Escherichia coli DNA Polymerase III Holoenzyme Footprints Three Helical Turns of Its Primer*

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Escherichia coli DNA polymerase III holoenzyme in the presence of ATP and E. coli single-stranded DNA-binding protein forms an initiation complex on a primed template capable of rapid and highly processive DNA replication. DNase I digestion of initiation complexes demonstrated that holoenzyme protected 27–30 nucleotides of primer. Like the formation of initiation complexes, this protection required both ATP and E. coli single-stranded DNA-binding protein.

Initiation complexes assembled with core DNA polymerase III (α, ε, and θ subunits), γ-complex (γ, δ, δ', χ, and ψ) and the β subunit produced a footprint identical to that formed with intact holoenzyme, indicating that initiation complexes formed with reconstituted enzyme and those formed with holoenzyme were equivalent. The presence of the τ subunit in reconstituted initiation complexes did not alter the DNase I footprint. Preinitiation complexes (γ-complex plus β subunit) assembled onto primer-template in an ATP-dependent reaction protected a larger region of the primer than did holoenzyme. The addition of core DNA polymerase III to preinitiation complexes restored the 30-nucleotide footprint observed with intact holoenzyme. These results suggest that holoenzyme subunits rearrange during initiation complex formation.

Replication of the Escherichia coli chromosome requires the multisubunit replicative complex, DNA polymerase III holoenzyme (holoenzyme). This enzyme contains core DNA polymerase III (α, ε, and θ subunits), β, processivity factor, the γ and τ products of the dnaX gene plus δ, δ', χ, and ψ subunits (McHenry and Kornberg, 1977; McHenry and Crow, 1979; McHenry, 1982, 1988; McHenry et al., 1986; Maki and Kornberg, 1988; O'Donnell and Studwell, 1990). During chromosomal replication, holoenzyme binds to an RNA-primed template with the concomitant hydrolysis of ATP to form a highly stable initiation complex (Wickner and Kornberg, 1973; Wickner, 1976; Johanson and McHenry, 1982; Burgers and Kornberg, 1982a; O'Donnell, 1987). Once loaded onto DNA by this mechanism, holoenzyme becomes highly processive (Fay et al., 1981), capable of replicating at least 150 kb without dissociating (Wu et al., 1992). In the absence of the auxiliary subunits, the core polymerase incorporates only 10–15 nucleotides per association event (Fay et al., 1981, 1982).

Reconstitution studies using the individual components of holoenzyme have provided evidence that assembly of holoenzyme at the initiation site can be biochemically divided into two stages. In the first stage, the γ-complex (γ, δ, δ', χ, and ψ) is involved in an ATP-dependent reaction that transfers β to the initiation site to form the preinitiation complex (Wickner, 1976; O'Donnell and Studwell, 1990). In the second stage, core DNA polymerase III binds the preinitiation complex and forms an initiation complex capable of rapid and highly processive DNA replication (Wickner, 1976; O'Donnell, 1987).

The study of holoenzyme subassembly forms has provided an opportunity to identify the specific roles of the subunits within the initiation complex. Analysis of one of the subassembly forms, pol III', led to the prediction that holoenzyme exists as a dimer. Molecular weight and subunit stoichiometry determinations indicate that pol III' contains two units of core pol III and two τ subunits (McHenry, 1982; Studwell-Vaughan and O'Donnell, 1991). The presence of τ increases the core polymerase processivity ~4-fold (Fay et al., 1981, 1982); however, ATP does not influence pol III' activity (Reems et al., 1991).

To gain further insight into holoenzyme subunit interactions, we initiated a structural study to identify holoenzyme-primer contacts at the initiation site. We determined the length of primer protected from DNase I by holoenzyme and identified DNA contacts made by preinitiation complexes. Our data indicate that a dynamic rearrangement of the subunits occurs during initiation complex formation.

