Coordination between the Polymerase and 5'-Nuclease Components of DNA Polymerase I of Escherichia coli*

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The polymerase and 5'-nuclease components of DNA polymerase I must collaborate in vivo so as to generate ligatable structures. Footprinting shows that the polymerase and 5'-nuclease cannot bind simultaneously to a DNA substrate and appear to compete with one another, suggesting that the two active sites are physically separate and operate independently. The desired biological end point, a ligatable nick, results from the substrate specificities of the polymerase and 5'-nuclease. The preferred substrate of the 5'-nuclease is a “double-flap” structure having a frayed base at the primer terminus overlapping the displaced strand that is to be cleaved by the 5'-nuclease. Cleavage of this structure occurs almost exclusively between the first two paired bases of the downstream strand, yielding a ligatable nick. In whole DNA polymerase I, the polymerase and 5'-nuclease activities are coupled such that the majority of molecules cleaved by the 5'-nuclease have also undergone polymerase-catalyzed addition to the primer terminus. This implies that the 5'-nuclease can capture a DNA molecule from the polymerase site more efficiently than from the bulk solution.

The DNA polymerase I (Pol I)* enzymes of eubacteria function in DNA repair and in the removal of RNA primers from Okazaki fragments during lagging strand replication (1). To facilitate the formation of ligatable structures, the bacterial Pol I enzymes usually have nuclease activity, which degrades the downstream DNA or RNA strand. Originally described as a 5'-3' exonuclease, this activity is now recognized to be a structure-specific nuclease with specificity for the junction between a DNA duplex and a 5'-single-stranded tail (or flap) and is therefore better described as a 5'-nuclease (2). On a nicked DNA duplex, polymerase-catalyzed primer extension continuously regenerates the substrate for the 5'-nuclease, so that the polymerase effectively drives nick translation (3). The 5'-nuclease activity of Pol I is present on an independent structural domain encoded by the first one-third of the structural gene, which can be separated from the polymerase by proteolysis or by recombinant DNA manipulations (4–6). The structure specificity is inherent to the 5'-nuclease domain itself and does not rely on the presence of the polymerase domain (3, 6, 7). Indeed, some bacteriophages encode separate nucleases, having a high degree of homology to the N-terminal region of the bacterial Pol I enzymes, which act in concert with the relevant bacteriophage polymerase (8–10). In eukaryotes and archaeabacteria, the structure-specific cleavages required in replication and repair are carried out by “flap endonucleases” (11–13), which exist as independent protein subunits and are therefore able to collaborate with a variety of polymerases. The flap endonuclease family of proteins shows only a modest level of sequence similarity to the bacterial and bacteriophage 5'-nucleases (14), but the similarity in three-dimensional structures (14–18) and in the reactions carried out by these two families of 5'-nucleases leaves little doubt that they are functionally analogous to one another.

In vivo, the polymerase and 5'-nuclease of bacterial Pol I must collaborate so as to leave a nick that can be sealed by DNA ligase. Two extreme scenarios can be envisaged. The Pol I molecule might adopt a structure that brings the two active sites into close proximity so they can bind simultaneously to the DNA substrate. Alternatively, the polymerase and 5'-nuclease activities might operate essentially independently of one another, perhaps even with the DNA traveling from one active site to the other via dissociation into free solution. This latter scenario fits well with the existence of a separate 5'-nuclease in some systems and is analogous to the relationship between the polymerase and 3'-5' exonuclease (editing) functions of Klenow fragment, where the two active sites are separated by about 30 Å, with a substantial amount of the transfer between them occurring via dissociation (19). Close juxtaposition of polymerase and 5'-nuclease active sites might be difficult to achieve; the polymerase active site is located at the base of a cleft (20), and the 5'-nuclease domain is structurally complex, possibly requiring threading of the unpaired 5' end through an arch or loop of the protein (16–18). However, recent structures of polymerases with DNA bound at the polymerase site suggest a dislocation of the downstream DNA template beyond the site of synthesis (21, 22), and such a dislocation might provide a way to accommodate both polymerase and 5'-nuclease active sites in reasonably close proximity. Only two polymerase crystal structures (both of Taq DNA polymerase) indicate a 5'-nuclease domain. The first structure showed the polymerase and 5'-nuclease sites separated by about 70 Å at opposite ends of a rather extended molecule, arguing in favor of separate and independent active sites; however, x-ray scattering measurements suggested that the polymerase may fold more compactly in solution (15). A more recent crystal structure of Taq DNA polymerase complexed with an Fab showed the nuclease domain (which was remote from the Fab location) close to the fingers subdomain of the polymerase (23). Although the active sites were still separated by almost 40 Å, this location brings the 5'-nuclease much closer to the downstream portion of a

