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Probing The Structure And Function Of Transcription Complex Of RNA Polymerase II With TFIIF At Single Molecular Level

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Previously, we have developed a bio-conjugation method that allows us to specifically label the C-terminus of any subunit of a RNA polymerase. Here, we reconstitute a RNA polymerase II (Pol II) with TFIIF (IIF), in which the C-terminus of a RNA polymerase subunit is labeled with Cy3 and an amino acid of the largest subunit of TFIIF is labeled with Cy5. By measuring FRET at single molecular level, we are able to probe the structure information of Pol II/IIF in the absence of X-ray structure. By using single molecular FRET and tethered particle motion method, we are also carrying out transcription of Pol II in the presence or absence of IIF to identify the role of IIF in RNA elongation at various stages.

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Agaerye

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In order to ensure stable expression of an endogenous protein, the cell has to regulate each step of gene expression. Starting with transcription, we here observe in live cells the real-time transcription of an endogeneous gene, β-actin. To achieve this, we use a transgenic mouse model where RNA stem loops knocked into the untranslated region of β-actin mRNA are bound to a fluorescent MS2 reporter protein.

We monitor β-actin transcription levels in single fibroblast cells, either as a response to serum induction or in conditions of basal expression (constant serum level). This way, we are able to describe the kinetics of β-actin transcription over a long period. We observe that the cell responds to serum induction within minutes, and falls back to basal levels after ~1h, in agreement with data on fixed cells. We then compare how levels of transcription vary within one cell (between the alleles), and from cell to cell within a cell population. These findings uncover how extrinsic causes (influence of the environment within the cell) and intrinsic causes (inherent stochasticity of transcription at one gene locus) respectively contribute to genetic expression noise of an essential endogeneous gene. Supported by NIH grant EB2060.

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Metal Preference At The Second Metal Binding Site Of E. coli NikR

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Escherichia coli NikR (EcNikR) regulates cellular nickel uptake by binding free nickel ions at high affinity metal binding sites in the protein, which induces EcNikR binding to the *nik* operon - a process that leads to suppression of the gene encoding the nickel uptake transporter, NikABCDE. A structure of the EcNikR-DNA complex suggests that a second metal binding site is present in addition to the high affinity sites, and raises the question of which metal occupies this second site under physiologic conditions: K^+, which is present in the crystal structure, or Ni^{+2}. To determine which ion is preferred at the second metal binding site, and the reason for any preference of one ion over another, we calculated the electrostatic free energy of EcNikR binding to DNA with either K^+ or Ni^{+2} in the second site. While the interaction between EcNikR and DNA is more favorable when the second site contains Ni^{+2}, the large desolvation penalty associated with moving Ni^{+2} from solution to the relatively buried second site offsets this favorable interaction. Consequently, our data suggest that EcNikR binding to DNA is more favorable when the second site contains a K^+ ion. Moreover, additional calculations indicate that the second site is best suited for an ion having the size of K^+ and not Ni^{+2}, suggesting that the second site is optimized for K^+. Taken together, our data suggest that the second metal binding site contains K^+ and explain why K^+ is preferred over Ni^{+2} at this site.

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Force-Dependence of Lac-Repressor Mediated DNA Loop Formation

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Protein-mediated DNA looping is a ubiquitous motif in transcriptional control schemes. Formation of these loops is driven by thermal fluctuations of the substrate DNA, which in turn are known to be exquisitely sensitive to mechanical constraints on the DNA. Because DNA in vivo is subject to a complex micromechanical environment, it is intriguing to study the effect of mechanical tension in the substrate DNA on the formation of these regulatory loops to investigate the role of mechanics in controlling gene regulation. For this purpose, we measured the formation and breakdown rates of lac repressor-mediated DNA loops under tension using constant-force axial optical tweezers.

We observed that an incremental force of less than 100 femtonewtons is sufficient to reduce loop formation rate about sevenfold in a construct with an interoperator spacing of 305 bp. This result suggests the possibility of mechanical pathways to control gene expression with forces that are two orders of magnitude lower than other typical intracellular forces acting on DNA, such as the forces exerted by RNA polymerase and molecular motors. Moreover, we developed a model that quantifies the relation between the force sensitivity of the loop formation rate and the angle between the incoming and outgoing DNA strand in the loop as a way to infer loop topology from our micromechanical measurements. We conclude that the LacI-mediated DNA loop prefers an anti-parallel loop topology over a parallel conformation.

