Instituto Juan March
de Estudios e Investigaciones

94 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Mechanisms of Homologous Recombination and Genetic Rearrangements

Organized by

J. C. Alonso, J. Casadesús, S. Kowalczykowski and S. C. West

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Introduction

J. C. Alonso, J. Casadesús,
S. Kowalczykowski and S. C. West

Cells are constantly facing the challenge of repairing alterations in their genetic material. DNA damage occurs under normal physiological conditions and can be exacerbated by environmental factors. Cells have evolved several distinct mechanisms of DNA repair and DNA-damage tolerance, which help to maintain the structural and informational fidelity of their genome. A large body of evidence supports the idea that, when those mechanisms fail, accumulated genetic changes lead to a number of diseases, including the development of neoplasia.

The process of genetic recombination continues to shape and reshape the genomes of all organisms, thereby enhancing the variation generated by mutations and increasing the polymorphism of natural populations. Recombination is also used widely as a tool, for both genetic analysis of biological processes and engineering of new gene constructs and transgenic organisms. A knowledge of recombination is therefore crucial to many areas of basic biology, biotechnology and medicine. The analysis of molecular reactions involved in recombination has witnessed significant developments in the last decade. A large number of recombination genes and proteins have been identified; in a number of cases, their activities have been associated with specific reactions.

At the cellular level, failure to repair damaged DNA is associated with sensitivity to radiation, increased mutation rates, DNA and chromosomal aberrations, defects in cell division, and reduced viability. At the clinical level, human diseases with known or suspected defects in repair show a range of symptoms, including a high incidence of malignancy, photosensitivity, immunodeficiency, neurological disorders, growth retardation, premature aging, and death. The contributions presented in the workshop highlighted the major role of homologous recombination in the maintenance of the structural and informational fidelity of DNA.

The participants at the workshop represented an international group of prominent investigators with diverse interests, backgrounds, research strategies and viewpoints. This diversity, together with the precise focus of the meeting, permitted a critical evaluation of recent progress in the field. On the other hand, the breadth and depth of the topics presented, the small size of the meeting, the presence of young motivated participants, and the intimate and stimulating atmosphere of the Juan March Institute combined to produce a highly informative workshop with lively and inspiring discussions.

J. C. Alonso, J. Casadesús, S. Kowalczykowski and S. C. West

Session 1

Chair: Nancy Kleckner

Early Steps of Genetic Recombination in Prokaryotes, Archaea, and Eukaryotes.

Stephen Kowalczykowski

Both genetic recombination and the recombination-dependent repair of broken chromosomes are initiated at either double-strand DNA (dsDNA) breaks or dsDNA with ssDNA gaps. In all organisms, the first steps of recombination are the processing of this dsDNA interruption into a substrate suitable for the action of a DNA pairing protein, and the subsequent homologous pairing. Processing is mediated by the combined action of a helicase and nuclease. Though the ssDNA produced by this processing event is in principle a substrate for the DNA strand exchange protein, competitive binding by a single-stranded DNA binding (SSB) protein may preclude assembly of the DNA strand exchange protein on this ssDNA. Thus, common to all recombination systems is a mechanism to ensure assembly of the DNA strand exchange protein in the face of competition from the SSB protein. The functional similarities of this important step in different systems will be discussed.

In *E. coli*, processing is mediated by one of two DNA helicases: either the multifunctional RecBCD helicase/nuclease or the RecQ helicase. The RecBCD enzyme binds to a dsDNA break. It then degrades the strand 3'-terminal at the entry until a site is encountered, whereupon its degradation ceases, the polarity of strand degradation switches, and a weaker degradation of the 5'-terminal strand commences. Importantly, the translocating -activated RecBCD enzyme then directs the loading of RecA protein onto the -containing ssDNA to the exclusion of SSB protein, ensuring the success of this strand in the subsequent invasion of a supercoiled homologue.

A second pathway of homologous recombination in *E. coli* is the *RecF*-pathway. Initiation is mediated by the RecQ helicase, in concert with the RecJ nuclease. Though the RecQ helicase can effectively process dsDNA into a substrate for RecA protein, it does not load RecA protein onto ssDNA. Instead, the RecOR proteins provide the functional equivalent of RecA protein-loading by facilitating the displacement of SSB protein.