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DNA molecule bound at the polymerase active site. At the very least, the structural data suggest a flexible linkage between the 5'-nuclease and the rest of the polymerase molecule, and this could allow the two domains to be closely associated in an active complex.

In this work, we have investigated two aspects of the collaboration between the polymerase and 5'-nuclease components of *Escherichia coli* Pol I: the way in which the substrate preferences of both activities are biased so as to produce a ligatable nick and the extent to which polymerase and 5'-nuclease are coupled so that both reactions take place within the same DNA binding event.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA oligonucleotides were synthesized by the Keck Biotechnology Resource Laboratory at Yale Medical School and purified by denaturing gel electrophoresis after either 5'- or 3' end-labeling. Radiolabeled nucleotides were from Amersham Pharmacia Biotech. DNase I was from Cooper Biomedical. Restriction enzymes were from New England Biolabs and were used according to the manufacturer’s recommendations. Standard molecular biology protocols were used throughout (24).

**Enzyme Purification**—Derivatives of Pol I and Klenow fragment, all containing the D424A mutation that eliminates the 3'-exonuclease activity (25), were purified to homogeneity as described (26). Wild type and mutant derivatives of the 5'-nuclease domain were purified as described (6), with the addition of a final gel filtration column.

**DNase I Footprinting**—Footprinting of the 5'-labeled 112-mer (see Fig. 1a) was carried out as described (27), except that samples were analyzed on polyacrylamide gels containing 7 M urea and 40% (v/v) formamide in order to denature completely the hairpin structure of the oligonucleotide. Markers were generated by restriction enzyme digestion of the 112-mer and by partial chemical degradation at G residues (28). The data were quantitated by phosphorimaging on a BAS 2000 Bio-Imaging Analyzer (Fuji) using the MacBAS Image Analysis software (Fuji).

**Kinetic Assay of the 5'-Nuclease**—The 5'-nuclease activity was measured on the substrates illustrated in Fig. 1. The 5'-end-labeled DNA (0.03 nM) and the wild-type 5'-nuclease (0.5 μM) were preincubated in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA. The nuclease reaction was initiated by the addition of MgCl2 to a final concentration of 5 mM. Samples, removed at appropriate time intervals, were analyzed as described previously (6).

**Gel Mobility Shift Assay of Klenow Fragment**—The procedure was essentially as described previously (29), except that binding was carried out in 50 mM Tris-HCl, pH 7.5, 2 mM MgCl2, 10% (v/v) glycerol, 50 μg/ml bovine serum albumin, and the gel was run in 50 mM Tris borate, 2 mM MgCl2, 0.2 mM EDTA.

**Substrate Preference of the 5'-Nuclease**—The 63-mer oligonucleotide (Fig. 6) was labeled at the 3’ end by Klenow fragment-catalyzed extension with [α-32P]dCTP to give the S5 substrate or with unlabeled dGTP and [α-32P]dATP to give S4. These labeled DNAs were gel-purified and then elongated with one unlabeled nucleotide (giving S5 and S4, respectively) using Klenow fragment, which was subsequently inactivated by heating. Mixtures containing about 25 nM of each substrate were made by combining S5 with S5A and S4 with S4C, and were incubated at 23 °C with the wild-type 5'-nuclease domain in 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 100 mM NaCl. The S5/S4 reaction was incubated with 10 μM 5'-nuclease for 5 min, and the S5/S5 reaction was incubated with 2 μM enzyme for 30 s; these conditions were chosen to limit the reaction so that less than 20% of the substrate was converted to product. The reaction mixture was quenched by adding EDTA to 20 mM, and then fractionated by electrophoresis on an 8% polyacrylamide gel containing 7 M urea and 40% (v/v) formamide. Regions of the gel corresponding to cleaved and uncleaved DNA were located by autoradiography, excised, eluted in 10 μM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 μM ammonium acetate, and concentrated by ethanol precipitation. Because the 5'-nuclease cleavage did not always occur at a unique position, the cleaved fraction often consisted of several bands on the gel, which were eluted and processed together. The DNA samples were digested with MluI, fractionated on a 15% polyacrylamide-urea gel, and quantitated by phosphorimaging. Analysis of the data is described in the legend to Fig. 6.

