272-Pos Counting mRNA Copy Numbers In Single *E. Coli* Cells Reveals Non-Poissonian mRNA Distributions

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Board B105

Detecting mRNA in a single cell is essential to understanding cell-to-cell variations (noise) in gene expression of a genetically identical population and transcription mechanisms. Since most bacterial genes express low copy number of mRNAs, single molecule detection sensitivity is required to measure the mRNA copy numbers. Here we use "digital PCR" to count specific mRNA molecules at low copy numbers in individual *E. coli* cells. The high sensitivity of real-time PCR allows detection of a single copy of mRNA molecule within a single-cell. By counting genomic DNA together with mRNA, we removed the heterogeneity of mRNA copy number due to the variation in gene copy numbers. This allowed us to map the distribution of mRNA molecules expressed from a constitutive promoter (P_{A1}) and *lac* promoter (P_{lac}) among a cell population. In contrast to the Poissonian distribution observed under highly repressed conditions, we consistently observed non-Poissonian mRNA distributions under the fully induced conditions. To shed light on the underlying transcription mechanism, we compared the data with different models for transcription.

DNA Replication, Recombination & Repair

272.01-Pos A Computational Study Of The Non-covalent Interactions Between The K-ras Gene And Some (BPDE And Acrolein) Carcinogens

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Board B105.01

The K-ras gene is intimately related to cancer as it modulates various intracellular signal pathways that control, among other things, cell growth. Some types of cancer are linked to mutations in the K-ras gene. In some human cancers, codons 12, 13 and 61 of the K-ras gene are frequently mutated. Benzo[α]pyrene, a polycyclic aromatic hydrocarbon, and acrolein, an aldehyde, are two known carcinogens. Benzo[α]pyrene is found in cigarette smoke and automobile exhaust fumes and it needs to be metabolized before it can react with DNA. (+)-anti benzo[α]pyrene diol epoxide is the ultimate carcinogenic form of benzo[α]pyrene. Acrolein can also be found in cigarette smoke and, being a most abundant, reactive and mutagenic aldehyde, needs not be activated to damage DNA. We have minimized free energy functions with the aid of genetic algorithms to compute the non-covalent interactions between the exon 1 of the K-ras gene and the carcinogens BPDE and acrolein. For both carcinogens, we found important potential binding (docking) sites for which the Van der Waals interaction is dominant. Some of these docking sites are related to potential DNA damage after the covalent bondings have taken place.

272.02-Pos The Role of γ-phosphate Binding in the Catalytic Mechanism of dUTPase

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Board B105.02

dUTPase is the unique enzyme that catalyses the pyrophosphorolysis of dUTP, thus regulating the extent of uracil incorporation into DNA. Massive uracil incorporation into DNA leads to cell death, dUTPase has therefore been recognized as a high-potential drug target in cancer. The atomic structure of several different dUTPase isoforms is known; however, detailed solution studies on the mechanism of eukaryotic dUTPases were completely missing and thus we endeavored to fill this gap.

The present work focuses on the catalytic mechanism of human dUTPase, and is a continuation of our recently published study which revealed the fundamental steps of the enzymatic cycle and provided a quantitative model for the mechanism. Our specific interest now is in deciphering the structural-functional reasons that lead to the hydrolysis of the α-β-phosphate linkage only and specifically in the presence of the γ-phosphate. We seek to understand why dUDP is not hydrolyzed by dUTPase despite the presence and similar coordination of the α-β-phosphates in dUDP and dUTP. To address the above issues, several transient kinetics and equilibrium enzymological as well as spectroscopic methods were employed using active-site mutant human dUTPase enzymes. We engineered active-site mutants specifically to disrupt interactions between the protein and the γ-phosphate moiety of the nucleotide. We found that perturbation of the p-loop-like γphosphate binding-site of dUTPase resulted in a similar K_d for dUTP and dUDP and that it prevented an isomerization event at the active-site. Enzymatic activity is reduced to various extents depending on the specific mutation and correlates with the residual ability of the protein to bind the γphosphate moiety. Based on our observations we now hypothesize that binding of the γ-phosphate causes a relatively slow isomerization of the active site, which develops strain within the nucleotide phosphate-chain aiding catalysis.

