

Single Molecule Investigations of Tn5 Transposase – DNA Interactions

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Single DNA molecule micromanipulations provide a unique means for the study of protein – DNA interactions. Here we report the development of a single molecule Tn5 transposase DNA binding and synaptic complex formation assay. The method utilizes a long DNA tether where biotin is bound to one end of the molecule while dioxigenin is bound to the other end. Streptavidin-coated magnetic beads and anti-dioxigenin functionalized glass or polystyrene beads tether the single molecules of DNA for manipulation. Magnetic tweezers are used to extend and contract the DNA tether. A 51 kb DNA tethering molecule containing a 1.3 kb Tn5 transposon has been constructed. This tether is used to study Tn5 transposase – DNA interactions. Initial results of the Tn5 transposase's interactions with non-specific and specific DNA are discussed. Salt affects on the binding of the transposase to DNA and other transposases are described. A model describing the observed results is presented.

Tn5 transposase as a target identifies compounds that inhibit HIV integration in cells

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HIV-1 integrase is an underutilized drug target for treatment of human immunodeficiency virus infection. A limiting factor for drug development is the lack of co-structural information. The Tn5 transposase is a structurally well-characterized, related protein that may serve as a useful surrogate for inhibitor discovery and development. Little data exist, however, on inhibitor cross reactivity between Tn5 and HIV-1. To address this question, we screened 16,000 compounds and identified twenty compounds that inhibit Tn5 transposase-DNA complex assembly. Six were found to also inhibit HIV-1 integrase activity. Most surprising is the discovery of several cinnamoyl derivatives that also inhibit HIV integration in cells. These compounds lack the pharmacophoric hydroxyl groups and are less toxic than several previously reported members of this inhibitor class. We further identified several coumarin dimers and benzoic acid inhibitors. Based on these results, we believe that the Tn5 system is well suited for the development of therapeutics targeting HIV-1 integrase.

Hot Start, Fidelity and Cloning Improvements for PCR

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We demonstrate two ways to achieve a hot start for PCR.

(1) Precipitate the magnesium with “too much” phosphate; the mineral resuspends during thermal cycling and starts the PCR.

(2) Cold-sensitive mutations of KlenTaq DNA polymerase have been identified which have reduced activity during bench temperature reaction setup, yet are active and stable at PCR temperatures of 65-95°. The mutants cluster at the hinge point (“knuckle”) of the fingers that are believed to move at each polymerase incorporation cycle. The mutant enzymes provide an automatic hot start for PCR, and the fidelity of the resulting amplicons is slightly improved.

Fidelity of DNA polymerase is increased in non-standard buffers and dNTP concentrations (Eckert & Kunkel); we show progress in determining how extreme the conditions can be, while still allowing effective PCR-amplification of 4 kb of DNA (with lacZ) which is of resulting higher fidelity. Betaine (1.3 M) does not hurt fidelity.

To clone and recombine the PCR products, we start with “riboprimers” which have one 3'-ribo base. We have identified conditions and RNases that can cleave the single ribo base when it is surrounded by double-stranded DNA. After removal of the primer, 3'-sticky ends of the length of the primers (25-30) are left, and they readily recombine *in vitro* with the vector which was prepared the same way with complementary riboprimers. Using long and accurate PCR, our largest vector and target combination so far has been 12 kb vector and 4 kb target.

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High-throughput production of selenomethionine-labeled proteins in 2-liter PET bottles using an auto-inducing medium

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The production of protein labeled with selenomethionine (SeMet) has become a standard approach for determining experimental phases and structures by X-ray crystallography. CESG has implemented a high-throughput protocol for the routine production of SeMet-labeled proteins that provides high yield cell growth, maximizes expression and solubility, and gives a high percentage incorporation of SeMet. The protocol uses a 2-liter PET beverage bottle as the growth vessel and the Met auxotroph and codon-adapted strain *Escherichia coli* B834 pLacIRare as the expression host. An initial expression of the unlabeled protein in a medium containing tryptone and induced using 1mM IPTG has been used to assess target-dependent properties such as cell mass yield, protein expression level, solubility, purity after semi-automated purification, proteolysis from protein tags upon treatment with tobacco etch virus protease, stability of the cleaved protein, and identification of crystallization conditions. Cumulative favorable results from these successive pipeline steps triggers production of the SeMet-labeled protein, which is undertaken in a supplemented minimal medium containing SeMet, all other amino acids except Met, vitamins, trace metals, and the auto-inducing mixture of glucose and lactose. The complete cell growth process from inoculation of the PET bottles to cell harvest takes ~24 h, with protein expression commencing in the auto-inducing medium after ~9 h. The cell mass yields are target-dependent, with an average 7.2 ± 1.5 g of wet cells obtained per liter of culture medium ($n = 29$ different protein targets). Evaluation of the CESG database shows a strong correlation between the yield and purity results obtained in either the unlabeled or the SeMet-labeled proteins. ESI mass spectral analyses of SeMet-labeled proteins shows $90 \pm 5\%$ substitution of SeMet for Met. Recent examples of crystal structures solved from proteins expressed using this protocol will be presented. (This research funded by NIGMS GM645980).

**Screening and quantification methods for high-throughput production
of unlabeled and labeled soluble *Arabidopsis thaliana*
proteins in *Escherichia coli***

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Analytical procedures for detecting and assigning the results of expression trials are critical inputs to the operation, reproducibility, and stability of protein production operations in the high-throughput structural genomics environment. Here we present the details of denaturing gel electrophoretic evaluations of a high-throughput process for production of unlabeled, selenomethionine-, and ¹⁵N-labeled *Arabidopsis* proteins in *Escherichia coli* B834 pLacIRare. All recombinant proteins produced in this *E. coli* expression host were evaluated for total protein expression and the partition between soluble and insoluble fractions using a simple, three category evaluation scheme. The total protein content was normalized as the sum of soluble protein fraction plus the insoluble fraction re-dissolved to a normalized volume. Pre-treatment of the host cells with lysozyme followed by sonication in a multi-well device gave reproducible, optimal breakage of the expression cells obtained from a variety of growth conditions and containing a wide variety of expression targets. The scoring scheme for denaturing electrophoresis gels was based on the pattern and the intensity of both constitutive and recombinant protein markers present in the soluble fraction. UW CESG has found that two highly soluble *E. coli* proteins of ~45 kDa and ~27 kDa provide a useful correlation between the completeness of cell lysis and the fractionation of the recombinant target protein between soluble and insoluble fractions. Two other integral membrane *E. coli* proteins of 37 and 40 kDa provide reliable markers for the insoluble fraction. Examples of how this scoring system can be used to evaluate expression results in a high-throughput environment will be presented (funded by NIGMS GM645980).

End synopsis and replicative formation of IS911 transposition intermediates.

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Transposition of the bacterial insertion sequence IS911 involves the production of covalently closed circular intermediates which then undergo integration into a target DNA molecule. When the element is located on a plasmid a molecular species in the form of a figure-eight can be detected. This results from an initial single strand cleavage at one end (IR), liberating a 3'OH which acts as a nucleophile to attack the same strand three bases 5' to the other end.

We present the results of *in vivo* kinetic experiments which follow the formation and disappearance of this species. These demonstrate that the figure-eight is the precursor of the free IS circle. We have developed *in vitro* systems which reproduce figure-eight formation and circle integration. We have been unable, however, to reproduce the formation of the circular IS from figure-eight intermediates *in vitro*. This suggested that specific host factors might be implicated in this reaction.

The *in vivo* kinetic data not only show that the figure-eight gives rise to a circular transposon form but also indicate that this step does not require IS911 transposase. It must therefore involve host factors. We present experiments demonstrating that the primase, DnaG, is essential for this step, implicating replication in this conversion. Experiments using ³H thymidine incorporation and plasmids with a temperature-sensitive replication apparatus demonstrated that the process does not require replication from the plasmid replication origin.

