Defining the Interactions between DNA and the Exonuclease Domain of DNA Polymerases

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In this communication we reveal a method for probing the nature and location of the interactions between DNA utilizing enzymes and the phosphodiester groups of their DNA substrates. The current study focuses on the interaction of DNA with the exonuclease domains of three enzymes; the Klenow fragment of DNA polymerase I (KF), T4 DNA polymerase (T4DP), and T7 DNA polymerase (T7DP). The results show that the exonuclease domains of these enzymes share common features with respect to the binding of the phosphate groups of DNA. This information is then integrated into a model for the editing function of these DNA polymerases.

DNA replication and repair is an important element of cell growth and reproduction. These tasks are mediated by DNA polymerases, which elongate DNA from the 3′ end of a primer strand by a process of template-directed phosphodiester bond formation.[1] The accuracy of this process is enhanced by a polymerase-encoded 3′ exonuclease ("proof reading") activity, which catalyzes the excision of misincorporated nucleoside monophosphates from the 3′ end of the primer. The mechanism by which polymerases switch back and forth from a polymerization mode to a proofreading mode is still unknown.

While extensive work has gone into defining the specificity of DNA-binding proteins with regard to nucleoside base recognition,[2] much less work has been directed at verifying the importance of interactions between these proteins and the phosphate groups of their DNA substrates.[3] Evidence for the existence of protein–phosphodiester contacts in both the polymerase[4] and exonuclease[5] domains of DNA polymerases is supported by X-ray crystallographic studies on KF. The two crystal structures of DNA bound to the exonuclease domain are reported to 3.1 and 3.8 Å resolution. However, these structures show some disorder beyond the second nucleotide. Recent work from our laboratory shows that the polymerase-catalyzed elongation of DNA may be compromised by the introduction of neutral internucleosidic 3′ *O*-sulfonate or 3′ *N*-sulfonamide linkages (in the place of normal phosphodiester linkages) at specific locations on either the DNA primer[6] or template strand.[7] Sulfonates and sulfonamides bear close structural homology to phosphodiesters in that all three are tetrahedral and have similar steric bulk. Thus DNA containing these linkages may be used to probe DNA–protein interactions.

Seven homododecamers of thymidine containing a single sulfonate linkage replacing a phosphodiester at one of the first seven positions were synthesized as described.[8] (Position 1 is the initial phosphodiester bond that undergoes exonuclease-catalyzed hydrolysis.) At all temperatures tested, KF appeared incapable of degrading oligomers containing sulfonate linkages at positions 1 or 2 (Figure 1A). Cleavage of the oligomer containing a sulfonate at the 3 position was also retarded at 0 or 15 °C. However, at 25 °C, KF was capable of degrading this oligomer rather well. Oligomers with sulfonates at the fourth and fifth positions were accepted as good substrates by KF, while oligomers with sulfonates at the sixth and seventh positions were poorer substrates. Similar results were obtained upon analysis of T4DP and T7DP, except that no long range interactions (past phosphate 3) were observed.

These experiments define the position of the interactions between the exonuclease domains of the three DNA polymerases and the phosphodiesters of their DNA substrate. In each case, the exonuclease domains interact with three phosphodiester linkages; the one that is being cleaved (necessarily) as well as the next two. In addition, the interference caused by the sulfonate substitution is always strongest at the penultimate phosphodiester, suggesting that binding to this phosphodiester group is particularly important.[9] KF shows two additional interference points at positions 6 and 7 of the oligomer. This interference must arise from longer range interactions of the oligonucleotide with the enzyme. Since the DNA-binding region of the exonuclease domain is too small to accommodate a heptanucleotide, it seems reasonable to suppose, *a priori*, that these long-range interactions occur in the polymerase domain of the enzyme. If this is true, then sulfonate substitutions at positions 5 and 6 of a template-primer duplex strand should interfere with interactions at the polymerase site. (Positions 5 and 6 for elongation of a primer correspond to 6 and 7 for editing, since the polymerase adds a new residue prior to editing.) In fact, sulfonate substitutions at positions 5 and 6 of a primer strand do interfere with DNA elongation catalyzed by KF but not by T4DP or T7DP.[6] A model depicting the proposed binding mode is shown in Figure 2. This model, which requires the duplex DNA to unwind in order to reach the exonuclease site, is similar to one recently proposed by Steitz and co-workers.[4]