**RESULTS**

**Klenow Fragment and the 5'-Nuclease Compete for Binding to a DNA Substrate**—We investigated the spatial relationship between the DNA binding sites for the polymerase and 5'-nuclease domains of Pol I by DNase I footprinting. The substrate was a 5'-labeled 112-mer double-hairpin oligonucleotide with a single-base gap and a 5’-displaced strand (Fig. 1a). Because the footprinting procedure was carried out in the presence of Mg2+ (which does not support the 5'-nuclease reaction), we used 5'-nuclease derivatives carrying inactivating mutations in the conserved carboxylates of the nuclease region (6) to prevent degradation of the substrate. We believe that these mutant derivatives provide a good model for DNA binding by the wild-type 5'-nuclease because similar results (not shown) were obtained with the wild-type enzyme in Ca2+ (20). The overlap (region b) was centered on the one-base gap and the junction with the 5'-displaced strand; Klenow fragment bound also to the DNA duplex upstream of the primer terminus (a), whereas the 5'-nuclease protected more of the DNA downstream of the 5’ extension (c).

At low enzyme concentration, the footprint of whole Pol I (Fig. 2B) resembled that of Klenow fragment, with no apparent protection of the 5’-nuclease binding site. The partial protection of the 5’-nuclease binding region that was observed at higher Pol I concentrations (Fig. 2B, region c) probably corresponds to nonspecific binding; these high concentrations of Pol I gave substantial protection outside of the normal footprint region, particularly on the hairpin loops.

**DNase I Protection using Mixtures of the Separate Klenow Fragment and 5'-Nuclease Domains**—We performed experiments in which the concentration of Klenow fragment was kept constant and increasing concentrations of 5’-nuclease were added to either a singly-gapped (112-mer) or a nicked (113-mer)
DNA. As the concentration of the 5'-nuclease domain was increased, its binding site became protected; however, there was a corresponding loss of the polymerase-specific portion of the footprint, suggesting that binding of the two domains is mutually exclusive; compare lane 5 (Fig. 3, left panel) with lane 2 (Klenow fragment alone) and lane 6 (5'-nuclease alone). The balance of polymerase and 5'-nuclease binding affinities was different on the two substrates such that binding of the 5'-nuclease was more readily observed, at the concentrations tested, on the nicked substrate. The antagonism between the polymerase and 5'-nuclease modes of binding was also demonstrated kinetically (Fig. 4). The presence of Klenow fragment inhibited cleavage by the 5'-nuclease of the single-base-gapped substrate but had very little effect (at the same concentration) on cleavage of the nicked substrate (on which Klenow fragment competes less effectively with the exonuclease) or of a bifurcated oligonucleotide (data not shown), which does not contain a primer terminus to serve as a binding site for the polymerase.

Both Klenow Fragment and the 5'-Nuclease Can Distinguish between Gapped and Nicked Substrates—As demonstrated, the balance between polymerase and 5'-nuclease is influenced by whether the DNA substrate has a gap or a nick adjacent to the 5'-displaced strand. To achieve this result, either or both of the proteins could exhibit preferences for one DNA substrate over the other. A gel mobility shift experiment (Fig. 5) demonstrated that the polymerase domain can discriminate between nicked and gapped DNA molecules. Klenow fragment formed a relatively stable complex with the DNA oligonucleotide having a one-base gap, whereas the dissociation constant was higher for the nicked substrate, and the complex was less stable in the gel, as indicated by the smearing between the bound and free species. The bifurcated DNA substrate, which lacks a primer terminus to serve as a binding site for the polymerase, bound Klenow fragment even more weakly than the nicked DNA. The slowly migrating complexes formed at high concentrations of Klenow fragment appear to be due to nonspecific binding, particularly to the loops of the hairpin structures.