To investigate this potential replicative pathway for circle production more closely, we have used the *E.coli* replication terminator sequence, *terC*, which when bound by the Tus protein, inhibits replication forks in a polarised manner. When cloned within the transposon in an appropriate orientation, *terC* was found to inhibit figure-eight resolution and circle formation in the presence of Tus but not in its absence.

These results provide strong support for a model in which IS911 circle intermediates are derived from the figure eight forms by a replicative mechanism. The results of studies using single molecule technologies to investigate synaptic complex formation between both transposon ends will also be presented.

Bug Transposons

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We study the transposition of 2 cut & paste transposons, Tn7 from E. coli and Hermes from the house fly, both hosts being bugs although of different sorts. Tn7 is a member of the widespread Retroviral Integrase Superfamily and Hermes of the hAT family that includes McClintock's Ac element.

The Hermes system is relatively simple: the transposase is comprised of a single polypeptide. We have established that recombination proceeds via double strand breaks that excise the element from the donor site, followed by joining to the target DNA. Interestingly, the breaks occur as in V(D)J recombination with hairpins being formed on the flanking donor DNA. The exposed 3'OH ends of the transposon then join to the target DNA. We have also established a Hermes transposition system in Saccharomyces cerevisiae and have used a genetic screen to isolate transposase mutants that promote high frequencies of transposition. We have also used site-directed mutagenesis to probe structure-function relationships of the transposase

The Tn7 transposition system is far more elaborate. Tn7 insertion into its preferred chromosomal site attTn7 requires 4 Tn7-encoded proteins TnsABC+D and an essential ATP cofactor. We have found that after the DNA breakage and joining events that result in transposition have occurred, the transposition product is still included in an elaborate nucleoprotein complex. Notably, the gaps that flank the ends of the newly inserted transposon are inaccessible to a repair polymerase in this complex. We are using a variety of methods to probe this complex.

Antibiotics, antibiotic resistance, and the meaning of life.

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I joined the UW Biochemistry Department in 1967 and started studying two lines of research: antibiotic mode of action with an emphasis on translation inhibitors and ribosome function, and mechanisms of resistance to antibiotics in bacterial pathogens. Ever since, my research has involved antibiotics (in one way or another). Small molecule biology is fascinating! Antibiotics represent only a minor segment of the massive structural diversity of these low-molecular-weight, biologically active compounds present in nature and made by all forms of life, having a wide variety of hormetic activities. Despite providing a profitable resource for research and pharmaceutical applications, the natural roles of this vast array of small molecules are largely unknown. Chemical ecology is as much of a mystery as microbial ecology, largely because it has not been studied properly. It is assumed that all microbes in the environment are involved in a constant arms race, simply because of the activities of a small number of microbial WMDs under laboratory conditions. This notion of aggression is enhanced by the identification of antibiotic resistance mechanisms in bacterial pathogens. In fact, resistance genes often have other roles in their natural hosts and have been hijacked to perform protective roles in various bacteria. It is suggested that antibiotic action and resistance determination may in fact be rare functions in the environment. A better understanding of all aspects of chemical ecology will likely be of benefit in terms of pharmaceutical discovery and drug applications. As for the meaning of life ...

Transposon-mediated Rescue Cloning of Unidirectional Deletion Libraries From Either End of Any Target DNA

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We describe a simple method for the construction of unidirectional deletion libraries from either end of any target DNA. The system utilizes a transposon containing a conditional origin of replication (R6K_{ori}) and a selectable drug resistance marker. After transposition into the target DNA, the transposition reaction products are amplified in a PCR reaction using a primer from the distal end of the transposon and a second primer from the desired end of the target. The PCR reaction products are end-repaired, self-ligated, and transformed into the appropriate bacterial host to generate the deletion library. It should be noted that the target DNA can be almost any DNA containing the desired sequence: an existing plasmid, cosmid, or BAC clone, a restriction fragment, or a PCR product from genomic DNA or any other source.

By combining the above approach at the DNA level with the use of a specialized expression transposon carrying a T7 promoter and RBS, it is possible to obtain unidirectional amino and carboxyl deletions at the protein level. These could be valuable for epitope mapping, domain mapping, and protein engineering.

Studies on the Conjugative Transposon CTn DOT

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Conjugative transposons (CTns) are self-transmissible elements that are normally integrated into the bacterial chromosome. The first step in transfer is the excision of the CTn from the donor chromosome to form the circular transfer intermediate. A single strand of the intermediate is transferred to a recipient where it integrates into the recipient's genome. The focus of our work is on a *Bacteroides* CTn, CTnDOT. CTnDOT is found in many human colonic *Bacteroides* strains. It carries genes that confer resistance to the antibiotics tetracycline and erythromycin. Previously we developed an *in vitro* system for the integration reaction. We also constructed a miniature (mini-element) form of CTnDOT that contains only the ends of CTnDOT and the CTnDOT integrase (IntDOT). Excision of the mini-element *in vivo* requires IntDOT and an operon that carries four genes whose gene products can function *in trans*. More recently we have developed an *in vitro* excision system. In this system, the left and right junctions of CTnDOT, *attL* and *attR*, are provided on separate plasmids. The excision reaction produces a cointegrate that contains the *attDOT* site (the joined ends of CTnDOT) and the chromosomal *attB* site. Optimal recombination frequencies were obtained from reactions that contained IntDOT and a crude extract from a *B. thetaiotaomicron* strain that carries the excision region of CTnDOT on a plasmid. Deletion analyses have determined the minimal DNA sequences of *attL* and *attR* that are necessary for recombination. Mutants that contain mutations in *attL* and *attR* that affect the efficiency of recombination have been isolated. The mutations may affect binding sites for IntDOT.

Analysis of a Tn5 Insertion Library in MG1655

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Transposition is a recombination event mediated by transposable DNA elements such as the Tn5 transposon and can lead to inversions, insertions, deletions and duplications in a genome. Here we report the generation of a Tn5 insertion library ($\sim 10^5$ single insertion events) in the *E. coli* strain MG1655 and subsequent analysis for *in vivo* transposition activity. Mutants that displayed an aberrant activity level (either hyper- or hypoactive) were selected for further analysis and sequencing. The goal of this work is to identify host genes that affect Tn5 transposition.

Stress signaling pathways

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Effective mechanisms to deal with stress are vital to all living organisms. To maintain cellular viability, stress signaling pathways and their responses must be rapid, sensitive and robust. In *E. coli*, separate responses cope with stress in the cytoplasmic and envelope compartments of the cell. Each response is carried out by an alternative σ factor. A signal transduction cascade that has proteases as central mediators controls the activity of each σ . During normal cell growth, the envelope stress response is induced primarily by unassembled outer membrane porins (OMPs) in the envelope compartment. σ^F directs this response and its activity is controlled by RseA, a membrane spanning anti-sigma factor. I will discuss the design principles of the proteolytic cascade that carries out regulated proteolysis of RseA.

The Impact of Host Factors on Tn10 Transposition: The Story Unfolds

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Transposons are mobile DNA elements. They are highly abundant in most organisms and impact significantly on the organisms in which they reside. Not surprisingly host proteins are often involved in regulating DNA transposition reactions. For example, in bacteria Tn10 transposition is modulated by the nucleoid binding protein IHF. This host protein helps in the assembly of the Tn10 recombination complex (or transpososome). It does so by creating a bend at each transposon end sequence. This permits the transposase protein to contact the end sequence at two distinct sites and to stabilize this sequence in a folded conformation. However, the transposon ends must be unfolded at a later stage to permit the capture of a target site for an intermolecular strand transfer event – it is thought that one of the folded transposon arms blocks the entry of an intermolecular target DNA into the transpososome. If IHF is not ejected, and the transposon arm is not unfolded, the transposon ends are constrained in such a way that primarily intramolecular strand transfer events occur. The predominant intramolecular transposition product is an unknotted transposon inversion circle.