The interference with DNA hydrolysis caused by the introduction of single sulfonate linkages supports the idea that there are specific loci for the interaction between the protein and the phosphodiester backbone of the DNA. The nature of these interactions is not known, although X-ray crystallographic studies of KF containing DNA bound to the exonuclease site suggest that these interactions consist of H-bonding to the three 3′ terminal phosphates (in agreement with our interference data) as well as hydrophobic interactions with four of the DNA bases.[5] To test the possibility that there are specific H-bonds to phosphodiesters at positions 2 and 3 of the DNA, we synthesized and assayed oligomers containing *sulfonamide* linkages in place of the sulfonate linkage.[10] This secondary alteration was made with the following rationale: The sulfonamide linkage is isosteric to the sulfonate linkage but is a better H-bond acceptor, even though it is neutral. Thus, if the protein–DNA binding is mediated by formation of specific H-bonds, we might expect the sulfonamide-containing DNA to be a better substrate than the sulfonate-containing DNA. Indeed, in almost every case, replacement of the sulfonate with a sulfonamide linkage enhanced activity (Figures 1B–D).

By limiting the number of enzyme–DNA contacts at the 3′ end of the primer strand, the binding of the 3′ terminus in the polymerase site becomes largely dependent on the formation of a stable duplex with the template strand, thus ensuring that mismatched DNA will be edited efficiently.

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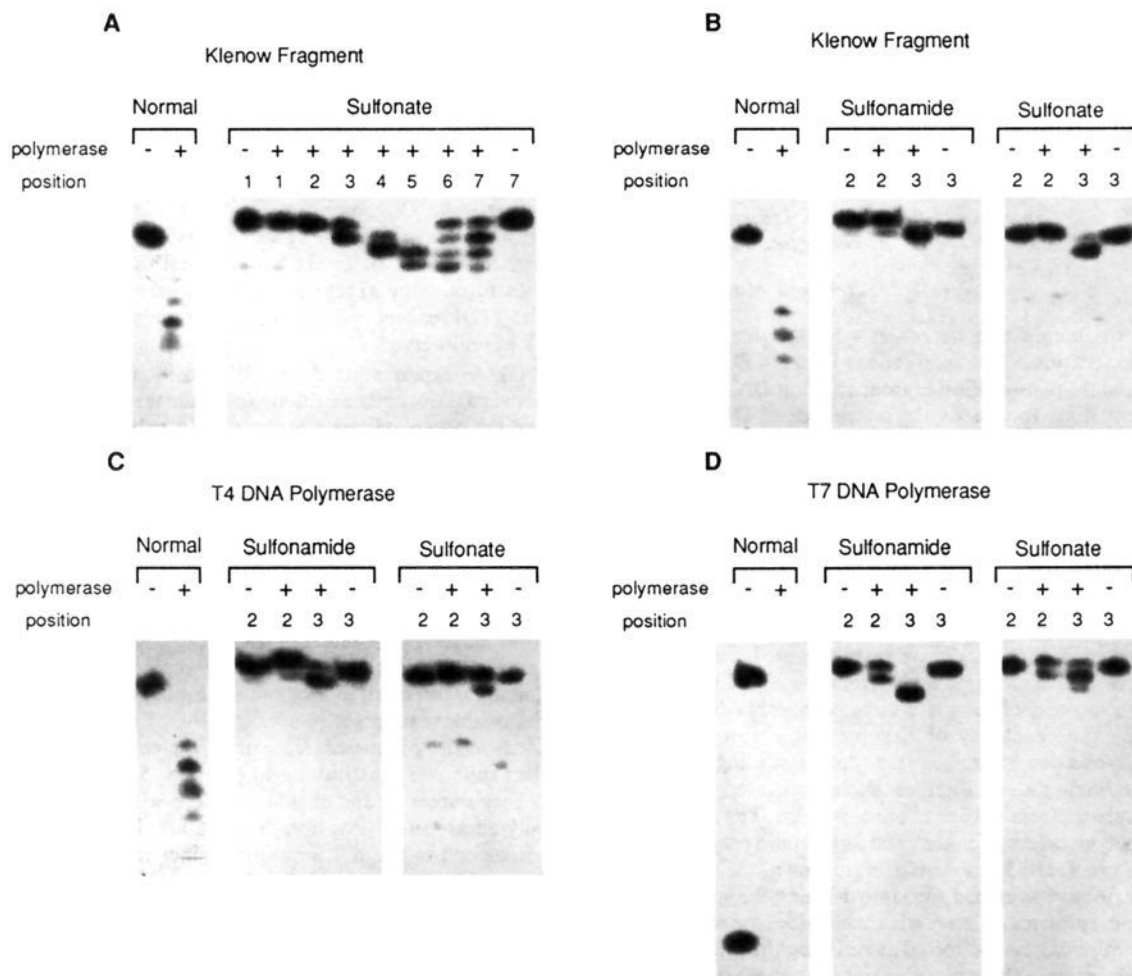

