymerase III core enzyme on a randomly primed fd DNA template (Fay et al., 1981). On the other hand, stimulation of both rate of synthesis and the extent of processive DNA synthesis was observed for the holoenzyme in the presence of either spermidine or SSB. One or more of the holoenzyme auxiliary subunits were presumably responsible for the change in response to these compounds.

As shown in Fig. 1A, spermidine stimulates reaction rate for both DNA polymerase III' and polymerase III*. In the case of DNA polymerase III*, a lower concentration of spermidine is required for stimulation. Thus, the γ subunit, present in polymerase III' and polymerase III*, but lacking in the core enzyme, appears to confer the capability of stimulation by spermidine.

The inclusion of SSB in a similar assay has disparate effects on the DNA polymerase III' and DNA polymerase III* enzyme forms (Fig. 1B). SSB coating of the template (approximately 1 μg of SSB/800 pmol of fd DNA) results in no stimulation of the polymerase III'. In fact, higher levels of SSB than those required for complete coating are inhibitory to DNA synthesis. The synthetic activity of polymerase III* is stimulated after coating of the template with SSB. The stimulation is similar to that previously observed with the holoenzyme. Thus, holoenzyme subunit(s) present in polymerase III* which are lacking in polymerase III', presumably the γ and δ subunits, confer upon the core enzyme the capacity to utilize an SSB-coated DNA template.

Processive DNA Synthesis by DNA Polymerase III' and DNA Polymerase III*—Both enzyme forms exhibit a moderately processive mode of DNA synthesis in the presence of an excess level of randomly primed fd DNA (Table I). The values obtained in the absence of spermidine or SSB are intermediate to those previously reported for the core enzyme, which adds about 11 nucleotides, and the holoenzyme, which adds over 100 nucleotides each time it binds a primer terminus (Fay et al., 1981).

When the fd DNA template is coated with SSB, the processivity of polymerase III* remains unchanged. On the other hand, processivity of DNA polymerase III' is increased about 4-fold compared to the value in the absence of SSB. Thus, an SSB-coated template, while having no effect on the translocation of polymerase III', allows polymerase III* to form a more stable enzyme-DNA complex capable of synthesizing DNA over much longer distances in a single binding of the polymerase to the primer template.

The above results demonstrate that each stable form of DNA polymerase III makes a distinct class of product sizes in the absence or presence of effectors of synthesis, which is indicative of the particular enzyme form.

When conditions are changed so that there are saturating levels of DNA polymerase with respect to the primer template, the total distance over which synthesis occurs at each 3' end.

### Table I

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Processivity*</th>
<th>No. of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymerase III'</td>
<td>37.5 ± 4.5</td>
<td>5</td>
</tr>
<tr>
<td>+Spermidine*</td>
<td>60.5 ± 9.8</td>
<td>7</td>
</tr>
<tr>
<td>+SSB'</td>
<td>38.5 ± 9.4</td>
<td>3</td>
</tr>
<tr>
<td>Polymerase III*</td>
<td>50.2 ± 11.4</td>
<td>7</td>
</tr>
<tr>
<td>+Spermidine (1 mM)</td>
<td>48.2 ± 9.2</td>
<td>6</td>
</tr>
<tr>
<td>+Polymerase (4 mM)</td>
<td>30.8 ± 5.4</td>
<td>3</td>
</tr>
<tr>
<td>+SSB</td>
<td>189.1 ± 22.1</td>
<td>5</td>
</tr>
</tbody>
</table>

* Standard deviations are shown with these values.
  The concentrations of spermidine used in this experiment were 2 mM and 4 mM; results were indistinguishable and, therefore, were combined.
  The concentration of SSB used in the above experiments and in those presented in Table II was 1.25 μg of SSB/nmol of fd DNA (as nucleotide).
  Antibody (3.1 μg) to the β subunit of holoenzyme was included in some determinations. The results obtained in its presence were indistinguishable from those in its absence and, therefore, were combined.
terminus is similar for both DNA polymerase III' and polymerase III in the absence or presence of spermidine (Table II). The values obtained represent the average amount of synthesis which has taken place at each 3' terminus after repeated interactions of the DNA polymerase with such termini until no more nucleotides can be added. Since excess levels of E. coli DNA polymerase I can add about 1000 nucleotides to each 3' terminus of this primed fd DNA template (data not shown), the values obtained are not determined by the physical length of the template, but, instead, determined by some sequence or structure in the template which prevents extensive synthesis by the two enzyme forms.