A screen for additional host factors that participate in bacterial DNA transposition has revealed that a second nucleoid binding protein called H-NS also plays an important role in Tn10 transposition. We provide evidence that H-NS acts in opposition to IHF to unfold the transposon ends in preparation for intermolecular target capture. We show that H-NS has a high affinity for the folded form of the transpososome and upon binding induces transpososome unfolding. H-NS also binds with a high affinity to a pre-formed target capture complex. It is known that target capture in Tn10 transposition involves bending of the target DNA, and given the known preference of H-NS to bind bent DNA structures, we speculate that H-NS binding to target DNA may serve to stabilize the target capture complex. Importantly, addition of H-NS to an in vitro Tn10 transposition reaction significantly increase the amount of intermolecular transposition product observed. The mechanism by which H-NS facilitates the unfolding of the Tn10 transpososome is currently being investigated. The involvement of two highly abundant nucleoid binding proteins in Tn10 transposition provides an excellent example of how a mobile element has co-evolved with its host.

Pyrophosphate Mimetics: a Mechanism Based Approach to Antiviral Drug Discovery

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Despite little or no sequence homology, enzymes that catalyze phosphoryl transfer reactions share a common active site architecture involving two divalent metal ions with a conserved inter-metal ion distance. Viruses encode a variety of essential phosphoryl transferases including polymerases, ribonucleases and integrases. We have identified highly potent and specific inhibitors of integrase from the human immunodeficiency virus (HIV-1). These compounds are the first inhibitors of integrase to demonstrate activity against HIV-1 both in vitro and in vivo. Extensive characterization of these inhibitors has elaborated the mechanism of action as direct sequestration of the active site divalent metals in integrase. Elements of the inhibitors which interact with the divalent metal ion have been identified and the structure of the minimal pharmacophore is consistent with the observed spatial orientation of the metal ions in the active site of integrase as well as other enzymes in this class. These inhibitors of HIV-1 integrase therefore present an entirely new approach to develop novel agents to treat HIV-1 infection and provide an important proof of concept for a general pharmacophore that can be exploited to inhibit phosphoryl transferases through this conserved mechanism. Consistent with this hypothesis, extensive screening of related compounds has led to the identification of specific inhibitors for a wide variety of viral targets, including RNA and DNA polymerases for a diverse number of human pathogenic viruses.

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**Gene Regulation by
Tetracyclines:
Mechanisms and Applications**

Hillen.pdf

Gene regulation by the tetracycline (tc) inducible Tet repressor (TetR) is not only a paradigm for understanding the properties of a regulatory protein, but also a widespread tool for conditional gene expression in most organisms, including bacteria, fungi, plants, invertebrates and mammals (1). TetR is a homodimer of about 46 kDa containing one helix-turn-helix DNA recognition motif and one tc binding site per subunit. Extensive X-ray analysis has produced detailed models of the conformational changes associated with induction (2). *In vitro* evolution approaches yielded TetR mutants with altered DNA sequence and inducer recognition properties (1), and also a TetR inducing oligopeptide. Recently we have also succeeded in reversing the TetR allosterical response by obtaining TetR variants for which anhydro-tc, the inducer of the wild type, is a corepressor (3). The implications for binding site plasticity and conformational changes in allosterical proteins will be discussed.

For eukaryotic applications a suitable TetR variant is usually fused with a readout domain. We have used fusions with VP16 and KRAB to construct HeLa cell lines in which procaspase-2 or t-Bid can be expressed under doxycycline (dox) control. Although both cell lines show dox inducible apoptosis, a rather large amount of procaspase-2 must be expressed to induce apoptosis, whereas the much more active t-Bid must be tightly controlled to avoid uninduced apoptosis. Two different regulatory strategies accomplishing these needs are described (4).

To analyse the influence of DNA structure on enhancer activity, we have fused TetR with various oligomerisation domains and flanked an SV40 enhancer with binding sites for that construct. As a result, we can completely regulate SV40 enhancer activation of a downstream TATA box by reversibly separating it from the promoter in a DNA loop.

For random gene targeting, we have constructed an insertion DNA element carrying a tc regulated outward promoter and used it for random mutagenesis of *E. coli*. Transposase driven insertion of this element turns out to be random and yields about 4% of the mutants with a tc inducible gene knockout phenotype. Thus, this construct allows the random generation of conditional knockout mutants throughout bacterial chromosomes.

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CREB Hypoxia and Cancer

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Tumor microenvironment controls the selection of malignant cells capable of surviving in stressful and hypoxic conditions. The transcription factor, cAMP responsive element binding protein (CREB) activated by multiple extracellular signals, modulates cellular response by regulating the expression of a multitude of genes. We have demonstrated that two cysteine residues, at the DNA binding domain of CREB, mediate activation of CREB-dependent gene expression at normoxia and hypoxia. The construction of a dominant-positive CREB mutant, insensitive to hypoxia cue (substitution of two cysteine residues at position 300 and 310 to serine, in the DNA binding domain) and of a dominant negative CREB mutant (addition of a mutation in serine133) enabled a direct assessment, in-vitro and in-vivo, of the role of CREB in tumor progression. In this work, we demonstrate, that CREB controls hepatocellular carcinoma (HCC) growth both in-vitro and in-vivo, supports angiogenesis and renders resistance to apoptosis. Along with the identification, by DNA array, of at least 77 genes mediated by CREB at hypoxia, we suggest that in parallel to other hypoxia responsive mechanisms, CREB plays an important role in hepatocellular carcinoma (HCC) tumor progression.

Gene Silencing in Arabidopsis

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Chromatin is well known to play an important role in silencing and activation of gene expression. Histone modifications play many roles in this process, including modulating chromatin condensation, providing binding sites for specific factors, and targeting of DNA methylation. Purification of histones from *Arabidopsis* and analysis by nHPLC- μ ESI- LTQ-FT mass spectrometry has allowed us to obtain a relative quantitation of most of the acetylation and methylation sites on the histone H3 N-terminus. We find distinct differences between the abundance of active and silencing modifications on the two histone H3 variants and a very low abundance of both types of modifications on the same histone. As methylation of lysines can involve one, two or three methyl groups, we have also determined the relative abundance of each of these variations present in the two variants. In conjunction with these studies, we have also analyzed histone modifications in a mutant line lacking a functional KRYPTONITE gene (SU(VAR)3-9 homolog). Using western analysis, ChIP and mass spec, we find that KRYPTONITE is the primary enzyme responsible for dimethylation of histone H3K9. As this mutant was isolated as a suppressor of an epigenetic mutation in the SUPERMAN gene and resulted in a decrease in CNG methylation, we can conclude the dimethylation of H3K9 is the critical mark for both directing CNG DNA methylation and gene silencing¹.

1. Dimethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in *Arabidopsis thaliana*

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Architecture of the Hin synaptic complex before and after DNA exchange

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The Hin recombinase catalyzes a site-specific DNA inversion reaction in the chromosome of *Salmonella* that regulates the alternative expression of two different flagellin genes. Although Hin is able to synapse the two *hix* recombination sites, the chemical steps leading to the exchange of DNA strands requires the formation of a higher order “invertasome” structure that contains the Fis-bound recombinational enhancer sequence. The invertasome is assembled at the base of a supercoiled DNA branch, which ensures that only *hix* sites on the same molecule recombine to invert the intervening DNA. We have isolated a series of hyperactive Hin mutants that no longer require Fis, the enhancer, or DNA supercoiling to promote recombination. These mutants have enabled development of a simplified in vitro system where reactions on oligonucleotide substrates can be studied. Synaptic complex intermediates can be isolated that contain 4 Hin protomers, each covalently associated with the 5' ends of the cleaved recombination sites through a phosphoserine linkage. These complexes can be chased into ligated products in which the DNAs are in either the parental or recombinant structures. Kinetic analysis reveals that synapsis, DNA exchange, ligation and resolution/disassembly are fast relative to the postsynaptic step(s) leading to DNA cleavage. Site-directed crosslinking between strategically-placed cysteines has been used to probe the configuration of Hin subunits within the synaptic complex during recombination. The results localize the interface between Hin dimers and demonstrate that the *hix* DNA segments are located on the outside of the complex. These experiments also provide evidence supporting a conformational change, such as a helix-coil transition, within the C-terminal end of the dimerization helices that may be critical for DNA attack by the active site serines. Crosslinked recombinants from differentially-tagged Hin homodimers demonstrate that heterodimers are formed upon DNA exchange. These results provides the first direct evidence for the exchange of protein subunits during recombination and are consistent with earlier experiments following topological changes in the DNA that occur upon recombination. The architecture of the Hin synaptic complex forces us to modify earlier structural models for the Fis-activated invertasome complex.