In the eukaryote, *S. cerevisiae*, the recombinational repair of broken dsDNA requires coordination of the proteins defined by the *RAD52*-epistasis group, as well as others such as the replication protein-A (RPA). The DNA strand exchange protein is the Rad51 protein. However, much like in the prokaryotic system, DNA strand exchange by Rad51 protein is blocked by prior binding of RPA to ssDNA. This inhibitory effect of RPA is alleviated by an analog of the RecOR proteins, the Rad52 protein. Rad52 protein associates with the RPA-ssDNA complex and recruits Rad51 protein to this complex, resulting in the subsequent rapid assembly of a presynaptic complex. Thus, efficient homologous pairing is ensured.

In the archaeon, *Sulfolobus solfataricus*, the DNA strand exchange protein is RadA protein and the ssDNA binding protein is related to RPA. In several ways, the behavior of the archaeal proteins parallels that of the their eukaryotic counterparts.

Further parallels and distinctions between prokaryotic, archaeal, phage, and eukaryotic systems will be developed.

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Allostery and the control of the activity of recombination proteins. Oleg Voloshin, Vlad Malkov, Ning Ma, Igor Panyutin*, Victor Zhurkin*, Peter Romanienko and R. Daniel Camerini-Otero, Genetics and Biochemistry Branch, NIDDK, *Clinical Center and *Laboratory of Experimental and Computational Biology, NCI, National Institutes of Health, Bethesda, MD 20892-1810.

A fundamental problem in homologous recombination is how the search for homology between the two DNAs is carried out. In all current models a homologous recombination protein, such as the prototypical *E. coli* RecA protein, loads onto a single-strand DNA generated from one duplex DNA and scans another duplex to form a synaptic (pairing) complex. Eventually, DNA strands are exchanged and a new heteroduplex is formed. A novel technique developed by us in collaboration with Igor Panyutin of the Clinical Center, radioprobing of nucleoprotein structures with iodine-125, has allowed us to trace the spatial arrangement of the three DNA strands in the RecA protein mediated synaptic complex. The synaptic complex represents a poststrand exchange late intermediate in which the heteroduplex is located in the center and the outgoing strand forms a relatively wide and mobile helix intertwined with the heteroduplex. The structure implies that homology is recognized in the major groove of the duplex by the two extended DNAs. Efforts are underway to trap and characterize earlier intermediates in the pairing reaction.

In order to understand the mechanism and structures involved in greater detail we have endeavored to miniaturize the reaction. In the past, we have shown that short oligonucleotides can be used as the substrates (Ferrin, and Camerini-Otero (1991), Science 254, 1494; Hsieh *et al.* (1992), PNAS USA 89, 6492). Recently, we have determined that a 20 amino acid peptide that includes loop L2 of RecA can promote the key reaction of the whole RecA protein: pairing (targeting) of a single stranded DNA to its homologous site on a duplex DNA (Voloshin *et al.* (1996), Science 272, 868). In the course of the reaction the peptide binds to both substrate DNAs, unstacks the single-stranded DNA, assumes a beta structure and self-assembles into a filamentous structure like RecA (Wang, L. *et al.* (1998), J. Mol. Biol. 277, 1). It is possible that two DNAs align and pair on an extended beta-sheet of L2s in the whole RecA. In order to understand the function of L2 we have generated by site-directed mutagenesis all possible point mutants of residues 193-212 in the whole RecA protein (380 mutants). The *in vivo* phenotype of these mutants with respect to recombination and UV and mitomycin resistance was determined (Hörtnagel *et al.* (1999), J. Mol. Biol. 286, 1097). An analysis of these results suggested that L2 may be involved in most aspects of RecA function. For example, as RecA is an ATP-dependent DNA binding protein and a DNA-dependent ATPase, we asked whether the loop might be directly involved in these allosteric interactions. We have been able to show that ATP, but not ADP, interacts with the arginine (Arg196) within L2 peptides and that this interaction induces the active beta-structure conformation of the peptides. Experiments with mutant RecA proteins indicate that Arg196 binds to both DNA and the gamma-phosphate of ATP and is essential for the cooperativity between DNA and ATP binding. We suggest a mechanism for ATP hydrolysis by RecA that is similar to those proposed for heterotrimeric G proteins. The

role of DNA in the stimulation of the ATPase activity of RecA is similar to the role of the recently described RGS (Regulators of G protein Signaling) proteins in activating the GTPase of heterotrimeric G proteins and consists in stabilizing the highly mobile region involved in hydrolysis. Thus, other biopolymers in addition to proteins, such as DNA, can act to stimulate nucleotide hydrolysis by similar stereochemical regulatory mechanisms. Finally, we are investigating whether others domains of RecA are interacting with L2 and are responsible for regulating (posifively or negatively) the efficiency of some of the biochemical activities of this loop.