The inclusion of SSB results in a marked increase in the ability of the polymerase III' and polymerase III in to utilize this template, presumably by removing regions of secondary structure. Assuming approximately five primers per single-stranded fd DNA molecule (6408 nucleotides in length), it appears that SSB allows the two enzyme forms to utilize 70 to 80% of the lengths of fd DNA templates available for synthesis adjacent to each 3' terminus.

Product Size Determinations on a Poly(dA)-Oligo(dT)10 Template—This defined homopolymeric template has previously been used to quantitate the lengths of DNA products synthesized by the DNA polymerase III core and holoenzyme (Fay et al., 1981). The method allows for direct determination of processivity, as indicated by the length of the product made when primers of known length are extended after one binding event of the DNA polymerase to each reacted primer terminus. To ensure that each reacted terminus has interacted only once with a DNA polymerase molecule, the DNA is present in large excess over the enzymes, such that after the reaction, the total number of nucleotides incorporated is approximately the same as the number of primer termini in the reaction.

Furthermore, this analysis has the advantage that if more than one class of products are made simultaneously, each class can be observed and quantitated. Additionally, changes in product size distributions can be detected.

After the reaction, primer size determination is made from the position of elution of the labeled primers, compared to oligonucleotides of known length, from an alkaline agarose column. Quantitative values of product size can be estimated for products ranging from 1 to greater than 100 nucleotides. Products even greater in length are completely excluded from the resin, such that an exact size estimation cannot be made.

The number of nucleotides added onto each oligo(dT)10 primer after reaction with polymerase III was determined in the absence and presence of spermidine and SSB (Fig. 2). Extension involved, on the average, the addition of 30 to 50 nucleotides each time the polymerase binds to the primer terminus in the reaction with no additions (Fig. 2A), and a similar profile was obtained in the presence of SSB (Fig. 2C). The addition of spermidine (Fig. 2B) shifted the extended oligo(dT) primer to the left, indicating a greater length of processive primer extension (approximately 70 to 90 nucleotides).

Reactions on this template using DNA polymerase III' (Fig. 3) resulted in the elongation of the oligo(dT) primer by 40 to 60 nucleotides (Fig. 3A). The presence of sufficient anti-β antibody (3.1 μg) to inhibit 30 units of DNA polymerase III holoenzyme activity, as measured using the G4 assay (Johanson and McHenry, 1980), resulted in a similar elution profile (not shown). When spermidine was present at a concentration of 1 mM (Fig. 3B, closed circles), no apparent change in the elution profile was observed, while at 4 mM (Fig. 3B, open circles), the average size of the product was slightly diminished. The inclusion of SSB in the reaction resulted in a heterogeneous distribution of products (Fig. 3C), with many products over 100 nucleotides long.

As is the case with results obtained on the primed fd DNA template, the products generated by DNA polymerase III' and polymerase III' are intermediate to those made by the core enzyme and the holoenzyme. On the poly(dA) template, the core enzyme produced a single size class of product 8 to 11 nucleotides long, whereas products attributable to the intact holoenzyme were large enough to be completely excluded from the resin (more than 100 nucleotides in length) (Fay et al., 1981). Since intermediate enzyme form products are sufficiently distinct from the core and holoenzyme products, conversions from core to intermediate or from intermediate

### Table II

<table>
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<tr>
<th>Conditions</th>
<th>No. of nucleotides polymerized*</th>
<th>No. of determinations</th>
</tr>
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<tbody>
<tr>
<td>Polymerase III'</td>
<td>178.6 ± 8.7</td>
<td>3</td>
</tr>
<tr>
<td>+Spermidine (2 mM)</td>
<td>189.7 ± 15.5</td>
<td>4</td>
</tr>
<tr>
<td>+SSB</td>
<td>771 ± 26</td>
<td>2</td>
</tr>
<tr>
<td>Polymerase III'</td>
<td>200.7 ± 29.9</td>
<td>4</td>
</tr>
<tr>
<td>+Spermidine (1 mM)</td>
<td>218.9 ± 24.1</td>
<td>4</td>
</tr>
<tr>
<td>+SSB</td>
<td>810 ± 151</td>
<td>4</td>
</tr>
</tbody>
</table>

* Standard deviations are shown with these values.
Products of Escherichia coli DNA Polymerase III Subassemblies