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Malleable machines in transcription regulation: the Mediator complex

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The Mediator complex provides an interface between gene-specific regulatory proteins and the general transcription machinery including RNA polymerase II (RNAP II). The complex has a modular architecture and cryo-EM analysis suggested that it undergoes dramatic conformational changes upon interactions with activators and RNAP II. These rearrangements were proposed to play a role in the assembly of the pre-initiation complex and also contribute to the regulatory mechanism of Mediator. In analogy to many regulatory and transcriptional proteins, we reasoned that Mediator might also utilize intrinsically disordered regions (IDRs) to facilitate structural transitions and transmit transcriptional signals. Indeed, a high prevalence of IDRs was found in various subunits of Mediator from both *Saccharomyces cerevisiae* and *Homo sapiens*, especially in the Tail and the Middle modules. The level of disorder increases from yeast to man, although in both organisms it significantly exceeds that of multi-protein complexes of similar size. IDRs can contribute to Mediator's function in three different ways: they can serve as target sites for partners with variable structure; they can act as malleable linkers connecting globular domains that impart modular functionality on the complex; and they can also facilitate assembly and disassembly of complexes in response to regulatory signals. Short segments of IDRs, termed molecular recognition features (MoRFs) distinguished by a high protein-protein interaction propensity, were identified in 16 and 19 subunits of the yeast and human Mediator, respectively. In *Saccharomyces cerevisiae* the functional roles of 11 MoRFs have been experimentally verified and those in the Med8/Med18/Med20 and Med7/Med21 complexes were structurally confirmed. The arrangement of disordered regions and that of the embedded interaction sites are similar in *Saccharomyces cerevisiae* and *Homo sapiens* yet their sequences are weakly conserved. All these data suggest an integral role for intrinsic disorder in Mediator's function.

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Structure/function Correlations in P. aeruginosa DNA Ligase LigD

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The ATP-dependent DNA ligase D (LigD) performs a major role in non-homologous end-joining (NHEJ) pathway. *Pseudomonas aeruginosa* LigD (PaeLigD) contains a N-terminal phosphoesterase domain (PE) domain followed by a ligase domain and a C-terminal polymerase domain. The PE domain (187 residues) possesses manganese dependent phosphodiesterase and phosphomonoesterase activities as it sequentially removes the 3′-ribonucleoside from the primer strand of the RNA primer-DNA template duplex and subsequently hydrolyzes the 3′-PO_4 to a 3′-OH group (1).

PaeLigD-PE belongs to a class of unique 3′- end-processing enzymes as it cleaves the primer strand to a point at which a single ligatable ribonucleotide remains (2).

Extensive mutagenesis studies have identified critical residues required for ribonuclease and 3′-phosphatase activities (1). Multiple active sites present in

the enzyme lead to the belief that this enzyme possesses some unique motifs. However, in the absence of atomic level structural information clear structure/function correlations are lacking. We are currently working towards obtaining a high-resolution structure of PaeLigD-PE using solution NMR methods.

Reference:
1) Zhu, H., and Shuman, S. (2006) J. Biol. Chem. 281, 13873-13881.
2) Zhu, H., and Shuman, S. (2008) J. Biol. Chem. 283, 8331-8339.

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Zero-Mode Waveguides for Real-Time Observation of Single Nucleotide Incorporation

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Single-molecule fluorescence studies on the incorporation of fluorescently labeled nucleotides by DNA polymerizing enzymes typically operate at nucleotide concentrations well below their Km values. While this is inevitable given the femtoliter observation volumes accessed via conventional fluorescence microscopy, the biological relevance of the insights gained into enzymatic kinetics may be compromised. Zero-mode waveguides (ZMWs), sub-wavelength holes in a thin metal film, provide an excellent solution to this problem by greatly reducing the observation volume [M. J. Levene, et al., 2003, Science 299, 682-686].

We have successfully designed and fabricated ZMWs of about 100 nm in width. In addition we have developed a surface treatment protocol based on PEG functionalization to make the ZMWs biocompatible, and to facilitate the controlled tethering of biomolecules [A. Crut, et al., Nanotechnology, in press (2008)]. In these structures, we have observed the real-time observation of single nucleotide incorporation at biologically-relevant concentrations in ZMWs, The latest scientific results will be presented.

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Formation Of RecA Filament During The Mechanical Unzipping Of dsDNA To ssDNA: Competition With SSB Differentially Controls RecA Mediated SOS Response And Replication Repair

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RecA and its eukaryotic homologs Rad51 and Dmc1 carry out many DNA transactions: recombinational DNA damage repair, genome integration of incoming DNA, programmed recombination during meiosis, and stalled DNA replication forks processing, all reactions initiated forming a RecA ssDNA nucleoprotein filament. ssDNA/RecA interactions activate the ''SOS'' response through RecA co-protease activation, raising RecA concentration. RecA/ssDNA binding must be regulated such that it only occurs in specific situations: a feature involved in modulating specificity is competitive binding of SSB and RecA to ssDNA. We studied this competition using a magnetic tweezers assay system in which we follow the formation of RecA filaments, in the presence and absence of SSB, on a single ssDNA molecule obtained by mechanical unzipping of dsDNA. We examined various buffer conditions and the effects of several relevant nucleotides. When RecA and SSB tetramer are equimolar, SSB wins the competition and no stable RecA filament is observed; when RecA is in a 20-fold molar excess, stable RecA filament forms. At intermediate molar ratios mixed situations are observed. These results provide information on the competition dynamics between RecA and SSB at the single DNA molecule level. Our results confirm previous ensemble studies: RecA and SSB affinities for ssDNA provide an intrinsic differential control for RecA mediated DNA repair and recombination functions, independently by a RecA loading machinery. SOS response increases RecA concentration giving a molar ratio of RecA:SSB of ~30:1 outcompeting SSB without need for loading factors. These studies show that a ssDNA/RecA filament is stable for much longer than any ssDNA/SSB complex examined. This could imply that the free energy for bound RecA is much lower than that for SSB.