HoxB-13 in Prostate and Colorectal Cancer Development

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Hox homeobox genes are known to function as a set of transcription factors, merely due to the presence of DNA-binding homeodomain. *Hox* genes function during development to regulate axial regional specification during embryonic development. Therefore, *Hox* genes are expressed in a tissue-specific and frequently stage-related fashion. The *hox-13* paralog is especially important to the development of the caudal extent of the body axis, including the prostate and colon. Hoxb13 has been recently found to limit proliferation and activate programmed cell death in secondary neural tube and caudal mesoderm. In mesoderm-derived prostate gland, Hoxb13 is highly expressed from the embryonic stage to adulthood and Hoxb13 knockout mice retain swollen prostate gland. However, precise role of Hoxb13 in this organ and molecular mechanisms how Hoxb13 functions are entirely unknown.

In prostate, expression pattern of HOXB13 seems to correlate with androgen-receptor (AR), suggesting that HOXB13 may be involved in AR signaling to modulate the growth of normal and cancerous prostate cells. In fact, HOXB13 interacts with AR to suppress androgen-mediated AR signaling and subsequently inhibits the growth of AR-positive LNCaP cells. HOXB13-mediated growth suppression can be counteracted by the overexpression of hormone-activated AR. In AR-negative PC3 cells, however, HOXB13's growth suppressive function is accomplished through the transcriptional inhibition of T cell factor-4 (TCF-4). Accordingly, HOXB13 suppressed TCF-4 mediated transactivation and the expression of its responsive gene, *c-myc*. Therefore, HOXB13 seems to regulate AR, TCF-4, or both signaling pathways, depending on the status of AR. In other words, HOXB13 and AR may need to coexist and may functionally counteract each other in regulating the growth and progression of prostate cancer cells. Although no difference is found in HOXB13 expression between tumors and matching normal tissues, over 70% of colorectal tumors lost the expression of HOXB13. Considering that mutations in APC/ β -catenin signaling cascade is a decisive event for colorectal tumorigenesis and involved in over 90 % of colorectal cancers, loss of HOXB13 may be an another step for colorectal cell to acquire growth advantage during the transformation process.

Our ultimate goal is to understand the mechanistic and functional connection between HOXB13 and AR and/or TCF-4 signaling pathways. Achieving this goal will further explain how HOXB13 can play a role as a mediator to regulate these two important growth signals.

Site-specific Recombination and Transposition Mediated by the Novel Piv/MooV DNA Recombinases

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The Piv/MooV family of DNA recombinases includes DNA transposases and site-specific invertases from a rapidly increasing number of prokaryotic systems. We have previously demonstrated that the completely conserved amino acid motifs from the Piv/MooV recombinases are required for catalytic activity of the site-specific DNA invertase, Piv. Piv controls phase variation of type IV pili in *Moraxella lacunata*, mediating inversion of a 2.1 kb chromosomal segment that encodes the pilin subunit. Although Piv apparently utilizes a conservative recombination mechanism for DNA inversion, molecular modeling of Piv revealed a RNaseH-fold in the amino-terminal third of the protein with conserved acidic residues positioned to coordinate at least one divalent metal cation. This structural motif is found in nucleases, Holliday junction resolvases (RuvC family), and the DDE-motif retroviral integrases/transposases. We have pursued the roles of each of the conserved acidic residues D9, E59, D101 and D104, which the predicted Piv tertiary structure places in the catalytic pocket. In addition, the function of a predicted RuvA-like helix-hairpin-helix motif in the central region of Piv is currently under investigation. A model based on these structure/function studies and characterization of recombination intermediates and products will be presented.

A Possible Role for Sigma Factor C in *Mycobacterium tuberculosis* Latency.

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Mycobacterium tuberculosis remains the leading cause of morbidity and mortality by a bacterial pathogen. Annually 6.8 million suffer from active tuberculosis leading to 2.4 million deaths globally. It is estimated that 1/3 of the world's population is latently infected with the bacterium, each individual carrying a 10% lifetime risk of developing active tuberculosis or a 10% annual risk if co-infected with the HIV virus. The metabolic fate of the bacilli inside the host during latent infection is unknown. One possibility is that the bacilli enter into a metabolically-inert state to avoid being killed by granulomas that form around infected macrophages to prevent bacterial dissemination. Another possibility is that the pathogen "hides out" by replicating slowly in other cell types such as lung epithelial cells. In either scenario, the mycobacteria persist for months to decades until an immune-compromising event allows the bacilli to emerge from the "latent state" and cause active disease.

Some of the non-primary sigma factors likely enable *M. tuberculosis* to sense and alter gene expression in various host environments. I have created a family of secondary sigma factor knockout mutants in the pathogenic *M. tuberculosis* strain H37Rv and have utilized in vitro and animal models to identify sigma factors required for virulence. Of those examined to date, a mutant defective in the production of sigma factor C possesses phenotypes consistent with SigC functioning as an important regulator of persistent infections. SigC is not required for growth in rich medium, for entry or survival inside of the human macrophage cell line THP1, or for initial replication in the lungs of guinea pigs infected by aerosol. However, a SigC mutant strain is less effective than the parent, H37Rv, at disseminating the infection to the spleen by 6 weeks post-infection. The mutant is less able than H37Rv at maintaining a persistent infection; titers of the mutant strain in the lungs and spleen drop to undetectable levels by 20 weeks post-infection. Animals infected with the SigC mutant also exhibit reduced pathology by possessing fewer and smaller lung granulomas than animals infected with H37Rv. Experiments to identify the SigC-regulated factors that enable *M. tuberculosis* to be a successful pathogen are underway.

Bacteriorhodopsin Biogenesis: from Polypeptide to Membrane Crystal.

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The integral membrane protein bacteriorhodopsin (BR) is the first-discovered member of a superfamily of microbial rhodopsins that function as light-driven ion pumps and phototaxis sensors in archaea, bacteria and eukaryotes. Because its structure is known at atomic resolution, BR is attractive for studying events in integral membrane protein biogenesis, including insertion into the membrane, folding and assembly into multimeric structures. BR consists of a polypeptide (bacterioopsin) containing seven transmembrane α -helices that provide a binding pocket for vitamin A retinal, which is covalently bound to the molecule and is responsible for its purple color. In *Halobacterium salinarum*, which produces BR, the protein assembles with unique membrane lipids in extensive two-dimensional crystals known as the purple membrane.

We seek to understand each step in BR biogenesis at the molecular level. In earlier work, we demonstrated that bacterioopsin is inserted into the *H. salinarum* membrane as it is translated. External loops of the protein appear outside the cell in the same order as they occur in the primary sequence, in accord with current models of membrane protein integration. We also identified genes that are essential for retinal production in *H. salinarum*, including some that are related to known carotenoid biosynthetic genes and others that may be specifically required for the assembly of retinal with the polypeptide chain of microbial rhodopsins. Finally, we showed that specific amino acid residues in transmembrane α -helices contribute to purple membrane assembly and stability.

More recently, we used semi-random mutagenesis in *H. salinarum* to identify the structural features of BR that determine its biogenesis. We targeted substitutions to regions of ~15 amino acids throughout the protein with a doped oligonucleotide PCR strategy designed to yield an average of five substitutions in each mutant protein. The sequence of the bacterioopsin gene in 873 unique mutants that yielded purple colonies indicated that most residues could be altered with little effect on BR formation. Eight of the 238 residues targeted were unaltered in any mutant and ~15 residues tolerated only conservative changes. Most of these residues are in the retinal binding site where mutations are known to influence BR formation and the color of the protein. However, several residues outside of the retinal binding pocket were identified that may be essential for BR formation. We conclude that BR insertion and folding is generally independent of the amino acid sequence except in the retinal binding pocket. The implications of these results for membrane protein biogenesis will be discussed.

