We have quantitatively assessed each individual step of the Rad51-ssDNA interaction (i.e. nucleation, filament extension and disassembly). Moreover, we investigate the mechanical coupling between the ssDNA template and the reaction kinetics of filament by varying the tension on the DNA molecule. Hence, we have obtained new insight into the reaction pathway of this essential biological system.

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Isothermal Amplification and Quantification of Nucleic Acids Using Intrinsic Fluorescence of Primers

Besik Kankia.
The Ohio State University, Columbus, OH, USA.

Real-time polymerase chain reaction (RT-PCR) is widely used to amplify, detect and quantify nucleic acids. Current RT-PCR specific probes (Molecular Beacons, TaqMan, Scorpions) use complicated mechanisms based on fluorescence resonance energy transfer, and require costly synthesis and considerable effort to achieve optimal sensitivity. Typically, a fluorophore-quencher pair is attached to the ends of a probe oligonucleotide, which doesn't fluoresce when free in solution. Upon probe hybridization to a target sequence, the fluorophore is separated from the quencher and a signal is released. Temperature cycling is another limitation of PCR since it requires expensive instrumentation for thermocycling and complicates rapid detection of pathogens in the field and at point-of-care.

We developed a new method, quadruplex priming amplification (QPA), which uses intrinsic fluorescence of primers for quantification of DNA products and can proceed under isothermal conditions. A key feature of QPA is that after polymerase elongation, the specifically designed guanine-rich primers are capable of forming a quadruplex structure with significantly more favorable thermodynamics than the corresponding DNA duplexes. As a result, target sequences are accessible for the next round of priming since their complementary strands are trapped in a quadruplex conformation and DNA amplification proceeds under isothermal conditions. In addition, 2-aminopurine (2Ap), which is part of the primers and quenched before polymerase elongation, regains its maximum emission upon quadruplex formation, which allows simple and accurate detection of product DNA. The advantages of QPA over traditional quantification methods and its thermodynamic bases will be discussed.

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Binding Affinity and Displacement Synthesis Activity of Pol I DNA Polymerase on Different Gapped DNAs

Yanling Yang, Vince J. LiCata.
Louisiana State University, Baton Rouge, LA, USA.

Gapped DNAs, intermediates in excision repair, can be filled by DNA polymerase I (Pol I) and sealed by ligase. Understanding the binding preferences of Pol I for different gaps and how their binding affinity correlates with displacement synthesis is helpful for investigating repair mechanisms *in vivo*. The roles of the 5′- or 3′-phosphate, and of magnesium, in the binding of Klenow and Klentaq polymerases to gapped DNAs, differing in the size of the ssDNA gap (0, 2, and 10 nt), were examined using a fluorescence anisotropy binding assay. 5 mM Mg^{2+} does not significantly alter the binding of gapped DNAs to Klenow, but Mg^{2+} weakens the binding of gapped DNAs to Klentaq. For Klentaq, but not Klenow, a 5′-terminal phosphate increasingly weakens the binding as the gap size increases. Under the same conditions, $Mg^{2+}$and 5′-phosphate do not alter the displacement synthesis ability of Klenow with gap2 and gap10 substrates, but do alter the activity of Klentaq. Conversely, a 3′-phosphate in the gap significantly weakens the binding of Klenow, but not Klentaq. We hypothesize that Klentaq can bind either the 5′- or 3′- end of the gap, while Klenow binds preferentially at 3′- end of the gap due to the 3′-end being pulled into the editing site. The binding affinity of Klenow, but not Klentaq, to different gaps increases as the size of the gap increases, and this correlates with the displacement synthesis ability of Klenow on gaps versus nicks. Klenow binds primed-template DNA substrates with 2-3 kcal/mol tighter affinity than gap0 or gap2 substrates, while Klentaq shows only a slight preference (0.7 kcal/mol) for primed-template over gapped DNAs, indicating that Klenow more significantly prefers replication over repair substrates.

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Conformational Dynamics of a DNA Polymerase At the Single-Molecule Level

Joshua Gill, David Millar.
The Scripps Research Institute, La Jolla, CA, USA.