**MATERIALS AND METHODS**

Proteins and Enzymes—Holoenzyme (700,000 units/mg) was prepared by the method of Oberfelder and McHenry (1987). Pol III (400,000 units/mg) and core pol III (850,000 units/mg) were purified by the methods of McHenry (1982) and McHenry and Crow (1979), respectively. The γ-complex purified by A. J. Hughes, Jr., of this laboratory contained the γ, δ, δ', χ, and ψ subunits. β subunit was isolated by standard procedures (Johanson and McHenry, 1980). SSB was isolated from an E. coli overproducer, RLM727 (gift from Roger McMacken, Johns Hopkins) and purified by a modification (Griep and McHenry, 1989) of the method described by Meyer et al. (1980). The following enzymes were purchased: Sequenase version 2.0, T7 DNA polymerase (U.S. Biochemical Corp.), terminal deoxynucleotidyl transferase (Amer sham Corp.), T4 polynucleotide kinase (New England Biolabs), and deoxyribonuclease I (2306 units/mg) (Worthington).

Nucleotides and Nucleic Acids—Adenosine 5'-triphosphate (dATP, >5000 Ci/mmol) was purchased from Amer sham Corp. The single-stranded DNA template,
M13mp19 was isolated as described by Johanson and McHenry (1984). DNA concentrations for primer-template were expressed in terms of primer 3'-hydroxyl termini.

Oligonucleotide Synthesis and Purification—The primer used in these studies was complementary to M13mp19 at positions 991–1041 (Yanisch-Perron et al., 1985). The 50-mer primer, which contained a 3'-terminal ribonucleotide, was synthesized on a Biosearch 8600 DNA synthesizer. The 3'-terminal ribonucleotide was incorporated into the primer using a cytidine-linked controlled pore glass support (Milligen/Biosearch). Cleavage of the 50-mer from the glass support, depurination, and purification of the primer were carried out as described by Hagerman (1985). The oligomer was cleaved from the support and depurinated after treatment with concentrated ammonium hydroxide for 5 h at room temperature followed by an additional 24 h of incubation at 55–60°C. The 50-mer was then purified on a 15% polyacrylamide-8 M urea denaturing gel followed by binding to a DE52 (Bio-Rad) column and elution with 3 M potassium acetate. The DNA concentration of each fraction was determined spectrophotometrically by measuring the absorbance at 260 nm, using an extinction coefficient of 7370 (μM nucleotide)⁻¹ (Berkowitz and Day, 1971). Peak fractions were dried in a Savant Speed Vac, suspended in 95% ethanol, and evaporated a second time. The primer was dissolved in 10 mM HEPES (pH 7.5), and 1 mM EDTA, and stored at -20°C.

Radiolabeling the Primer-Template Using Sequenase—The 50-nucleotide primer (500 nm) was hybridized to M13mp19 (400 nm) in 50 mM HEPES (pH 7.5) and 200 mM NaCl (total volume 25 μl) at 75°C for 5 min and then allowed to cool slowly to room temperature (1.5°C/min). Using a modification of the procedure described by Tabor and Richards-Jones (1987), the 3'-primer terminus was radiolabeled using Sequenase. Primer-template (100 nm) was incubated with [α-32P]dATP (1.0 μM) and 5 units of Sequenase/μmol of primer-template in 10 μM magnesium acetate, 50 mM HEPES (pH 7.5) (total volume 100 μl) for 30 min at 37°C. Enzyme was thermally inactivated (10 min, 65°C). Excess unannealed primer and unincorporated nucleotide were separated from the 3'-end radiolabeled primer-template by gel filtration on a Bio-Gel A-5m column (1.0 × 3.0 cm) (Bio-Rad) equilibrated in 50 mM HEPES (pH 7.5), 100 mM potassium glutamate, 1 mM EDTA. The length of the labeled primer was 51 nucleotides. The fractions from the first high molecular weight radioactive peak were pooled and the DNA concentration was determined as described above.

Protection by Holoenzyme—To determine the length of primer protected by holoenzyme, we used 51-nucleotide exonuclease-resistant primer annealed to a single-stranded cir-

RESULTS

E. coli DNA polymerase III holoenzyme forms a highly stable initiation complex with a dnaG-primed template in the presence of ATP (Wickner and Kornberg, 1973; Wickner, 1976; Burgess and Kornberg, 1982a, 1982b; Johanson and McHenry, 1980, 1982). To investigate the protein-DNA interactions that occur within the initiation complex, holoenzyme-primer-template complexes were subjected to DNase I footprint analysis. Preliminary studies performed with a 5'-end radiolabeled DNA primer annealed to a single-stranded circular template showed that both DNase I-treated and untreated holoenzyme-primer-template complexes produced identical digestion patterns. Further investigation revealed that the holoenzyme 3'→5'-exonuclease activity was responsible for the DNA digestion pattern observed for the holoenzyme-primer-template complexes not treated with DNase I (data not shown).