While homologous pairing and strand exchange are the earliest contacts between two parental DNAs mediated by RecA and its eukaryotic homologues, Rad51 and Dmc1, homologous recombination is initiated by DNA double-strand breaks (DSBs). The protein that catalyzes DSB formation in meiosis in the budding yeast, *Saccharomyces cerevisae*, is the product of the *SPO11* gene. Disruption of this gene results in meiotic arrest, spore lethality and a lack of meiotic recombination. Spo11 homologues have been identified in other eukaryotes and archaebacteria resulting in the identification of a new family of proteins related to DNA topoisomerase IIs. We have identified and cloned Spo11 homologues in *D. melanogaster*, mouse and man. In order to study structure –function relationships, we constructed chimeras of yeast and fly spo11 to see whether they could rescue the Δspo11 spore-lethal meiotic defect seen in *S. cerevisiae*. In addition, in collaboration with Brian Oliver (LCDB in NIDDK) we have generated a number of transgenic flies to study the role of DmSpo11 may play in the recombination-less meiosis seen in male flies.

Recombinational DNA repair: Coordinated action of the RecA, RecF, RecO, and RecR proteins. Michael M. Cox, Qun Shan, Kerry MacFarland, Julie Bork, Tanya Arenson, and Ross B. Inman, Department of Biochemistry, University of Wisconsin, 433 Babcock Drive, Madison, Wisconsin 53706 USA

The major function of homologous genetic recombination in *E. coli* is the repair of stalled replication forks. When replication forks stall at a DNA lesion, the repair that ensues requires the deposition of a RecA protein filament in the resulting DNA gap.

The mechanism of RecA protein-promoted DNA strand exchange involves at least three phases. First, a RecA filament is assembled on ssDNA or a gapped duplex. Second, there is a DNA pairing phase in which a single strand is aligned with a homologous duplex within the RecA filament groove. ATP hydrolysis is not required for this phase, which can routinely yield hybrid duplexes of 1 kbp or more (2-5, 8). These nascent hybrid DNA regions can be extended in a third phase that relies on ATP hydrolysis. In the second phase, RecA protein exhibits properties consistent with a motor function that is coupled to unidirectional extension of the hybrid duplex. Many different strands of investigation lead to this picture, including an examination of filament state at various reaction stages, characterization of the strand exchange reaction observed in the absence of ATP hydrolysis, and the direct testing of predictions derived from molecular models.

We have been characterizing the filament assembly and disassembly processes. Following nucleation, RecA filaments assemble in the 5' to 3' direction, growing at one end. These filaments also disassemble in the 5' to 3' directions, with RecA dissociating from the end opposite to that at which assembly occurs (6). End-dependent disassembly requires ATP hydrolysis, but does not occur when dATP is substituted for ATP. End-dependent disassembly occurs at a maximum rate of 60-70 monomers per filament end per minute (1).

RecA filaments on ssDNA hydrolyze ATP with a k_{cat} of about 30 min^{-1}. ATP is hydrolyzed on all RecA monomers uniformly through these filaments. Although DNA binding is cooperative, the ATP hydrolytic cycles of adjacent monomers are largely uncoupled. Some exchange of RecA monomers into and out of such filaments is observed, although this is suppressed entirely if dATP replaces ATP (7). In contrast, RecA filaments on dsDNA hydrolyze ATP with a k_{cat} of about 20 min^{-1}, and the ATP hydrolytic cycles of adjacent monomers are tightly coupled so that ATP hydrolysis proceeds unidirectionally through the filament in coordinated waves. The rate of RecA monomer exchange into and out of the filaments is increased (7).

RecA filament assembly and disassembly is modulated by the RecF, RecO, and RecR proteins. The RecO and RecR proteins act as a complex to facilitate the nucleation of RecA filaments on SSB-bound ssDNA (9). The RecOR complex also prevents end-dependent disassembly of RecA filaments from ssDNA (6). The RecR protein (and probably the RecO protein as well) remains associated with the RecA filament. The RecOR complexes do not simply facilitate repeated nucleation of filaments, but remain stably bound and prevent disassembly. The RecF and RecR proteins form an alternative complex that binds randomly to dsDNA (10). The extension of RecA filaments is attenuated when it encounters one of these complexes (11). The RecO and RecF proteins demonstrably compete for RecR protein binding. Together, the RecF,O, and R proteins act to constrain RecA filaments to DNA gaps.