Transposon-based innovative method for sequencing highly repetitive heterochromatic DNA in BAC/oriV clones. M. Mendez Lago^{1,2}, J. Wild¹, J.P. Abad², A. Martin-Gallardo³, A. Villasante², W. Szybalski¹. 1) McArdle Lab., University of Wisconsin, Madison, WI; 2) Centro Biología Molecular (CBMSO, CSIC-UAM), Madrid, Spain; 3) Servicio Interdepartamental de Investigación (SIdI) UAM, Madrid, Spain.

The contiguous finished sequence from rigorously assembled contigs is needed to understand the role of heterochromatin regions in chromosome behavior. However, when using the current aligning approaches, based on sequence overlaps, it has been hardly possible to align the newly acquired repetitive heterochromatic sequences, because they are often nearly identical. Moreover, some present methods are laborious and not open to the extensive mapping technologies currently employed by sequencing centers. Here we present a novel transposon (Tn)-based strategy for sequencing heterochromatic BACs that contain *oriV*, which permits DNA amplification in *trfA* hosts.

First, we modified Tn5/*oriV* by adding rare restriction sites, I-SceI and PI-SceI. Using this Tn, we constructed a library of BAC/*oriV* clones with two PI-SceI sites. One site was at a fixed position in the backbone of the BAC plasmid, while the other was in Tn, randomly inserted into the cloned heterochromatic DNA. In the next step, sequences (500-1000 nt) are to be collected, from the outward priming sites in Tn, from several hundreds of such clones. Finally, all Tns were precisely mapped using PFGE and appropriate hybridization probes from the BAC vector and transposon. Combined results of sequencing and determination of Tns precise positions and orientation will permit us to assemble the entire sequence of the heterochromatic clone, since the principle of our approach is the assembly of all the newly acquired sequences according to their physical positions instead of aligning by the conventional search for overlaps. This strategy, we hope, especially when automated by optical mapping with labeled probes, will become crucial for heterochromatin sequencing.

**Molecular basis and biological reasons of hyper-recombinogenic activity of
Pseudomonas aeruginosa RecA protein**

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RecA protein promotes DNA strand exchange by forming filaments on single-stranded DNA (ssDNA) followed by uptake of the duplex substrate. RecF, O, and R proteins help to RecA to polymerize on ssDNA covered by SSB proteins. A distinguishing characteristic of RecA protein from *Pseudomonas aeruginosa* (RecAPa) is its constitutive hyper-rec activity in *E. coli* cells which is manifested through a 6-8 fold increase in the frequency of recombination exchanges per DNA unit length. Relative to RecAEc (RecA from *E. coli*), RecAPa displaces SSB-Ec and SSB-Pa proteins (SSB from *E. coli* and *P. aeruginosa*, respectively) from ssDNA more rapidly, forms more salt stable and temperature stable presynaptic filaments, and possesses a greater affinity for ssDNA at its primary DNA binding site. In DNA strand exchange reactions, RecAPa promotes joint molecule formation more efficiently but is less effective in the generation of final products, reflecting its propensity to initiate DNA pairing rather than to complete strand exchange. RecAPa is also less dependent on the mediator activity of *E. coli* RecF, O, and R proteins and does not possess a 3' end bias in initiation of recombination by its presynaptic filament. All these properties suggest an enhancement of both ssDNA gap- and dsDNA break repair mechanisms promoted by RecAPa. The research was supported by a Fogarty International Research Collaboration Award (grant TWO 1319-O1A1).

HIV-2 Vpx: Modeling and Probing Structure/Function

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HIV-1 and HIV-2 have evolved somewhat altered mechanisms by which viral cDNA can be carried to the nucleus in preparation for host DNA integration. An ancient horizontal transfer event from HIV-1 has lent an additional gene to HIV-2, known as Vpx, which is homologous to Vpr in both viruses. However, the functions of Vpr as seen in HIV-1 appear to be split between Vpr and Vpx in HIV-2. A prominent function is a nuclear localization activity of Vpx. Here, we model a core structure of Vpx onto an HIV-1 Vpr NMR template. A flexible loop between helices two and three is predicted. We show that an insertion in this region is tolerated, resulting in active Vpx-containing preintegration complexes. The mutant protein is also correctly packaged into viral particles. The C-terminal region of Vpx remains unmodeled but likely adopts a polyproline II helix joined to the core via a flexible linker. We have probed this region for binding partners via phage display and present a few candidate genes. Finally, we find that the human invariant chain protein (important to MHC class II antigen presentation), when fused to Vpx, acts as a chaperone, making Vpx soluble and stable under a variety of conditions.

Nonsense-mediated mRNA decay in mammalian cells: Splicing-dependent degradation that occurs 5'-to-3' and 3'-to-5' as a consequence of a "pioneer" round of translation

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Nonsense-mediated mRNA decay (NMD) in mammalian cells is generally a splicing-dependent mechanism by which cells recognize and degrade mRNAs that prematurely terminate translation during a "pioneer" round of translation. The dependence on pre-mRNA splicing reflects the deposition of exon junction complexes (EJCs) on mRNA. These EJCs then recruit the Upf NMD factors. We recently demonstrated that NMD degrades mRNA from both 5' and 3' ends by recruiting decapping and 5'-to-3' exonuclease activities as well as deadenylating and 3'-to-5' exonuclease activities¹. In short, down-regulating the Dcp2 decapping protein, the PM/Scl100 component of the exosome, or poly(A) ribonuclease (PARN) was found to abrogate both nucleus-associated and cytoplasmic NMD. Consistent with these results, NMD factors Upf1, Upf2 and Upf3X co-immunopurify with the Dcp2 decapping protein, Rat1 and Xrn1 5'-to-3' exonucleases, PM/Scl100 and Rrp41 components of the exosome, and PARN. Additional studies of the enzymology of NMD will be presented.

The bulk of cellular proteins derive from translation of mRNA that is bound at the 5' cap by eukaryotic translation initiation factor (eIF)4E. However, our studies of NMD indicate that mRNA that is bound by cap binding protein (CBP)80, which is a precursor to eIF4E-bound mRNA, can also be translated during what we call a "pioneer" round of translation²⁻⁴. We find that the pioneer round, which can be assessed by measuring NMD, is not inhibited by 4E-BP1, which is known to inhibit steady-state translation by competing with eIF4G for binding to eIF4E. Therefore, at least in this way, the pioneer round of translation is mechanistically distinct from steady-state translation. eIF4GI, poly(A) binding protein (PABP)1, eIF3, eIF4AI, and eIF2 α co-immunopurify with both CBP80 and eIF4E, which suggests that each factor functions in both modes of translation. Consistent with roles for PABP1 and eIF2 α in the pioneer round, Paip2, which is known to destabilize PABP1 binding to poly(A) and inhibit steady-state translation, and inactive eIF2 α , which is also known to inhibit steady-state translation, also inhibit NMD. Polysome profiles indicate that CBP80-bound mRNAs are translated less efficiently than their eIF4E-bound counterparts.

As noted above, depending on the particular mRNA, NMD can take place in association with nuclei or in the cytoplasm. Differences between the two types of NMD will be discussed.

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Micromechanical study of proteins interacting with single DNA molecules

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I will discuss the use of single-DNA micromanipulation - studying elastic properties of individual molecules of roughly tens of kb in length - as a tool to investigate protein-DNA interactions. Many proteins effect dramatic physical changes in DNA organization, by folding, looping or otherwise modifying double helix conformation. Single-DNA experiments offer the possibility to watch these proteins interact with the double helix in a direct way, allowing observation of the random dynamics of small numbers of molecules which are usually inaccessible in bulk solution phase experiments. I will focus in particular on two studies recently done in our lab: measurements of changes in DNA mechanics resulting from binding of the DNA-bending proteins HU, NHP6A and HMGB1, and the assembly/disassembly dynamics of nucleosomes observed in *Xenopus* egg extracts.