To eliminate a digestion pattern due to the holoenzyme 3'→5'-exonuclease activity, we made two alterations in the substrate used for all subsequent studies. First, we synthesized an exonuclease-resistant primer by positioning a penultimate ribonucleotide and a terminal deoxyribonucleotide residue at the 3' terminus. These modifications reduced the exonuclease activity to stabilize holoenzyme-DNA primer-template interactions nearly 1000-fold (Griep et al., 1990). Second, we radiolabeled the 3'- rather than the 5'-end of the primer, such that any primer acted upon solely by the 3'→5'-exonuclease generated single nucleotide radioactive products; any product greater than a single nucleotide in length was non-radioactive. This approach prevented the production of radioactive fragments that obscured the DNase I footprint.

Protection by Holoenzyme—To determine the length of primer protected by holoenzyme, we used 51-nucleotide exonuclease-resistant primer annealed to a single-stranded cir-

![Figure 1](image-url)
Fig. 2. Holoenzyme does not protect unannealed primer. Reaction conditions were as described under “Materials and Methods.” Where indicated, saturating levels of holoenzyme and/or SSB were added.

Fig. 3. SSB does not protect annealed primer. Reaction conditions were as described under “Materials and Methods.” Saturating levels of SSB were added where indicated.

Fig. 4. Effects of ATP and SSB on the holoenzyme footprint. Reaction conditions were as described under “Materials and Methods.” ATP and saturating levels of holoenzyme and SSB were added as indicated.

Certain template derivative of M13. The 3'-end radiolabeled primer-template was incubated with ATP and saturating levels of SSB prior to the addition of holoenzyme. Protein-DNA complexes were allowed to form and then treated with DNase I. The nuclease digestion pattern of DNase I-treated initiation complexes showed a clear region of protection (Fig. 1, lane 1), whereas in the absence of holoenzyme, DNA fragments were present for the full-length of the primer (Fig. 1, lane 3). Based on the gel migration of radiolabeled standards, the protected region was identified in the area of the gel where DNA fragments from 1 to 29 nucleotides in length migrated. Initiation complexes not treated with DNase I showed only full-length primer (Fig. 1, lane 2). This latter result confirmed that products generated by the 3'-5' exonuclease did not interfere with the footprint. In the control lane lacking holoenzyme (Fig. 1, lane 3), three radioactive DNA fragments were missing between the 24- and 30-nucleotide radioactive standards. This imposed uncertainty in our determination that the holoenzyme footprint was 30 nucleotides in length since slightly smaller products would not have been visible. Thus the actual footprint is between 27 and 30 nucleotides.

To determine whether the dideoxynucleotide residue at the 3'-terminus of the primer influenced primer protection by holoenzyme, the dideoxy was replaced with a deoxynucleotide. Holoenzyme again protected between 27 and 30 nucleotides of the primer when a 3'-deoxy residue was coupled to the 3'-penultimate ribonucleotide (data not shown). Thus the 3'-hydroxyl group on the ribose did not influence the length of primer protected by holoenzyme.

DNase I Digestion of Unannealed Primers—Although unannealed primer was removed from the primed template by gel filtration prior to footprinting, the possibility remained that free primer became available during the reaction due to melting of the duplex region. To ensure that the holoenzyme footprint directly reflected enzyme interaction with primer-template and not with unannealed primer, we examined holoenzyme interactions with single-stranded primers. Additionally, because SSB is required in the replication reaction to maximize holoenzyme activity, we examined the role of SSB in protecting unannealed primer. Consistent with the work of Chrysogoles and Griffith (1982), SSB completely protected the entire length of unannealed primer (Fig. 2, lane 2). Further, holoenzyme did not alter the protection afforded by SSB (Fig. 2, lane 4). In the absence of SSB, the unannealed primer was digested (Fig. 2, lane 1) but with a pattern distinct from that of annealed primer (compare Fig. 1, lane 3, to Fig. 2, lane 1).
Fig. 5. Footprint analysis of reconstituted complexes using DNA polymerase III. A, reaction conditions were as described under "Materials and Methods." Components added to the primer-template are indicated. Where indicated, 12 units of pol III′, 13.5 units of γ-complex, and 93 units of β subunit/μmol primed template were added. B, densitometric scan of lanes 2 and 6.