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Mechanisms of genetic recombination in *Bacillus subtilis* 168: The RecN protein shows an endo-exonuclease activity

Juan C. Alonso[1], Rosario Sabariegos[1], Silvia Ayora[1], and Rudi Lurz[2].
Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain, and [2]Max-Planck-Institut für molekulare Genetik, Ihnestrasse 73, D-14195 Berlin, Germany

On the basis of their phenotype and on shared indirect suppressors, the Rec⁻ strains, other than *rec*A, were classified within five different epistatic groups (α, β, γ, ε and ζ groups). A mutation in the *rec*N gene, when present in an otherwise Rec⁺ *B. subtilis* strain, renders cells sensitive to the DNA damaging agent 4NQO, but it does not affect genetic exchange. The *rec*N (α) mutation increases the sensitivity of *add*A5 *add*B72 (β) and *rec*H (γ) cells to DNA damaging agents, but does affect the DNA repair capacity of *rec*U (ε) cells. The *rec*N mutation blocks genetic recombination of *rec*H cells and affects recombination in *add*A5 *add*B72 and *rec*U cells. The *rec*N mutation neither affects the DNA repair nor the recombinational capacity of *rec*F (α) cells (1, 2). These data indicate that the *rec*N gene product is required for DNA repair and homologous DNA recombination and that the *rec*F, *add*AB, *rec*H and *rec*U genes provide overlapping activities that compensate for the effect of single mutations proficiency.

The RecN protein (predicted molecular mass 64.4 kDa) was purified toward homogeneity. The RecN protein, which has a molecular mass of ~ 500 kDa, co-purified with short ssDNA segments. The RecN protein possesses a weak DNA-dependent ATPase and an endonuclease activity. RecN is an ATP-independent sugar-nonspecific nicking enzyme that produces 3'-hydroxyl and 5'-monophosphate termini. RecN nicks supercoiled DNA to yield at first relaxed circles and then linear DNA. The linear DNA is subsequently hydrolysed with a weak sequence specificity (5'-$^{G}/_{Y}C/_{G}CG\neg C/_{T}G/_{Y}C/_{R}C/_{T}A/_{T}$) to yield DNA fragments that differ from each other by ~ 20-nt, and 5'-phosphotetranucleotides, in dsDNA, and 5'-phosphodinucleotides, in ssDNA, as end-products. Linear DNA smaller in size of ~ 500-bp are poor RecN substrates. RecN degrades ssDNA distributively and nicks dsDNA processively. The ssDNA nicks generated by RecN may be relevant to reactions that occur during SOS induction. We propose that the RecN nicking activity processes a duplex circular DNA to circular or linear ssDNA that could be utilised in DNA strand exchange.

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The helicity of DNA in the region of homologous pairing.

Konstantin Kiianitsa and Andrzej Stasiak

Laboratoire d'Analyse Ultrastructurale, Université de Lausanne, CH-1015 Lausanne-Dorigny. Switzerland

The RecA protein of E.coli and its structural and functional homologues in eucaryotic organisms are known to initiate the process of homologous recombination by forming helical filaments on single stranded DNA. These filaments then progressively bind homologous double-stranded DNA molecules so that homologous regions of single- and double-stranded DNA molecules become aligned in register while presumably winding around common axis. Earlier structural studies of RecA-dsDNA complexes revealed the double-stranded DNA in such complexes is unwound from 10 to about 18 base pairs per turn *(1,2)*. Electron microscopy studies of RecA-ssDNA complexes showed that single stranded DNA in these complexes seems to adopt a conformation with ca. 18 nucleotides per turn *(3)*. However the question remained - what is the helicity of double stranded DNA when it interacts with homologous ssDNA within RecA synaptic filaments?

To answer this question we devised a topological assay permitting measurement of dsDNA unwinding upon pairing with RecA covered single-stranded oligonucleotides of different size. Obtained results demonstrate that unwinding of duplex DNA is homology dependent and is consistent with a previous model postulating that all three DNA strands aligned within one RecA filament have the same helicity of ca 18-19 base pairs *(4)*. Additional implications of our finding for the molecular mechanism of homologous recombination will be discussed as presented in our recent publication(5).

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Session 2

Chair: Miroslav Radman

New insights into the link between recombination and DNA replication from mutations affecting RNA polymerase activity.