Fig. 3. Products made by DNA polymerase III* on the poly(dA)-oligo(dT)10 primer-template. Primers extended by DNA polymerase III* were generated in a standard reaction described under "Materials and Methods." Results are presented as in Fig. 2. A shows a profile of product size for the polymerase III* reaction which incorporated 64.1 pmol of dTMP or 1.7 pmol of dTMP/pmol of 3' terminus. In B, the polymerase reaction was supplemented with spermidine-HCl at a concentration of 1 mM (closed circles) or 4 mM (open circles). In the presence of 1 mM spermidine, the polymerase incorporated 49.1 pmol of dTMP or 1.3 pmol/3' terminus; at 4 mM spermidine, the enzyme incorporated 18.9 pmol of dTMP or 0.5 pmol/3' terminus. In C, the reaction was supplemented with SSB (2 μg). This reaction incorporated 67.5 pmol of dTMP or 1.8 pmol/3' terminus. The polynucleotide markers are those used in Fig. 2.

to holoenzyme forms should readily be detected after the addition of individual auxiliary subunits (see below).

Reconstitution of Holoenzyme Product Generation in a Reaction Containing DNA Polymerase III* and the β Subunit—To further substantiate that the size class of products excluded from the column resin resulted from primers extended by the intact holoenzyme, reactions were performed in which the polymerase III* was supplemented with varying levels of the free β subunit. Results from this analysis are presented in Fig. 4. In Fig. 4A, a sufficient amount of β was added to restore holoenzyme-like activity using the bacteriophage G4 (single strand to replicative form) assay in the presence of excess DNA polymerase III* (McHenry and Kornberg, 1977). This level of β resulted in only a small fraction of the primers elongated to the larger (excluded) size class; the primary size class still represents products synthesized by polymerase III*. When increased amounts of β are added (Fig.

4, B and C), the proportion of excluded material increases relative to the products attributable to the polymerase III*.

Previous analyses of the synthetic products of the holoenzyme using this template revealed a mixture of products (Fay et al., 1981), presumably resulting from partial dissociation of the holoenzyme into other DNA polymerase III forms. The above results demonstrate that only the largest of the product size classes observed after gel filtration (that is, those excluded from the resin) derive from the intact holoenzyme.

Furthermore, these results show that reconstitution of holoenzyme activity can readily be detected and even quantitated either by the disappearance of the class of product which derives from DNA polymerase III* or the appearance of products which derive from the holoenzyme.

DNA Synthesis from a Preformed Initiation Complex on the Poly(dA)-Oligo(dT)10 Template—The preceding experiments reflect the distribution of product size classes resulting from partial reconstitutions of holoenzyme activity. Previous experiments using the intact holoenzyme on the poly(dA) template also resulted in the appearance of some products in a size class which eluted in fractions included in the column resin, with an apparent size of 10 to 30 nucleotides. In the latter case, the small size class was attributable to a contamination of endogenous core DNA polymerase III activity present in the holoenzyme preparation (Fay et al., 1981). This activity presumably results from the breakdown of the labile holoenzyme complex.

In this section, we report on the size class of products made when the only polymerase form capable of synthesis is the intact holoenzyme. In these experiments, an initiation complex consisting of the holoenzyme prebound to the poly(dA)-oligo(dT) primer-template is formed and isolated by gel filtration. A similar complex using a poly(dA)-oligo(dT) template.

FIG. 4. Reconstitution of the holoenzyme from DNA polymerase III* plus the β auxiliary subunit. Holoenzyme activity was reconstituted using free β protein added to the standard DNA polymerase III* reaction described under "Materials and Methods." In A, 48 units of β were added to the standard reaction which incorporated 27.3 pmol of dTMP or 0.73 pmol of dTMP/3' terminus. In B, 360 units of β were present. This reaction incorporated 36.4 pmol of dTMP or 0.97 pmol of dTMP/3' terminus. The elution profile present in C resulted from a reaction containing 1200 units of β. This reaction incorporated 52.5 pmol of dTMP or 1.4 of dTMP/3' terminus.
was first described by Wickner and Kornberg (1973). Formation of the complex required spermidine and ATP in the preincubation mixture. Gel filtration of the preincubation mixture separates the template DNA, to which is bound the intact holoenzyme (initiation complex), from any endogenous core polymerase and auxiliary subunits. Additionally, the polyamine is also removed from the initiation complex at this step.

Product size classes of DNA synthesized by the mixture before and after gel filtration are presented in Fig. 5. The elution profile in panel A represents the products made prior to gel filtration of the mixture. Two distinct classes of products are observed. The excluded size class, attributable to holoenzyme activity, represents only a small portion of the total activity. The included size class has a length consistent with core enzyme products. The high proportion of this product size class may result from a combination of endogenous core polymerase present in the holoenzyme preparation and the generation of core enzyme and other forms during the reaction to form the initiation complex. After the initiation complex is isolated by gel filtration, a single size class of product, excluded from the resin, is the only product synthesized (Fig. 5B). Therefore, purification of the holoenzyme prebound to the primer-template in the form of an initiation complex is an effective means of removing other forms of DNA polymerase from the reaction, thereby eliminating the included product size class.