The substrate preference of the 5'-nuclease was examined by determining the extent to which nicked or gapped molecules were selected when the nuclease was presented with an approximately 1:1 mixture of the two (Fig. 6). The gapped substrate, S_{GA}, and the nicked substrate, S_{GA}, were generated by appropriate additions to the 3' end of the 63-mer oligonucleotide. After limited degradation by the 5'-nuclease, the cleaved DNA was separated from unreacted starting material by gel electrophoresis. Because cleavage by the 5'-nuclease did not occur at a unique site, the presence of an extra base at the 3' end could not be determined simply by examination of the cleaved pool on a denaturing gel. Instead, MluI digestion was used to determine the proportion of each pool derived from the nicked and the gapped substrates (Fig. 6). We consistently observed a small bias (~1.3-fold) in favor of cleavage of the nicked DNA, such that the product derived from S_{GA} was over-represented in the cleaved DNA pool, whereas the S_{G}
substrate accumulated in the pool of unreacted substrate. In these experiments, the $S_{GA}$ substrate usually contained a small amount of a longer species, presumably containing an additional (mismatched) A residue at the 3' end. This species, which can be described as having a double-flap structure with both 3' and 5'-single-stranded extensions, was degraded by the 5'-nuclease in preference to the nicked and gapped molecules and, thus, appeared almost exclusively in the product pool even at short reaction times. To investigate this phenomenon further, we converted the labeled nicked DNA, $S_A$, into $S_{AC}$ (which has the potential to form a double-flap structure) and determined that $S_{AC}$ is preferred by at least 14-fold over $S_A$ (Fig. 6). We deduced that the $S_{AC}$ DNA behaves as a double-flap structure with the 3' C residue unannealed, rather than the alternative nick structure that could be formed by branch migration, because the $S_{AC}$ substrate behaved like the $(n+2)$ species containing a 3' mismatch (in the $S_U/S_{GA}$ experiment), and did not behave like the bona fide nicked substrates. This reasoning is supported by similar studies on the Taq 5'-nuclease (7).

The Position of 5'-Nuclease Cleavage Is Substrate-dependent—The presence of a single-base gap, nick, or 3'-flap adjacent to the 5'-displaced strand influences the location of the 5'-nuclease cleavage site (Fig. 7). The gapped substrate, $S_G$, was cleaved at several positions along the 5' tail, with the predominant cleavages on either side of the first paired base; these would result in a mixture of one-base and two-base gaps in the product molecules. The nicked substrate, $S_A$, was also cleaved on either side of the first paired base, giving a mixture of nicked and singly-gapped molecules, with the latter predominating (~60% of the mixture). The double-flap substrate, $S_{AC}$, was cleaved much more rapidly than the other two substrates. Moreover, about 80% of the cutting occurred at a single position, between the first two paired bases; after renaturing of the 3'-terminal base, this would give a product with a ligatable nick.

DNA Transfer between the Polymerase and 5'-Nuclease Sites—Since Klenow fragment and the 5'-nuclease do not bind simultaneously to a DNA substrate, the DNA must travel from one active site to the other for Pol I to carry out its biological functions. We designed an experiment to determine whether transfer of the DNA substrate occurs intramolecularly, within a single enzyme-DNA complex, or whether it requires dissociation and re-binding. If DNA were to move from one active site to the other without dissociation, the two activities should be tightly coupled, and the products from a Pol I-catalyzed reaction should contain a larger proportion of molecules that have undergone both reactions than would be expected on a purely statistical basis.