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Control of DNA Replication by Anomalous Reaction-Diffusion Kinetics

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DNA replication requires two distinct processes: the initiation of pre-licensed replication origins and the propagation of replication forks away from the fired origins. Experiments indicate that these origins are triggered over the whole genome at a rate I(t) (the number of initiations per unreplicated length per time) that increases throughout most of the synthesis (S) phase, before rapidly decreasing to zero at the end of the replication process. We propose a simple model for the control of DNA replication in which the rate of initiation of replication origins is controlled by the interaction with a population of rate-limiting proteins. We find the time set by reaction-diffusion kinetics for such proteins to find, bind to, and trigger a potential origin. The replication itself is modeled using a formalism resembling that used to study the kinetics of first-order phase transitions. Analyzing data from Xenopus frog embryos, we find that the initiation rate is reaction limited until nearly the end of replication, when it becomes diffusion limited. Initiation of origins and hence I(t) is suppressed when the diffusion-limited search time dominates. We find that, in order to fit the experimental data, the interaction between DNA and the rate-limiting protein must be subdiffusive. We also find that using a constant nuclear import of the limiting proteins leads to a more accurate description of the experimental data.

Protein-Nucleic Acid Interactions I

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Single-molecule Study of Site-specific DNA Recombination by γδ Resolvase

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γδ resolvase is a serine recombinase coded by γδ transposon, which catalyzes DNA recombination between two *res* sites (114 bp) on a negatively supercoiled circular DNA, resulting in two catenated DNA circles. Each *res* site contains 3 different resolvase binding sites - site I, II and III, and each binds to a resolvase dimer. We have developed a single-DNA based system whereby synapsis and recombination should lead to torsional relaxation of a single supercoiled DNA. DNA relaxation catalyzed by γδ resolvase occurs at much higher rate on DNA substrate containing 2 res sites than those containing 1 or 0 res site. Furthermore, reactions on a 2-res-site substrate show a characteristic ~200 nm relaxation consistent with the +4 ΔLk observed to be associated with the recombination reaction in bulk experiments. We also have observed topoisomerase activity of γδ resolvase on the non-specific (0 res) DNA substrate.

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Thermodynamics of Interactions between Histone-like Proteins from *Escherichia coli* (HU and IHF) and Intact Duplex DNA

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The structural homologs $HU_{\alpha\beta}$ and IHF are major nucleoid associated proteins (NAPs) of *Escherichia coli*, which organize chromosomal DNA and facilitate numerous DNA transactions. Using isothermal titration calorimetry (ITC), fluorescence resonance energy transfer (FRET) and a series of DNA duplex lengths (8, 15, 34, 38 and 160 base pairs) we establish three different nonspecific binding modes for both $HU_{\alpha\beta}$ (Koh et al., 2008) and IHF. Both the NAP:DNA mole ratio ([NAP]/[DNA]) and DNA length dictate the dominant NAP binding mode. On sufficiently long DNA, at low [NAP]/[DNA], both HU and IHF populate a noncooperative 34 bp binding mode with a binding constant of ~ 10^7 M^{-1} at 0.082 M Na^+. With increasing [NAP]/[DNA], both HU and IHF bound in the noncooperative 34 bp mode progressively convert to two moderately cooperative modes with site sizes of 10 bp and 6 bp and smaller binding constants. As DNA length increases at low [NAP]/[DNA], fractional population of the 34 bp mode increases. The 34 bp mode of IHF exhibits a large negative $\Delta C_{p,obs}^{\circ}$ and an exothermic ΔH_{obs}° (15 - 25°C), similar to previous observations for the specific complex of IHF and wrapped H'-DNA, whereas a small positive $\Delta C_{p,obs}^{\circ}$ and an endothermic ΔH_{obs}° were observed for the 34 bp mode of HU. From these and parallel studies at various salt concentrations we propose that DNA is wrapped on the body of IHF in the nonspecific 34 bp mode like the specific complex of IHF and H'-DNA, whereas DNA is bent but not wrapped in the 34 bp nonspecific HU-DNA complex. Other structural features of the binding modes of HU and IHF deduced from these studies are also discussed.

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Novel Techniques for Study of the Nucleosome Core Particle Ionic Atmosphere and Its Role in Electrostatically-Driven DNA Packing

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The nucleosome core particle (NCP) is the primary mechanism for DNA compaction. While the wrapping of the DNA around the histone core is thought to be at least partially sequence dependent, the packing of the nucleosome core is believed to be almost entirely electrostatic in nature. Using novel techniques to probe the ionic atmosphere, we hope to elucidate details of this compaction and