Genetics as a source of emerging resistance to antibiotics

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Mutations, plasmid/transposon/integron are the source of accumulation of mechanisms of resistance and appearance of novel mechanisms of resistance. Mutation is a source of acquired resistance mostly from naturally-occurring genes that are either modified or overexpressed. Mutation combined with plasmid acquisition is also a frequent mechanism for acquisition of novel mechanisms of resistance. Transposon and integrons play a significant role for transfer of antibiotic resistance genes and accumulation of antibiotic resistance genes. A series of examples illustrating these mechanisms of emerging antibiotic resistance will be taken from clinically-significant gram-negative bacteria.

RNA Processing Regulation of Immunoglobulin Gene Expression

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Mammalian genomes have been found to encode a surprisingly small number of genes. However, as many as 60% of all genes may be alternatively processed to generate a much greater diversity of proteins. Therefore, to fully understand gene expression and the generation of protein diversity, we must understand how the complex process of alternative RNA processing is regulated. From studying model RNA processing substrates, we know that this regulation is likely to be complex and the result of multiple protein-RNA and protein-protein interactions. In addition, it has become clear in recent years that RNA processing and transcription elongation are functionally coupled reactions and thus, factors that modulate transcription may also affect alternative RNA processing decisions. We have been studying the immunoglobulin M (Ig μ) gene, one of the first to be described to be alternatively processed, as a model system to better understand RNA processing regulation. This gene contains competing cleavage-polyadenylation and splice reactions which lead to the production of two different mRNAs whose abundance varies during B lymphocyte maturation. We have demonstrated that these two reactions are in direct competition and that their efficiencies must be balanced. Also, since another gene with a similar structure was regulated in cell lines and in a transgenic mouse, we have concluded that regulation must be mediated by factors that affect general cleavage-polyadenylation and/or splicing. We are currently using microarray analysis to identify genes whose expression changes when a B cell line is stimulated to differentiate. Some of the differentially expressed genes encode products relevant to RNA processing regulation and are candidate Ig μ expression regulators. Also, we have recently described a sequence element downstream from the internal poly(A) site that seems to be an RNA polymerase pause site. We are examining this pause site and the elongation properties of RNA polymerase on the μ gene to better understand Ig μ regulation as well as to gain insights into RNA polymerase elongation control in general.

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Shuttling between Membranes, a New Energy Transduction Paradigm in Gram Negative Bacteria

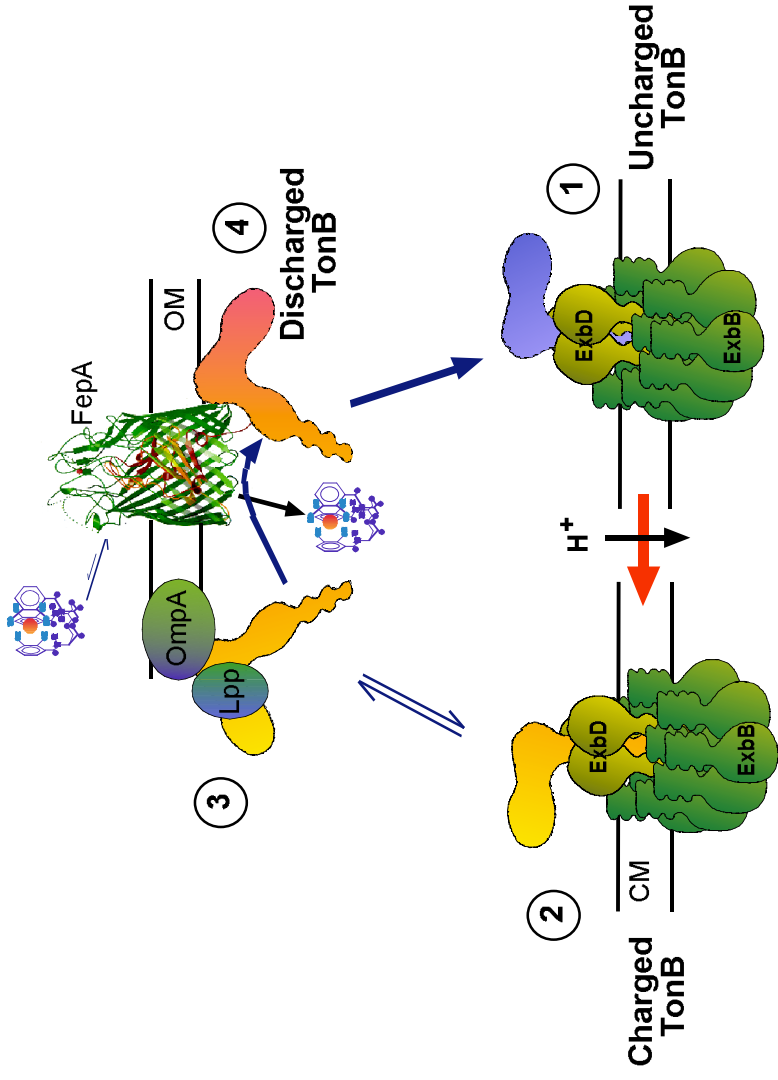
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Gram negative bacteria such as *E. coli* have both a cytoplasmic and an outer membrane. While processes in the cytoplasmic membrane can be energized by ion gradients or ATP hydrolysis, the outer membrane lacks access to these energy sources. Nonetheless, active transport of important nutrients occurs across the outer membrane. Surprisingly, the energy source for this transport turns out to be the protonmotive force (pmf) of the spatially separate cytoplasmic membrane. A large complex made up of two cytoplasmic membrane proteins--ExbB and ExbD--appears to harvest the cytoplasmic membrane pmf and use it to instill a third protein, TonB, with meta-stable conformationally stored potential energy. The energized TonB delivers stored energy to the outer membrane transporters, with subsequent movement of nutrients into the aqueous periplasmic space between the two membranes. From that point, the nutrients are retrieved by soluble binding proteins and actively transported into the cytoplasm by classical ABC transporter mechanisms.

Previous studies based on membrane fractionations suggested a model wherein TonB transduces energy by making sequential and cyclic contact with proteins in each membrane, a process called shuttling (1). A key feature of shuttling is that the amino terminal signal anchor (N-terminus in the cytoplasm) is released from the cytoplasmic membrane ExbB/ExbD complex as TonB becomes associated entirely with the outer membrane. To test this model *in vivo*, a sole cysteine was engineered in TonB at the extreme amino terminus. This cysteine could be labeled in whole cells by Oregon Green® 488 maleimide, but only under circumstances where TonB could shuttle to the outer membrane. Mutant TonB that could not shuttle also could not be labeled. In addition, more TonB was labeled under conditions where a greater proportion of TonB was found at the outer membrane (2). To our knowledge, this is the first description of a shuttle mechanism for energizing active transport.

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Model for Energy Transduction between Membranes of Gram-negative Bacteria



Uncharged TonB (1) is converted to charged TonB (2) by the passage of a proton through the ExbB/D complex. Charged TonB can shuttle to the outer membrane and dock with non-transporter proteins, Lpp and OmpA (3). Ligand binding to the TonB-gated transporter FepA results in a conformational change in FepA, that induces productive interaction with charged TonB, and release of the conformationally stored potential energy in TonB(4). Discharged TonB is recovered at the cytoplasmic membrane as uncharged TonB (1) by ExbB/D and begins a new energy transduction cycle.