In separate reactions, we used the 3' end-labeled $S_U$ and $S_A$ oligonucleotides (Fig. 6), which have either a single-base gap ($S_U$) or a nick ($S_A$) adjacent to the single-stranded tail that is to...
be accounted for merely by the substrate preference of the 5'-nuclease, which, as described above, will select a nicked molecule over a gapped molecule and a double-flap over a nick. To determine whether the substrate specificity of the 5'-nuclease could account for the bias seen in the Pol I-catalyzed reaction, we set up two control reactions in which the polymerase and 5'-nuclease functions were not covalently joined. One control used a mixture of Klenow fragment and the 5'-nuclease domain. The second control was similar except that the 5'-nuclease was present on a polymerase-defective Pol I derivative (D882N) that retains wild-type DNA binding affinity (because the DNA binding affinity of the 5'-nuclease domain is more than a 1000-fold lower than that of Klenow fragment (27, 29), we reasoned that the 5'-nuclease of intact Pol I might rely on the Klenow fragment portion of the molecule to facilitate DNA binding). The preference of the 5'-nuclease for molecules that had undergone polymerase-catalyzed addition at the 3' end was similar in both controls (Fig. 8B) and agreed with the results from the substrate preference experiments. For both substrates, the bias observed in the Pol I-catalyzed reactions was much greater than in the controls, indicating that the majority of 5' nuclease processing events are likely to be carried out by the same Pol I molecule that has just extended the upstream primer terminus.

**DISCUSSION**

To perform its functions in vivo, in excision repair and in lagging strand replication, Pol I must leave a ligatable nick. Achieving this end point requires a delicate balance between polymerase and 5'-nuclease activities; an imbalance will give either a gapped or a 5'-tailed duplex, neither of which is a substrate for DNA ligase. Our results imply that the DNA substrate cannot contact both polymerase and 5'-nuclease active sites simultaneously but, rather, must be passed back and forth between two autonomous and non-overlapping active sites. The correct end point, a ligatable nick, results from the substrate preferences of the two domains and the cleavage specificity of the 5'-nuclease.

**Polymerase and 5'-Nuclease Sites Are Separate and Operate Independently—**Our footprinting data show that the polymerase and 5'-nuclease domains, when present on separate molecules, cannot bind simultaneously to a DNA substrate. The slight overlap between the footprints of the separate domains accounts for the inhibition by Klenow fragment of both the binding and nuclease activity of the 5'-nuclease domain. The substrate specificities described below imply that the polymerase senses what is beyond the primer terminus and the 5'-nuclease senses the location of the upstream primer strand, and this fits with the observed footprints. When the polymerase and 5'-nuclease are covalently joined in whole Pol I, the polymerase mode of binding dominates, consistent with its greater DNA binding affinity (6, 27, 29). Failure to observe a Pol I footprint that is the sum of the footprints of the two separate domains suggests that no benefit results from the high local concentration of the 5'-nuclease domain when the polymerase is bound to DNA and argues strongly that the two domains do not cooperate so as to bind simultaneously to the DNA substrate. The apparent antagonism between the polymerase and nuclease binding modes probably accounts for the lower activity of the 5'-nuclease when present in whole Pol I (6).

**Formation of a Ligatable Nick—**Both polymerase and 5'-nuclease discriminate between related substrate structures in such a way as to increase the probability of generating a ligatable nick. The preference of the polymerase for gapped structures helps to ensure that gaps are filled, whereas rapid dissociation from a nick allows other enzymes to act. The preferred substrate of the 5'-nuclease is a double-flap molecule with an
unpaired base at the primer terminus. Cleavage of the double-flap DNA is focused almost exclusively to a single position between the first two paired bases of the strand with the 5′ overhang, generating a ligatable nick. The efficient processing of this substrate to yield the required product suggests that the double-flap structure may be the natural substrate for the 5′-nuclease. A preference for double-flap substrates has also been reported for the 5′-nucleases of Taq and Tth DNA polymerases and for eukaryotic and archaeobacterial FEN-1 enzymes (7, 30, 31).