DNA polymerases are essential components of the cellular machinery necessary for survival of an organism through the accurate replication of the genetic material. The replication pathway of DNA polymerases has been extensively studied to elucidate the structures and dynamics of the polymerizing and proofreading modes, but currently only a static collection of individual states has been extracted. With the advancements in modern single-molecule fluorescence technology, the potential now exists to examine all enzymatic processes and transitions during real time dynamic measurements. By employing the E. coli DNA polymerase I Klenow fragment (KF) as a model system, along with single-pair FRET labeling, we designed an experimental system to examine conformational dynamics during both nucleotide selection and proofreading steps. In the absence of nucleotides, the bound KF complex was observed to cycle repeatedly between two distinct conformations (open and closed). In contrast, just a single conformation (closed) was populated in the presence of a correct incoming nucleotide. In addition, with the presence of mismatches at the primer-template junction, the previously hypothesized intermolecular and intramolecular pathways were directly observed for transfer of a DNA substrate between the polymerase and exonuclease sites of KF. The evolution and continuous advancement of single-molecule FRET methodology has provided the opportunity to witness events and intermediates previously unobservable in standard bulk studies, leading to a more complete view of the enzymatic pathway. Supported by NIH grant GM44060.

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Topoisomerase IB Activity Investigated By Single Molecule Magnetic Tweezers: Mechanisms of Cytotoxicity

Jan Lipfert[1], Peter Jan Laverman[1], Iris Koster[1], Komaraiah Palle[2], Mary-Ann Bjornsti[3], Nynke H. Dekker[1].
[1]Delft University of Technology, Delft, Netherlands, [2]University of North Carolina, Chapel Hill, NC, USA, [3]University of Alabama at Birmingham, Birmingham, AL, USA.

Magnetic Tweezers (MT) are a powerful tool to investigate how single topoisomerase IB (topIB) molecules relax DNA supercoils [1,2]. MT studies have revealed that topIB activity is dramatically affected by the presence of camptothecin-class (CPT) inhibitors, used clinically as anti-cancer drugs [3]. In the presence of CPT, topIB remains covalently bound to DNA for much longer than in the absence of the drug (>100 s vs. ~ 2 s) and the rate of supercoil removal is significantly reduced, in particular for positive supercoils. The CPT-induced asymmetry in the rate of supercoil removal between positive and negative supercoils leads to an accumulation of positive supercoils in the G1 and S-phases in yeast cells in vivo [3].

Here, we present results on the G365C topIB point mutant, which exhibits CPT resistance and shows no accumulation of positive supercoils in vivo. In the MT assay in the absence of CPT, the G365C mutant shows activity similar to wt topIB. In the presence of CPT, G365C exhibits long-lived DNA-topIB complexes and slow supercoil removal for positive supercoils, similar to the wt enzyme. Surprisingly, for negative supercoils we found similarly long-lived complexes and slow supercoil removal for the G365C mutant. In contrast to the wt enzyme, the G365C mutant removes positive and negative supercoils with similar (slow) velocities in the presence of CPT. These results suggest that CPT cytotoxicity might be more strongly dependent on the asymmetry of the rate of positive vs. negative positive supercoil removal and the corresponding accumulation of positive supercoils than on the lifetime of the covalent DNA-topIB complex.

[1] Koster, et al. Nature 2005
[2] Lipfert, et al. Meth. Mol. Biol. 2009
[3] Koster, et al. Nature 2007

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Dynamics of An Archael DNA Polymerase Revealed By Single Molecule Fret

Xinghua Shi[1,2], Cheng Liu[2], Isaac K.O. Cann[2], Taekjip Ha[1,2].
[1]Howard Hughes Medical Institute, Urbana, IL, USA, [2]University of Illinois at Urbana-Champaign, Urbana, IL, USA.

In the archaeon *Methanosarcina acetivorans*, DNA replication is done by DNA polymerase BI. The processivity of this enzyme is greatly enhanced by a conserved cofactor known as PCNA, which plays a crucial role in orchestrating many replication-related processes. To understand the dynamics of these proteins, we have used single molecule FRET to examine the behavior of PolBI labeled with FRET donor on various DNA structures labeled with FRET acceptor and the effect of PCNA on the dynamics of PolBI. The binding of PolBI to DNA was observed in the low nanomolar concentration range as expected. Interestingly, this polymerase is highly mobile on the DNA structures with two nonadjacent primer strands that are complementary to two different regions in the template strand, 20 nucleotides apart, as evidenced by the frequent transitions between two long-lived FRET states exhibited in single molecule trajectories. To explore the nature of this spontaneous motion, we considered several possible mechanisms including translocations along single- or double-stranded DNA, polymerase binding orientation flipping, and polymerase active site switching. We observed that changes made downstream of the primer/template