To preclude reinitiation of the holoenzyme on a primer which had previously been extended by the initiation complex, anti-β antibody (3.1 μg) was included in this reaction. We have previously shown that the addition of this antibody to the primed poly(dA)·oligo(dT) template before addition of DNA polymerase III holoenzyme virtually eliminated generation of the product size class excluded from the resin, while the generation of products included by the resin was unaffected (Fay et al., 1981). Therefore, products excluded from the resin in the present experiment represent those made after one initiation event by the holoenzyme complex.

**DISCUSSION**

We have undertaken a mechanistic study of DNA polymerase III' and polymerase III*, two enzyme subassemblies whose structures are intermediate to the core and holoenzyme form of DNA polymerase III, in order to resolve the synthetic contributions made by the individual holoenzyme auxiliary subunits. Differential effects of spermidine and SSB on the initial rate of DNA synthesis and on the processivity of polymerization have been observed for each stable form of polymerase III. Spermidine or SSB inhibited synthesis catalyzed by the core enzyme while stimulation of both the initial rate of synthesis and processivity was observed with the holoenzyme (Fay et al., 1981). The initial rate of synthesis of DNA polymerase III' and polymerase III* is stimulated by spermidine. Thus, the τ subunit, present in the DNA polymerase III' and III* but lacking in the core enzyme, appears to confer the capability of stimulation by spermidine on a primed DNA template.

DNA polymerase III' and polymerase III* are easily distinguished if synthetic activity on naked DNA is compared with that on SSB-coated DNA. Although polymerase III' is not inhibited to the extent that the core enzyme is, neither is its activity stimulated by SSB. On the other hand, the activity of polymerase III* is stimulated on an SSB-coated template to a similar level previously observed with the holoenzyme. Thus, holoenzyme subunits present in polymerase III*, which are lacking in polymerase III', presumably the γ-δ complex, confer the capacity to utilize an SSB-coated DNA template.

Both DNA polymerase III' and III* exhibit a moderately processive mode of synthesis on primed DNA templates, with processivity values which are intermediate to the core and holoenzyme forms. Using a primed fd DNA template, polymerase III' and III* synthesize products about 40 to 50 nucleotides in length, respectively, in the absence of SSB or spermidine. A similar size class of product is observed using the homopolymer poly(dA)·oligo(dT) template.

Processive synthesis catalyzed by polymerase III' is enhanced about 1.5-fold in the presence of spermidine on both primed fd and the poly(dA) templates. A puzzling result is that the processivity of polymerase III* is unchanged as the spermidine level is raised to the value causing the maximum stimulation of the initial rate of synthesis. However, the processivity of polymerase III* is reduced by higher levels of the polyamine in a way that parallels the reduction in synthetic activity of this enzyme form.

Since the core DNA polymerase III adds only 10 to 15 nucleotides each time it binds a primer terminus, the association of the τ subunit with the core enzyme allows for a severalfold increase in processivity. The similarity of products, all 40 to 50 nucleotides long, derived from processive synthesis on uncoated DNA by the prime and star enzyme forms, indicates that the holoenzyme auxiliary subunits present in polymerase III' which are lacking in polymerase III* make no direct contribution to the lengths of DNA products synthesized on this template. However, when the primed template is coated with SSB, a marked difference in processivity of the prime and star forms is observed. The processivity of polymerase III' is unchanged from the value obtained in the absence of SSB on both the primed fd DNA and poly(dA) templates. On the other hand, processivity of polymerase III* increases about 4-fold when the fd DNA template is coated with SSB. Similarly, using the poly(dA) template, the presence of SSB
results in polymerase III* synthesizing a heterogeneous distribution of products with many greater than 100 nucleotides long. Since very stable secondary structure should not readily form on the homopolymeric template, SSB might directly interact with polymerase III* to form a stable replication structure not formed with polymerase III, which is capable of extensive DNA synthesis.

When the polymerase is in excess relative to the primed fd DNA, the enzyme can repeatedly interact with each primer terminus until no more nucleotides can be added. After such reactions, the maximum extent of synthesis at each 3' terminus can be quantitated. The values obtained for DNA polymerase III' and III* are similar to each other and intermediate to those values obtained with the core and holoenzyme (Fay et al., 1981). While spermidine is without effect on the total distance the enzymes can translocate during synthesis, SSB can increase these values about 4-fold, presumably by removing regions of secondary structure. SSB allows the intermediate enzyme subassemblies to synthesize over greater than 70% of the DNA templates adjacent to each 3' terminus.