In contrast to 5′-nuclease cleavage of the double-flap structure, the reaction with nicked and gapped substrates seems much less efficient and precise. The predominant cleavage site on a nicked or gapped substrate is between the first two paired bases of the downstream strand (Fig. 7) (2), which means that cleavage must be followed by at least one more round of polymerase addition before ligation can take place. Cleavage of nicked and gapped substrates often occurs at more than one position, and this could reflect formation (via branch migration) of several interconvertible structures. The extent to which a particular cleavage site is represented in the reaction products would be determined by the abundance of the relevant structure and the efficiency with which it is cleaved by the 5′-nuclease. Specifically, we suggest that the cleavages that apparently map within the single-stranded 5′ tail actually involve rearrangement of the DNA to give double-flap structures, usually having imperfect base pairing around the cleavage site. These double-flap and related structures need not be abundant in the substrate pool if they are strongly preferred as nuclease substrates. Similar reasoning has been invoked to explain the formation of a variety of cleavage products by the Taq 5′-nuclease (7). Examination of the structural model proposed for T5 5′-nuclease bound to its DNA substrate (16) suggests that there could be room for an additional base at the primer terminus; contacts between the active site and the 3′-unpaired base would then account for the preference of the 5′-nuclease for the double-flap substrate.

The potential for 5′-nuclease substrates (particularly those made by strand-displacement synthesis) to rearrange and form double-flap structures may account for some inconsistencies in the cleavage sites reported for these enzymes. For example, Lundquist and Olivera (3) consistently observed cleavage by the 5′-nuclease of E. coli Pol I apparently at the junction between the downstream duplex and the 5′ single strand, whereas other studies agree that the predominant cleavage position for the bacterial Pol I 5′-nuclease is one base 3′ to this position, i.e., between the first two paired downstream bases (2, 6, 32). Significantly, Lundquist and Olivera (3) made their DNA substrates by polymerase-catalyzed primer extension, whereas the other studies used synthetic oligonucleotides in which the possibilities for branch migration were more limited.

Our data suggest the following scenario for the processing of a DNA substrate by Pol I (Fig. 9). The polymerase extends the upstream primer strand, in some cases proceeding beyond the junction with the downstream DNA. Branch migration can then generate a family of interconvertible structures. Double-flap structures with a single frayed base at the primer terminus will be the most readily cleaved by the 5′-nuclease, generating a nick that discourages binding of the polymerase, allowing access of DNA ligase. Other conformations will lack the full complement of contacts to the 5′-nuclease domain and will therefore be cleaved less rapidly, allowing time for further rearrangement to a more optimal substrate or generating a product that can undergo additional cycles of extension and cleavage.

**Coupling of Polymerase and 5′-Nuclease**—When both polymerase and 5′-nuclease are covalently linked in whole Pol I, the fraction of product molecules that have undergone both reactions is greater than when polymerase and nuclease are on separate molecules, implying that both enzymatic reactions can take place within the same protein-DNA binding event. Given the greater binding affinity and reaction rate at the polymerase active site, the most likely scenario is that the polymerase acts first, giving a DNA intermediate that is then cleaved by the 5′-nuclease. Covalent linkage of the two domains delivers a high effective concentration of the 5′-nuclease, compensating for the rather weak binding of DNA to this domain. Nevertheless, the majority of the DNA molecules extended by the polymerase dissociate rather than become substrates for the 5′-nuclease, and this probably argues against an active mode of channeling the DNA intermediate from one active site to the other. Instead, coupling of the two activities...
may involve partial dissociation of the DNA from one active site followed by capture by the other active site before it is lost into the bulk solution.

Polymerase activity is also coupled to variable extents to some of the other auxiliary functions present in polymerases. Coupling between the polymerase and 3'-5' editing exonuclease activity is rather limited in Klenow fragment (19) but greater in T4 and T7 DNA polymerases, where the 3'-5' exonuclease reaction is more rapid and therefore competes more effectively with dissociation (34, 35). By contrast, there appears to be tight coupling between polymerase and RNase H activity in human immunodeficiency virus-1 reverse transcriptase (36). The important difference may be that in reverse transcriptase the nucleic acid substrate is able to contact both polymerase and RNase H sites simultaneously (37), whereas a DNA substrate has to transfer between the polymerase and editing sites of DNA polymerases. The coupling we have observed between polymerase and 5'-nuclease is not necessarily restricted to those systems in which the two activities are covalently linked. The activity of the bacteriophage T4 5'-nuclease (T4 RNase H) on the lagging strand may be integrated with the rest of the T4 replication complex through an interaction with the gene 32 single-stranded-binding protein (38), and the eukaryotic FEN-1 enzymes may be localized at the replication fork by association with the processivity factor, proliferating cell nuclear antigen (PCNA) (39).

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