Holoenzyme did not protect the unannealed primer in the absence of SSB (Fig. 2, compare lanes 1 and 3). Thus, the 30-nucleotide protection observed with holoenzyme is not an artifact of free unannealed primer.

SSB Does Not Protect Annealed Primer—Although SSB binds single-stranded DNA with an affinity that is at least 1000-times greater than for double-stranded DNA (Molineux et al., 1975), it was important to determine whether SSB was responsible for the 30-nucleotide protection observed on annealed primers. DNase I-treated primer-template in the presence or absence of SSB produced identical digestion patterns (Fig. 3), indicating that no apparent protection of the duplex region occurred due to SSB alone.

Influence of ATP and SSB on the Holoenzyme Footprint—Several subassembly forms of DNA polymerase III are known to interact with the primer-template. To confirm that the 30-nucleotide DNase I protection was due to holoenzyme and not subassemblies, we examined the influence of ATP and SSB on the holoenzyme-primer-template footprint. Maximum synthetic and 3'→5'-exonuclease activities for holoenzyme are achieved only when both ATP and SSB are present (Wickner and Kornberg, 1973; Wickner, 1976; Johanson and McHenry, 1982; Burgers and Kornberg, 1982a, 1982b; Grieb and McHenry, 1989; Reems et al., 1991). Simpler forms of DNA polymerase III (pol III, pol III′, and pol IIIa) are not affected by ATP; pol III and pol III′ are inhibited by SSB (Fay et al., 1981, 1982; Johanson and McHenry, 1982; Reems et al., 1991). As shown previously, DNase I treatment of initiation complexes formed in the presence of ATP and SSB generated a clearly defined 30-nucleotide footprint (Fig. 4, compare lanes 1 and 5). However, in the absence of ATP and/or SSB, this 30-nucleotide protection was not observed (Fig. 4, lanes 2–4). This dependence on ATP and SSB supports the conclusion that the 30-nucleotide footprint results from holoenzyme and not a simpler polymerase III form.

Protection by the Preinitiation Complex—To evaluate possible structural differences between the preinitiation and initiation complex, we treated preinitiation complexes with DNase I and compared the results to nuclease-treated initiation complexes. No interactions were detected for either the
**DNA Polymerase III Footprint**

![Diagram of DNA Polymerase III Footprint](image)

**Fig. 7. Model for the interaction of preinitiation and initiation complexes with the primer-template.** Preinitiation complexes interact with the full-length 51-nucleotide primer in an ATP-dependent reaction. Addition of either core pol III or pol III' to the preinitiation complexes induces rearrangement that leads to reduction in the length of primer protected to 30 nucleotides.

**DISCUSSION**

Identification of the molecular interactions that occur within holoenzyme-primer-template complexes is a prerequisite for understanding the mechanistic contributions of each holoenzyme subunit. As an initial step toward this goal, we determined the region of primer that is footprinted by holoenzyme within initiation complexes. Using DNAse I footprint analysis we found that holoenzyme directly contacts 27–30 nucleotides of primer. Assuming a B-DNA conformation, 30 nucleotides translates into approximately 3 helical repeats or 102 Å of duplex DNA (Fig. 7).

Based on data obtained analyzing holoenzyme pause-sites relative to secondary DNA structural barriers, we expect that the predominant region of DNA contact by holoenzyme is within the duplex region of the primer-template. Holoenzyme can approach a hairpin structure as close as 2–6 nucleotides (LaDuca et al., 1983). These results suggest that holoenzyme has limited contact with the single-stranded region of the DNA. Our observation that holoenzyme contacts 30-nucleotides of the primer correlates well with the 25-nucleotide primer contacts observed for both the T4 (44/62-43/45) and eukaryotic (Polδ-RFC-PCNA) replicative complexes (Munn, 1986; Tsurimoto and Stillman, 1991). Further, the major region of contact for both the T4 and pol δ replication complexes occur within the duplex region of the primer-template. Both T4 and δ extend 4–5 nucleotides downstream of the primer-template junction.