Tn5 transposase as a surrogate for HIV-1 integrase in drug screens

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The identification of small molecule inhibitors of a biochemical process can lead to useful compounds for studying intermediate steps in the overall process and, if the process in question is medically important, can lead to the development of pharmacologically useful compounds. High-throughput assays have been developed for Tn5 transposase with the goal of isolating compounds that can block (or enhance) various steps in the transposition pathway. In addition, since Tn5 transposase shares structural and mechanistic features with HIV-1 integrase, some of the identified compounds may also act on this important protein. 16,000 compounds were screened against Tn5 transposase and twenty compounds were found to inhibit transposase-DNA complex assembly. Six of these twenty were found to also inhibit HIV-1 integrase activity. One of the six was found to block HIV-1 integration within cells at concentrations significantly below the cytotoxic dose. This last compound was used as a lead to find two more inhibitors of HIV-1 integration. The structures of these compounds will be discussed.

Is there a role for Genomics in Crop Improvement?

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The availability of large-scale DNA sequence information and the associated genomic tools like large-scale transcript profiling has changed the way biological research is performed. However, it is still not clear what impact this information will have when applied to crop improvement. This seminar will focus on maize improvement starting with a description of what types of changes have been selected for by breeders over the last 70 years. This will be followed by a discussion of the ways in which genomic information can have an impact on crop improvement in several areas. First, a discussion of modifying metabolite flow to improve an agronomic trait will be described. Second, there will be a discussion of attempts to develop transgenic lines to improve a complex trait like abiotic stress tolerance. Finally, the possibilities and difficulties of using genomic information to improve breeding selection will be discussed.

Suffering the Slings and Arrows: Role of Host Cell Functions in Post-Integration Repair. René Daniel, Konstantin D. Taganov, James Greger, Richard A. Katz, and Anna Marie Skalka.* Fox Chase Cancer Center, Institute for Cancer Research, 333 Cottman Avenue, Philadelphia, PA 19111-2497 U.S.A.

The joining of retroviral and host DNA, mediated by the viral integrase, creates a discontinuity in the host cell chromatin. It appears that this discontinuity is sensed as DNA damage by the cell: our previous results have provided strong evidence that post-integration repair of this lesion is mediated mainly via components of the cellular non-homologous end-joining pathway. An ATM (ataxia- telangiectasia mutated)-dependent, alternative pathway has also been implicated, which we hypothesize may depend on components of the homologous recombination repair system. Finally, computational simulations as well as evidence of a requirement for ATR (ATM and Rad-related) function in post-integration repair suggest a link with cellular DNA replication. Based on these results we have proposed a model for post-integration repair in cycling cells. A brief description of the approaches used in these studies and key observations will be presented, along with further tests of this model. Knowledge of how retroviruses exploit the relevant host functions can provide new insight into cellular DNA damage response and repair mechanisms and may identify cellular targets for antiviral drug development.

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Role Of The Reactive Site Loop In The Function Of Maspin
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Epithelial cells, such as those from the mammary gland, prostate, skin and cornea, synthesize maspin, a member of the serine proteinase inhibitor family of proteins. In addition, the stromal cells of the cornea synthesize this protein. Upon malignant transformation of epithelial cells to carcinoma cells or activation of corneal stromal cells to the wound healing fibroblast phenotype, synthesis of maspin is down regulated. Addition of exogenous maspin to these cells stimulates adhesion to extracellular matrix molecules and inhibits invasion. An indirect mechanism is involved probably through regulation of gene expression.

The purpose of this study was to elucidate the portion of the maspin molecule required for stimulation of adhesion of cells to extracellular matrix molecules and inhibition of invasion. Chimeras were made between the active maspin molecule and the inactive homologous serpin, ovalbumin using a yeast secretion system. Replacement of the Reactive Site Loop (RSL) or the C-terminal peptide and the RSL of maspin with that of ovalbumin resulted in the loss of stimulation of adhesion of mammary carcinoma cells to fibronectin and corneal stromal fibroblasts to fibronectin, type I collagen and laminin. These mutants also lost their ability to inhibit invasion of the carcinoma cells through a Matrigel matrix. Maspin with the C-terminal ovalbumin peptide was fully active suggesting the C-terminal region is not involved in function. Transfer of the RSL of maspin to ovalbumin resulted in conversion of ovalbumin to a fully functional molecule. The maspin RSL peptide (P10-P5', residues 330-344) alone fully stimulated adhesion and inhibited invasion. This suggests the RSL peptide is sufficient for full activity of maspin.

Site directed mutagenesis of the putative P1 Arg 339 in the RSL of intact maspin to Gln had no effect on activity but replacement of this residue with Ala resulted in the loss of activity suggesting that the side chain of Arg forms a critical hydrogen bond with its target molecule. Replacement of Lys 334 at the P5' site with Ala also resulted in the loss of activity. Substitution of the Pro 337 at the P3 site with Val had no effect but mutation to Ala resulted in the loss of activity. This suggests that the rigidity of this Pro residue is not critical for the active conformation of the RSL. These mutagenesis studies imply that multiple residues in the RSL are important for function.

Maspin bound to the surface of the mammary carcinoma cells with a k_d of 367 ± 67 nM and $32.0 \pm 2.2 \times 10^6$ binding sites/cell. This binding was inhibited by the RSL peptide suggesting the RSL is involved in the binding of maspin to cells. Sufficiency of the maspin RSL for function suggests that the mechanism by which maspin regulates cell-matrix adhesion and carcinoma cell invasion does not involve the serpin mechanism of proteinase inhibition.

Identifying RNA binding sites on the *E. coli* Hfq protein

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Hfq is a RNA-binding protein originally discovered as a host factor required for Q β RNA virus replication in *E. coli*. The *hfq* gene is highly conserved among bacterial phyla and the protein has been shown to play an important role in regulating translation of a number of mRNAs. In *E. coli* it binds to small regulatory RNAs (e.g, DsrA, OxyS, RprA) and is required for the interaction of regulatory RNAs with their targeted mRNAs. Studies indicate that Hfq functions by enhancing bimolecular RNA-RNA pairing. Crystal structures of *S. aureus* and *E. coli* Hfq reveal that the protein forms a donut-like hexamer, and a structure of *S. aureus* Hfq with a U-rich RNA heptamer shows a RNA binding site that follows a circular path on one surface of Hfq. In this structure the highly conserved Tyr at position 42 of each Hfq subunit stack alternately between six of the bases that extend from the circular path of the RNA backbone. Comparative sequence analysis and structure modeling suggest the location of a second RNA-binding site. The path of this site involves a concentric ring of residues at a larger radius. In nearly all bacterial Hfq sequences (~160) Phe or Tyr occurs at position 39 forming a concentric ring of aromatic residues at the larger radius on the same Hfq surface. A small aliphatic group co-varies at position 12 adjacent to position 39 in the 3-D Hfq structure. In every instance (~ 6) where Phe or Tyr does not occur at position 39, Tyr replaces the small aliphatic group at position 12. To test the role of these residues, wild type *E. coli* Hfq and Hfq mutated at positions 12, 39, and 42 and other positions were over-expressed and their binding to rA₁₈ and several other RNA oligomers were examined by protein fluorescence quenching, gel retardation and fluorescence anisotropy. Results show that the F42A mutation (the inner ring) does not influence the binding of rA₁₈ to Hfq, whereas the F39A mutation (the outer ring) decreases the affinity of rA₁₈ to Hfq by 3-4 fold. Hfq binding affinity to rA₁₈ is recovered by the F39A/L12F double mutation. The results are consistent with a Hfq binding site for an A-rich sequence that involves the ring of aromatic residues at the radius of position 39.