272.03-Pos MutM Interrogating Normal Purine Bases in Its Active Site

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Board B105.03

MutM is a bacterial DNA glycosylase that specifically recognizes oxidatively damaged DNA bases and initiates the base excision

repair (BER) pathway to protect genome integrity. Locating the rare lesion sites among the vast span of normal DNA presents a formidable challenge to MutM, especially given the subtle changes of some damaged bases, for example, 8-oxoguanine (oxoG). Using disulfide crosslinking (DXL) strategy, we were able to capture late search states of Bacillus Stearothermophilus MutM interrogating undamaged purine bases in its active site. Together with the early search state structures, these new complex crystal structures delineate the base flipping pathways of normal nucleobases by MutM and provide basis for substrate specificity of MutM.

272.04-Pos Replication at a single-molecule level

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Board B105.04

Replication is a fast, accurate and highly coordinated process, conducted by several different proteins. The complex interplay between molecular motion, energy conversion and forces associated with the replication process is still largely unknown. Using a single-molecule assay that combines optical tweezers with fluorescence microscopy, we study the dynamics of T7-DNA polymerase and human mitochondrial Pol-Gamma, both members of the family-A DNA polymerases.

During replication in vivo, DNA polymerase needs to remove single stranded DNA binding proteins (SSB) in order to progress on the lagging strand. In our study we discuss the differences of the mechanokinetics of T7 and Pol-Gamma replication. Moreover we aim to understand the fine tuning between the binding strength of SSBs and the ability of DNA polymerases to remove roadblocks. We started this investigation by characterizing the binding dynamics and energetics of SSB on individual single stranded DNA molecules.

272.05-Pos New Insights into Redβ-mediated DNA Annealing using Atomic Force Microscopy

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Board B105.05

Redβ anneals DNA to initiate homologous recombination and has gained recent prominence through the development of the DNA engineering technology known as 'recombineering' or 'Red/ET'. It originates from the red-operon of λ phage where it is co-expressed in the early life cycle stage with Redα a processive 5′–3′ exonuclease and Redγ, a DNA mimetic and RecBCD inhibitor. Unlike RecA/RAD51, Redβ is not an ATPase and it's mechanism for initiating homologous recombination is poorly understood. To examine the structure and dynamics of Redβ complexes at sub-molecular resolution we performed tapping mode atomic force microscopy (AFM) of Redβ protein alone and in complex with DNA. Without DNA, Redβ forms a 'split lock washer' structure with a shallow right-handed helicity. Sequentially adding complementary ssDNA generates a stable left-handed helical filament. Importantly, the contour length of the helical filament equated linearly to the lengths of complementary ssDNA, giving the number of nucleotides per Redβ monomer. Additionally, the monomer width along the filament was quantified. These new quantities as well as the observed helical transition reveals new insights into the mechanism of DNA annealing mediated by Redβ and led us to suggest new mechanistic models.

272.06-Pos Real-time Observation of RecA-mediated Homologous Recombination at the Single-Molecule Level

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Homologous recombination, the exchange of strands between different DNA molecules, is essential for proper maintenance and accurate duplication of the genome. Using magnetic tweezers, we monitor RecA-driven homologous recombination of individual DNA molecules in real time. We resolve several key aspects of DNA structure during and after strand exchange. Changes in DNA length and twist yield helical parameters for the protein-bound three-stranded structure, in conditions where ATP was not hydrolyzed. When strand exchange was completed under ATP hydrolysis conditions that allow protein dissociation, a 'D-wrap' structure formed. Remarkably, the single-molecule analysis revealed that a region of only about 80 base pairs is actively involved in the synapsis at any time during the entire reaction involving a long (~1 kb) region of homology, indicating that strand invasion at one end and RecA dissociation at the other end occur at the same rate.