Figure 1. The exonuclease activities were monitored utilizing a 5′ ^{32}P labeled $(dT)_{12}$ oligomer (200 nM), containing a modified linkage, which was incubated with 1.0 unit enzyme in the presence of 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 7.5 mM DTT, for the time and temperature indicated. The reaction was quenched with formamide buffer containing 10 mM Tris-borate and 2 mM EDTA, at 0 °C. Reaction products were separated on a 20% polyacrylamide denaturing gel and visualized with a Molecular Dynamics phosphorimager. An unmodified $(dT)_{12}$ oligomer was used as the control. (A) KF utilizing an oligomer with a sulfonate linkage for 15 min at 15 °C. Oligomers with a sulfonamide linkage or a sulfonate linkage were incubated in the presence of (B) KF for 30 min at 15 °C, (C) T4DP for 5 min at 0 °C, and (D) T7DP for 30 s at 0 °C. The location of the sulfonamide or sulfonate is indicated in the panel. (Position 1 is the initial phosphodiester bond that undergoes exonuclease-catalyzed hydrolysis.)

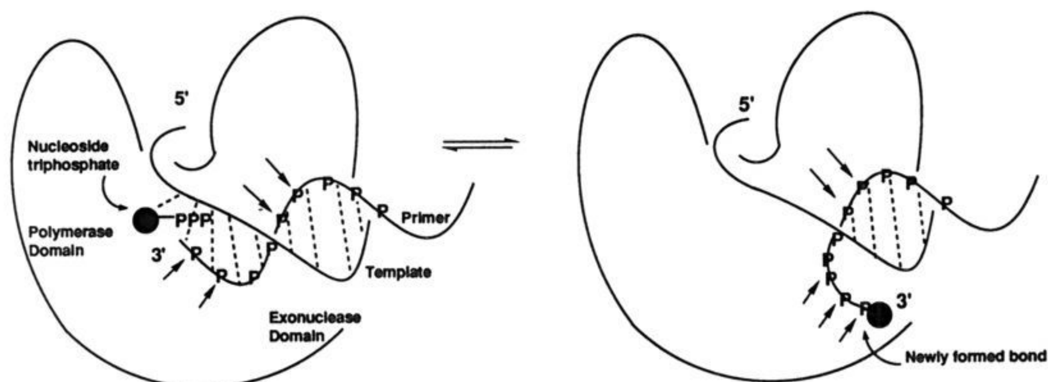

Figure 2. A model for how KF switches between editing and elongation modes. Arrows show positions of strong protein–phosphodiester contacts that have been established by sulfonate substitution. For KF, the newly extended primer is free to unwind up to the sixth phosphodiester whereas a strong contact exists in the polymerase domain. For T4 and T7 DNA polymerases these long-range contacts are absent.

Although the studies reported here are qualitative in nature, they indicate that the use of sulfonate- and sulfonamide-containing DNA can provide important information about the location, nature, and relative strength of interactions between proteins and the specific phosphodiesters of the DNA to which they bind.

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