The NAD⁺-dependent Sir2p Histone Deacetylase is a Negative Regulator of Chromosomal DNA Replication. Donald L. Pappas, Jr., Ryan Frisch and Michael Weinreich* Laboratory of Chromosome Replication, Van Andel Research Institute, Grand Rapids, MI 49503

The establishment of chromosome replication during S phase of the cell cycle requires a multi-step process and begins with the ordered assembly of a large protein complex at origins of replication during a narrow window from late mitosis to early G1 phase. The Origin Recognition Complex (ORC) determines the sites of DNA replication initiation by binding to origin DNA and recruiting additional initiation factors to the chromatin prior to DNA synthesis. The first step in this “chromosome replication cycle” is the formation of a large pre-replicative complex (pre-RC) at individual replication origins throughout the genome during exit from mitosis. Pre-RC formation occurs when Cdc6p(ATP) binds to ORC and facilitates the recruitment of the MCM DNA helicase to origins. It is not known how Cdc6p promotes MCM binding to origins or what role Cdc6p ATP binding or hydrolysis has during initiation. To better understand the genetic requirements for pre-RC formation, we selected chromosomal suppressors of a temperature sensitive *cdc6-4* mutant that altered a conserved residue in the Walker A ATP binding motif. We recovered loss-of-function mutations in the chromatin modifying genes *SIR2*, *SIR3*, and *SIR4* that suppressed the *cdc6-4* temperature sensitive lethality to varying degrees. This suppression was independent of the well-known transcriptional silencing roles for the SIR proteins at the *HM* loci, at telomeres, or at the rDNA locus. A deletion of *SIR2* gave the best suppression and uniquely rescued both the DNA synthesis defect of the *cdc6-4* mutant and its severe plasmid instability phenotype. A *SIR2* deletion suppressed additional initiation mutants affecting pre-RC assembly but not mutants that act subsequently. One model to explain these findings is that Sir2p promotes an inhibitory chromatin structure for pre-RC formation at replication origins. Thus, when Cdc6p function is compromised, a loss of Sir2p promotes a more “open” chromatin structure that allows loading of the MCM proteins. Since there are 7 *SIR2* orthologues in metazoans, a conservation of the ability to repress DNA replication from particular origins could afford one mechanism to establish patterns of DNA replication initiation within the developing organism or in response to cell autonomous signals.

Recombination Mediates Genetic Instabilities of Triplet Repeat Sequences

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The expansion of triplet repeat sequences is an initial step in the disease etiology of a number of hereditary neurological disorders in humans. Diseases such as myotonic dystrophy, Huntington's, several spinocerebellar ataxias, fragile X syndrome, and Friedreich's ataxia are caused by the expansions of CTG•CAG, CGG•CCG or GAA•TTC repeats. The mechanisms of the expansion process have been investigated intensely in *E. coli*, yeast, transgenic mice, mammalian cell culture, and in human clinical cases. Whereas studies from 1994-1999 have implicated DNA replication and repair at the paused synthesis sites due to the unusual conformations of the triplet repeat sequences, recent work has shown that homologous recombination (gene conversion) is a powerful mechanism for generating massive expansions, in addition to or, in concert with replication and repair.

We have recently focused on the recombinational properties of long GAA•TTC repeating sequences analyzed *E. coli* to gain further insights into the molecular mechanisms of the genetic instability of this tract as possibly related to the etiology of Friedreich's ataxia. Intramolecular and intermolecular recombination studies showed that the frequency of recombination between the GAA•TTC tracts was as much as 15 times higher than the non-repeating control sequences. Homeologous, intramolecular recombination between GAA•TTC tracts and GAAGGA•TCCTTC repeats also occurred with a very high frequency (~0.8%). Biochemical analyses of the recombination products demonstrated the expansions and deletions of the GAA•TTC repeats. These results, together with our previous studies on the CTG•CAG sequences, suggest that the recombinational hotspot characteristics may be a common feature of all triplet repeat sequences (TRS). Unexpectedly, we found that the recombination properties of the GAA•TTC tracts were unique, compared to CTG•CAG repeats, since they depended on the DNA secondary structure polymorphism. Our results demonstrate that sticky DNA exists and functions in *E. coli*.

***In vivo* Microarray Screening to Identify Uropathogenic *Escherichia coli* (UPEC) Genes Involved in Early Colonization and Infection in Murine Urinary Tract Infections**

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Uropathogenic *Escherichia coli* (UPEC) is the cause of over 70% of all urinary tract infections (UTIs). To identify genes involved in early colonization and infection of the bladder, we have implemented a screening technique that combines transposon mutagenesis and oligonucleotide microarray analysis. Insertions are generated using a transposon containing divergent T7 promoters and a kanamycin resistance cassette. A set of 2,068 Tn5 UPEC mutants (strain CFT073) have been generated and stored in 96-well format. As an attenuation control, a *fimH* knockout mutant was constructed and placed twice in each 96-well dish. Biotin-labeled RNA transcripts, generated from input (inoculum) and output (recovered) pools, are hybridized to custom designed oligonucleotide microarrays containing probes for both the top and bottom strands of the entire CFT073 genome, to determine insertion locations (gene identification) for individual mutants. Pools of 50 mutants are currently being screened in the murine UTI model where the bladders are harvested 6 hours post-inoculation. We will present a summary of these results and discuss mutant confirmation and follow-up strategies. This work will elucidate CFT073 mutants that are attenuated *in vivo*, and ultimately identify genes that are required for UPEC to colonize and/or survive in the bladder. Once identified, these genes may serve as potential therapeutic targets for treating bladder infections.

Dual Roles of *Bacillus subtilis* Glutamine Synthetase

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Glutamine synthetase (GS) catalyzes the ATP-dependent synthesis of glutamine from glutamate and ammonium. In the low-GC Gram-positive bacterium *Bacillus subtilis*, GS is not only required for the assimilation of ammonium, but it also plays a direct role in regulating gene expression in response to nitrogen availability.

The TnrA transcription factor controls the expression of a large number of *B. subtilis* genes involved in nitrogen metabolism. During nitrogen-limited growth, TnrA is active and can function as either a transcriptional activator or repressor. TnrA is inactive under conditions of nitrogen excess due to a protein-protein interaction with feedback-inhibited GS that prevents TnrA from binding to DNA.

One striking difference between GS from *B. subtilis* and enteric bacteria is that the *B. subtilis* enzyme is subject to feedback inhibition by glutamine. Kinetic experiments have demonstrated that glutamine inhibition is competitive with respect to glutamate. Additional studies have revealed that the binding of glutamine to *B. subtilis* GS only occurs in the presence of phosphate. These results, combined with structural studies of *Salmonella typhimurium* GS, argue that glutamine inhibits GS activity by binding to the active site.

A novel *B. subtilis* mutant with constitutive TnrA activity was isolated. This mutant is unique in that it crossfeeds Gln⁻ cells. Genetic mapping and DNA sequence analysis revealed that this mutant contains an alteration in *glnA*, the structural gene for GS, and replaces residue Ser-186 with phenylalanine. This amino acid residue is located within a β -strand that lines the active site. Enzymatic analysis revealed that the S186F GS is resistant to feedback inhibition. The mutant enzyme is defective in its ability to bind glutamine or inhibit TnrA DNA binding *in vitro*. The isolation and properties of this *glnA* mutant supports the model in which the feedback-inhibited form of GS plays a direct role in regulating TnrA activity *in vivo*.

Memory Formation in *Drosophila*

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Drosophila can be taught an olfactory-avoidance behavioral paradigm. Single-trial, repetitive massed, or repetitive spaced training recruit different signal transduction pathways, resulting in memories that have different durations. Spaced training uniquely activates transcription, including the transcription factor dCREB2, and this cascade of gene expression is needed for long-term memory. This universal requirement for acute gene expression around the time of training is independent of the type of behavior or animal species.

How do neurons strengthen specific synapses when cell-wide processes like transcription and translation occur? The synaptic tag has been hypothesized to mark recently active synapses, and allow them to exclusively utilize macromolecules sent ubiquitously. We are interested in the molecular/cellular properties of the tagging machinery. Our perspective is evolutionary—tagging is a sufficiently complex process, making it unlikely that neurons evolved a molecular solution *de novo*. Instead, they are more likely to re-deploy solutions that other "simpler" cell types have evolved to solve similar cell biological problems. The atypical protein kinase C protein is part of an evolutionarily conserved machinery used to exquisitely mark intracellular location. We will present recent data supporting the role of atypical PKC in the process of tagging, and argue that it is an important player in the process of marking recently active synapses.

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