272.07-Pos Disassembly Of Fluorescent Rad51 Recombinases Occurs In Bursts And Depends On Tension

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The central catalyst in eukaryotic homologous recombination is the Rad51 nucleoprotein filament, polymerized around single-stranded DNA. This filament searches for a duplex DNA segment homolo-

gous to this ssDNA and invades it. After this the nucleoprotein filament should disassemble in order to give way to additional proteins required to finish the recombination. To resolve the mechanism of Rad51 filament disassembly, we have combined optical tweezers with single-molecule fluorescence microscopy and microfluidics. We show that we can trigger filament disassembly of trapped single human Rad51-DNA complexes by activating ATP hydrolysis. We have found that disassembly can be slowed down and even stalled by applying tension to the DNA. We quantified the disassembly of discrete Rad51 patches and discovered that these disassemble in bursts interspersed by long pauses. Upon relaxation of a stalled complex, pauses are suppressed resulting in a large burst. We unite these observations in a model, in which tension-dependent disassembly takes place only from filament ends, after tension-independent ATP hydrolysis. *In vivo*, Rad51 filament disassembly is aided by auxiliary proteins. The complete molecular picture of spontaneous Rad51 filament disassembly that we present is crucial for a proper understanding of this essential step in homologous recombination.

272.08-Pos Molecular Basis of TTDA Disorder

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Board B105.08

General factor TFIIH opens the double-stranded DNA in the promoter region allowing polymerases I and II to initiate the transcription and in the damaged regions helping nucleotide excision repair (NER) factors to restore normal nucleotide sequence. After irradiation of eukaryotic cells by UV light the pool of TFIIH is redistributed towards NER. During NER process TFIIH recruits Tfb5 protein. The mechanism of Tfb5 recruitment is unclear. Here we show that Tfb5 interacts with the Tfb2 subunit of TFIIH. Crystal structures of the yeast Tfb5:Tfb2 complex reveals that two molecules form a symmetric dimer. The structure of each molecule resembles K homology (KH) domain. Based on the property of KH-like proteins to bind nucleic acid oligonucleotides (NA) we found that Tfb2 is NA-binding protein. NA can be displaced from Tfb2 by Tfb5. We introduced mutations found in patients with photosensitive form of inherited premature aging syndrome called Trichothiodystrophy onto yeast Tfb5 background. These mutations affect the Tfb5:Tfb2 affinity and the ability of Tfb5 to displace the NA from Tfb2C. The application of our findings for NER and general transcription are discussed.

272.09-Pos Specific DNA remodelling by the proteins initiating the Mismatch Repair mechanism in E.coli

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Board B105.09

Accumulation of errors in DNA is one of the factors associated with cancer. To avoid that, a specific mechanism (Mismatch Repair or MMR) identifies and repairs these errors, usually insertion, deletion or mismatches of a single base. The mismatch repair process is functionally conserved from bacteria to human. In E. coli the process is initiated by the MutS, MutL and MutH proteins. MutS detects the mismatch and binds to it, and subsequently recruits MutL. In the presence of ATP, this complex activates the MutH endonuclease. MutH incises the newly synthesized DNA containing the mismatch at a hemi-methylated Dam site (5′-GATC); the relevant portion of mismatched DNA is then specifically degraded by exonucleases loaded onto the nick and afterwards re-synthesized by DNA polymerase.

The precise molecular mechanism for the initiation of MMR has not yet been solved using classical biochemistry or genetics. In order to elucidate this problem, we use single molecule techniques (magnetic tweezers) to follow in real time the conformational changes and dynamics in a mismatched DNA induced by proteins MutS, MutL, MutH.

We observe for the first time that MutS and MutL, in the presence of ATP, induce DNA remodeling entirely dependent on the presence of a mismatch. These results can be interpreted as the capturing of thermal loops along DNA by a MutSL complex. We have also tested the role of ATP hydrolysis and multimerization by MutS, and these results are in agreement with bulk biochemical studies. Finally, addition of MutH to the system drastically increases the size of loops, implying a new mechanism other than thermal looping is at work. Quantitative analysis of the effects of MutH are currently under way.