There is a profound decrease in holoenzyme binding to the primer-template in the absence of ATP, as evidenced by the loss of the clearly defined 30-nucleotide footprint. This is consistent with previous studies in which holoenzyme was shown to use ATP to bind tightly with primed template (Wickner and Kornberg, 1973; Wickner, 1976; Johanson and McHenry, 1982; Burgers and Kornberg, 1982a).

In addition to ATP, we found that SSB was also required to maximize primer-template binding. A decrease in holoenzyme binding to the primer-template in the absence of SSB suggests that holoenzyme subunit-SSB interactions are important for stabilizing initiation complexes. Previous biochemical data support the structural evidence that holoenzyme subunit-SSB interactions are required for maximum enzyme activity. Both the elongation and proofreading activities of holoenzyme and a subassembly form of holoenzyme, pol III*, are stimulated by SSB (McHenry, 1980, 1982; Fay et al., 1981, 1982; Reems et al., 1991). Pol III* contains all the holoenzyme subunits minus the γ-complex or the β subunit alone in the presence of ATP (Fig. 5A, lanes 4 and 5). However, the preinitiation complex (γ-complex plus β) did protect the primer from DNAse I digestion (Fig. 5A, lane 2). The autoradiogram revealed a ladder of radioactive fragments with diminished band intensities relative to primer-template without enzyme (γ-complex and β) (Fig. 5A, compare lanes 2 and 6). A microdensitometer scan to quantify the intensity of each DNA fragment indicated that, with the exception of a single band at the 19-mer position, the entire length of the 51-nucleotide primer had diminished band intensities relative to a reference lane lacking enzyme (Fig. 5B). Thus, the footprint of the preinitiation complex was larger but weaker than that observed with reconstituted holoenzyme (Fig. 5A, lane 1). Together, these results indicated that the preinitiation complex interacted with a larger region of the primer than did holoenzyme.

**Protection by Reconstituted Holoenzyme**—To evaluate reconstituted holoenzyme-primer-template interactions, core pol III (α, ε, and δ subunits) and pol III' (α, ε, δ, and τ subunits) were separately added to preinitiation complexes that had already been assembled onto the primer-template. Pol III' alone did not visibly footprint the primer-template (Fig. 5A, lane 3). However, the addition of pol III' to preformed preinitiation complex generated the same 30-nucleotide footprint observed for intact holoenzyme (Fig. 5A, lane 1). In an analogous experiment, reconstituted initiation complexes formed with core pol III also protected the same length of primer in an ATP-dependent reaction (Fig. 6). These results demonstrate that reconstituted initiation complexes were structurally equivalent to initiation complexes formed with intact holoenzyme with respect to primer binding, and that the addition of the τ subunit when using pol III' instead of pol III does not alter the DNase I footprint.
DNA Polymerase III Footprint

β subunit. Pol III' and core pol III are inhibited by SSB (Fay et al., 1981, 1982; Reems et al., 1991). Since both holoenzyme and pol III* contain the γ-complex, whereas pol III' and core pol III lack this component, it appears that SSB interacts with some part of the γ-complex.

To characterize protein-DNA interactions that occur during the transition from preinitiation to initiation complex, individually purified proteins were assembled onto the primer-template. Footprint analysis demonstrated that the preinitiation complex protected almost the full length of the primer. With the addition of either core pol III or pol III’, the footprint contracted to the same 30-nucleotide stretch observed for intact holoenzyme (Fig. 7). Interestingly, the size of the footprint was not altered by the addition of the γ subunit when using pol III’. A reduction in the length of primer protected during the transition from preinitiation to initiation complex indicates that the subunits rearrange. Several scenarios could account for such a structural rearrangement. The preinitiation complex might, in fact, be larger than initiation complexes. Alternatively, the preinitiation complex might be less defined and the large but more weakly protected region might result from loading of multiple β subunits or from β sliding back and forth along the duplex region. The latter possibilities are consistent with previous observations that one γ-complex can load multiple β dimers onto a primer and that these β dimers are mobile, i.e. capable of sliding along duplex DNA (O’Donnell, 1987; Stukenberg et al., 1991; Kong et al., 1992).

REFERENCES

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