DNA polymerase III' and DNA polymerase III* each appear to make a distinct product size. It is important to point out several features.

1) The product corresponding to extents of processive synthesis for these enzymes on the poly(dA) template correspond in average size to those measurements made using the native fd DNA template. This suggests that the poly(dA) template is an appropriate model for native DNA.

2) Products made on the poly(dA) template are found in distinct symmetrical peaks, suggesting that each of the two intermediate enzymes is not contaminated with holoenzyme or core enzyme.

3) Intermediate enzyme form products are sufficiently distinct from holoenzyme and core enzyme products in average size that conversion from core to intermediate or from intermediate to holoenzyme forms can readily be detected.

4) There is insufficient difference in product size of DNA polymerase III' versus DNA polymerase III* in the absence of spermidine or SSB to clearly distinguish each enzyme. However, the presence of spermidine or SSB sufficiently changes the product sizes such that the enzyme forms can be distinguished by product size.

We have taken advantage of the distinct product size classes produced by the core-containing enzyme subassemblies to reconstitute the holoenzyme from polymerase III* and free β subunit. When products made by preparations of the holoenzyme were analyzed, two distinct size classes of products were observed (Fay et al., 1981). One size class eluted in an included column volume. We attributed these products to endogenous core polymerase activity, resulting from the breakdown of the labile holoenzyme complex based upon: 1) a similar size to those products made by purified core enzyme, and 2) suppression of this size class by SSB and spermidine, agents which stimulate holoenzyme activity. The larger size class, excluded from the resin, was attributed to the holoenzyme since its synthesis was suppressed by antibody to the β subunit.

When the free β subunit is added to polymerase III*, the resulting products made on the poly(dA)-oligo(dt)10 template are characteristic of those made by intact holoenzyme and polymerase III*. The proportion of holoenzyme products relative to polymerase III* products increases when increased levels of β are added to the reaction. The failure to obtain complete reconstitution of holoenzyme activity at very high levels of β indicates that β and polymerase III* may not readily reassociate to form an intact holoenzyme complex. This result is consistent with immunoprecipitation studies (Johanson and McHenry, 1980). When the holoenzyme was treated with β antibody, β is precipitated. Additionally, the α subunit of core polymerase also precipitates because of its physical attachment to β in the holoenzyme. However, if polymerase III* and β are mixed and subjected to a similar immunoprecipitation, only β precipitates.

In the presence of ATP, holoenzyme can form an isolatable initiation complex upon incubation with a primed single-stranded DNA template (Wickner and Kornberg, 1973; Johanson and McHenry, 1980). Gel filtration of the preincubation mixture separates the template DNA, to which is bound the intact holoenzyme, from any endogenous core polymerase. Formation of an initiation complex is blocked by antibody to the β subunit, while extensive elongation of a preformed initiation complex is insensitive to the antibody (Johanson and McHenry, 1980). Thus, β antibody can be used to block reinitiation so that processivity of the holoenzyme in the form of an initiation complex can be measured.

Prior to gel filtration, products made by the preincubation mixture indicate the presence of core enzyme activity. The relatively high level of this activity may result from breakdown of the holoenzyme structure during the incubation required for initiation complex formation. After gel filtration, only a single size class of product is made. The product, which is excluded from the resin, results from processive synthesis solely by the intact holoenzyme.

Recently, Huang et al. (1981) have demonstrated that ATP is required for the formation of a T4 DNA polymerase accessory protein-DNA template complex. This complex greatly increases the strength of binding between the T4 DNA polymerase and the primer terminus, resulting in a highly processive mode of synthesis.

From the study of intermediate holoenzyme subassemblies, we have concluded that: 1) a gradient of processivities exists from core enzyme through intermediate subassemblies to the holoenzyme, which reflects their structural complexities; 2) the τ subunit, when associated with the core enzyme, allows for stimulation of activity by spermidine and contributes to a processivity increase of severalfold; 3) those subunits present in polymerase III* but lacking in polymerase III', presumably the γ and δ subunits, allow the core enzyme to utilize an SSB-coated primed template for extensive DNA synthesis in one binding of the polymerase to the primer template; 4) the β subunit, in addition to its requirement for initiation, is required for maximum processive synthesis catalyzed by the holoenzyme; and 5) formation of an initiation complex results in the generation of products synthesized solely by the intact holoenzyme.

REFERENCES


Products of Escherichia coli DNA Polymerase III Subassemblies

250, 1972–1980