272.10-Pos A Kinetic Analysis of the DNA Binding and Annealing Activity of the Beta Protein of Phage μ

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Board B105.10

Genetic engineering in vivo by homologous recombination, also known as recombineering, is a well-established method allowing DNA alterations on any replicon in E. coli. Recombineering uses the bacteriophage λ Red recombination functions, Exo (a 5′ to 3′ double strand (ds)DNA exonuclease) and Beta (a single strand (ss)DNA binding and annealing protein). Recombineering with ss-oligonucleotides is more efficient than with dsDNA and requires only Beta activity. There is a higher recombination frequency with one of the two possible complementary oligonucleotides. The oligonucleotide of the same sequence as that at the replication fork lagging strand shows a higher recombination frequency compared with the leading strand. This suggests a mechanism by which the oligonucleotide bound beta is annealed directly to the complementary single strand region at the replication fork. For this to happen, single-stranded DNA binding protein (SSB) would need to be displaced during annealing of the Beta-oligonucleotide complex. We are investigating the mechanism by which Beta facilitates single strand annealing. We have used surface plasmon resonance (SPR) spectroscopy to demonstrate preferential binding of Beta to untethered 3′ ends (vs 5′ends) of ss-oligonucleotides as well as partial duplexes with free 3′ends. Beta does not bind to ds-oligonucleotides and binds more weekly to free 5′ ssDNA ends. This is consistent with work published by Li et al (JMB *276*, 733–744 (1998)). In addition we have used SPR spectroscopy and a fluorescence quenching approach to monitor the kinetics of Beta catalyzed annealing of complementary ssDNAs. Beta when bound to an oligonucleotide is able to displace SSB bound to the complementary oligonucleotide and facilitate annealing of the two strands.

272.11-Pos Real Time Study Of Replication Forks In A Living E. Coli Cell

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Board B105.11

DNA replication occurs with high efficiency and fidelity at replisomes where several proteins participate in the highly choreographed DNA synthesis. The major players of the replisome have been extensively studied in molecular biology and their crystal structures have been solved. Recent *in vitro* single-molecule real-time observations on reconstructed replisomes have revealed significant mechanistic insights into DNA replication (*Lee et al.* Nature 2006). However there have not been direct observations of the replication machinery in a living cell. Our group recently demonstrated single molecule studies of DNA-protein interactions and gene expression in living *E. coli* cells (*Elf et al*, Science 2007; *Yu et al*, Science 2006) Here we report a real time study of DNA replication in living *E. coli* cells. We labeled three components of the replication machinery, primase, ligase and single-strand binding protein (ssb) with a yellow fluorescent protein. Upon binding to the replication machinery localized at the center of the cell, individual primase and ssb molecules can be detected above the cell's autofluorescence background through spatial confinement of the molecules at the timescale of data collection. We observed repetitive fluorescence bursts that might be indicative of the formation of Okazaki fragments in real time.

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272.12-Pos Double- and Single-Stranded DNA Binding of Catalytic Alpha Subunit of *E. coli* Replicative DNA Polymerase III

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Board B105.12

Dual-beam optical tweezers experiments elucidate the properties of DNA binding proteins. Measuring the extension of DNA in the presence of a range of protein concentrations determines the mechanism of various binding modes. In these experiments, a single lambda DNA molecule is converted from its double-stranded to single-stranded form by applying force, a process that we refer to as force-induced melting. The α subunit of the replicative DNA polymerase III of *E. coli* is the active polymerase of the ten subunit bacterial replicase. The C-terminal region of α is predicted to contain an oligonucleotide binding-fold (OB-fold) domain. In a series of optical tweezers experiments, the α subunit is shown to have an affinity for both double and single stranded DNA. In the presence of the α subunit, we observe an increase in the DNA melting force as the DNA is stretched. However, upon relaxation, a significant fraction of the DNA does not reanneal, and subsequent stretches indicate that this fraction has been permanently melted. This suggests that the portion of the protein that binds to double-stranded DNA stabilizes the DNA helix, as protein binding must be at least partially disrupted to melt DNA. In addition, the single-stranded DNA binding component appears to be passive, as the protein does not facilitate melting, but binds instead to regions already separated by force, stabilizing the single-stranded form of

DNA. Constructs of segments of the α subunit show that the N-terminal region is responsible for dsDNA stabilization, while the C-terminal region binds to melted DNA.

272.13-Pos Direct Observation of Bacteriophage Mu Target Immunity with Single-Molecule Analysis

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Board B105.13

Transposons are mobile genetic elements that transfer DNA from one site within a genome to another site. Bacteriophage Mu is one of the transposon family members and the mechanism of DNA transposition is well-studied. Highly specific protein-DNA complexes are required for the DNA cleavages and joining involved in DNA transposition. MuA is the transposase, which binds to the Transposase binding sites at each end of the Mu genome and then assembles into a series of nucleoprotein complexes called transpososomes within which the DNA cutting and joining reactions take place. Another Mu encoded protein MuB is an ATP-dependent nonspecific DNA binding protein and in the presence of ATP, assembles into large oligomeric complexes on DNA, which serves as an efficient transposition target site. MuA stimulates ATPase of MuB, resulting in dissociation of MuB from DNA. Therefore, MuB accumulates on DNA that is not bound by MuA, resulting in a strong preference for transposition to occur at DNA sites at least 10 kb from MuA-bound regions. This phenomenon is called target immunity and thought to prevent destruction of the Mu genome by auto-integration. Although the detailed mechanism of target immunity still remains to be studied, recent analysis using enhanced green fluorescent protein (EGFP)-MuB shows that MuB oligomers accumulate at A/T-rich sequences and DNA looping between the MuA- and MuB-bound DNA sites is required for MuA-stimulated removal of MuB from DNA. In this study, we constructed fluorescent labeled MuA-DNA complex and used single-molecule fluorescence detection system to clarify the target immunity mechanism in more detail. We will show our present results and discuss the mechanism of target immunity.

272.14-Pos Insights in the transposition mechanism of the bacterial insertion sequence IS*911* revealed by Tethered Particle Motion

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Board B105.14

During the transposition process, the DNA segments called insertion sequences move between different loci in bacterial genomes. We focus on IS*911* a member of the widespread IS3 family of bacterial insertion sequences which follows a two-step transposition mechanism involving the formation of a circular transposon intermediate. IS*911* encodes itself for the transposase OrfAB responsible for this process and, like other insertion sequences, IS*911* is bordered by short imperfectly repeated sequences, IRL and IRR, in an inverted orientation. These are essential for the productive transposition, since they provide the specificity for both the binding of the OrfAB and the cleavage and strand transfer reactions required for the displacement of the element.

We follow the assembly of the synaptic complex occurring during this transposition process by using the single molecule techniques of Tethered Particle Motion which consists in tracking the movement of a bead tethered by a DNA molecule to the glass surface. It enables us to monitor the various protein-mediated looping states of the DNA consecutives to the interaction with the active transposase mutant OrfAB4M. New states are observed when compared to the results obtained with the inactive truncated form of the transposase OrfAB[149]. Moreover preliminary results show that in absence of Mg2+ the synaptic complex cannot be formed with the OrfAB4M. Additional experiments are in progress in order to elucidate these recent and interesting findings.

Ribosome & Translation

273-Pos Simulation of an Entire Cycle of tRNA Translocation of Ribosome Using Hybrid Elastic Network Interpolation

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Board B106

We first simulate the complete cycle of tRNA translocation during ratcheting motion of ribosome using hybrid elastic network interpolation (HENI) in which both rigid and flexible regions of a complex system can be represented as a spring network among rigid clusters and point masses. A 70S ribosome (2HGP and 2HGQ) structure was modeled as four rigid clusters (30S head, 30S body, L1 stalk, and 50S body) and point masses including all three tRNAs, mRNA, and coarse-grained residues or nucleotides nearby the interface region between 30S and 50S subunits. By adopting Frank's two-step mechanism for translocation (Nature, 2000), we applied group theory to compute the coordinates of hybrid and posttranslocation states of ribosome, respectively. HENI result for the first step from pretranslocation state to hybrid state demonstrates how A(P) site tRNA translocates from A(P) site to P(E) site of 50S subunit. Similarly, it is also observed that 30S subunit returns to the original position with respect to 50S subunit by relatively shifting A(P) site tRNA to P(E) site during the second step. Overlap calculation based on normal mode analysis of the same ribosome structure indicates not only that ratcheting motion is the most dominant mode during the cycle of tRNA translocation but also that other associated motions such as 30S head rotation, A site opening, and E site opening are crucial for explaining how mechanically tRNAs cross the bulge regions between A(P) and P(E) sites.