Robert G. Lloyd, Institute of Genetics, University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH (e-mail: bob.lloyd@nottingham.ac.uk)

The genome of an organism has to be duplicated once per cell cycle and accurately so as to preserve genetic integrity. Errors are made, and these fuel evolution, but they are kept in check by repair systems that detect and eliminate damage. Recombination is a vital component of the cell's armoury against damage, as is evident from the reduced viability, increased genetic instability and cancer predisposition of mutants defective in this process. It is becoming increasingly clear that recombination proteins are instrumental in maintaining DNA replication in times of stress by acting to preserve the structure of a replication fork stalled at a lesion in the template DNA, or to re-establish a fork following its collapse. Our understanding of the links between recombination and DNA replication, and of the lesions that block replication fork progression, stems mainly from studies of the *Escherichia coli* RuvABC proteins, which coordinate branch migration and resolution of Holliday intermediates, RecG, which drives branch migration of Holliday junctions and other branched DNA molecules, and PriA, which acts to load the replicative helicase DnaB at branched DNA structures (1 - 6).

Studies of genetic recombination and genome stability in bacteria in general, and in *E. coli* in particular, have focused on systems that operate during exponential growth under laboratory conditions. In nature, bacteria live a life of feast or famine and show a remarkable ability to adapt to changing circumstances by eliciting regulated responses that switch some genes on and others off. This adaptability depends on critical interactions between RNA polymerase and the signalling molecule, (p)ppGpp, the mediator of the stringent response to starvation synthesised by the RelA and SpoT proteins (7). I have found that increasing the cellular level of (p)ppGpp enables *E. coli* cells to promote recombination and DNA repair in the absence of the RuvABC proteins normally required to process Holliday intermediates formed by RecA. Thus, introducing the *spoT1* mutation, which reduces the (p)ppGpp 3' pyrophospho-hydrolase activity of the SpoT protein, into a Δ*ruvAC* strain dramatically increases resistance to UV light. It also makes recombination substantially more efficient. In contrast, the *ruv* phenotype is enhanced by reducing (p)ppGpp levels through inactivation of the RelA protein, and becomes very severe when (p)ppGpp is reduced to zero by eliminating both RelA and SpoT. (p)ppGpp modulates the promoter affinity of RNA polymerase and in its absence certain aminoacid biosynthetic operons and other genes cannot be expressed. Gene expression, and prototrophy, can be restored by mutations in RNA polymerase subunits that circumvent the need for (p)ppGpp. Certain specific mutations (*rpo**) mimic the effect of *spoT1* in that they also suppress the UV-sensitivity of *ruv* mutants.

The suppression of *ruv* by *spoT1* and *rpo** raises the possibility that one manifestation of the stringent response to starvation is the induction of DNA repair activities that circumvent the need

for Holliday junction processing by the RuvABC proteins. We are investigating this possibility. An alternative explanation is that a transcribing RNA polymerase molecule stalls at a lesion in the template DNA and prevents progression of the DNA replication fork. If the stalled fork collapses, recombination proteins, and Holliday junction processing, then become necessary to re-establish a fork. Replication resumes when RNA polymerase and the offending lesion have been cleared from the template. In this case, suppression of ruv is explained if RNA-polymerase interactions with the (damaged) template DNA are destabilised by increasing the level of (p)ppGpp (*spoT1*) or, in the absence of (p)ppGpp, by compensating mutations (*rpo**) that effect RNA polymerase directly. Either would reduce the number of roadblocks to replication fork progression and the risk of fork collapse, thus obviating the need for RuvABC. Evidence supporting this model will be presented. Suppression of *ruv* by *spoT1* and *rpo** mutations depends critically on RecG, a structure-specific DNA helicase that acts on recombination intermediates and R-loops, and which helps to link recombination to DNA replication by countering a potentially detrimental effect of PriA helicase activity (4, 5, 8). It is significant that RecG is encoded in the same operon as SpoT and other proteins associated with RNA metabolism. A possible model for RecG in maintaining the integrity of replication forks that encounter stalled RNA polymerase molecules on the DNA template is currently under investigation.

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Recognition and manipulation of DNA structure by junction-resolving enzymes

David M.J.Lilley *CRC Nucleic Acid Structure Research Group, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, United Kingdom. dmjlilley@bad.dundee.ac.uk*

Structure-selective DNA-protein interactions lie at the heart of the enzymology of DNA repair and recombination. In homologous genetic recombination, the penultimate step requires resolution of a branched four-way junction (Holliday) intermediate, and the manner of this determines the genetic outcome of the process. Enzymes that recognise and resolve four-way DNA junctions have been isolated from eubacteria and their phages, yeasts, and mammalian cells and their viruses, and are probably ubiquitous proteins.

Junction-resolving enzymes cleave DNA junctions with great structural selectivity. They are basic proteins that bind to four-way junctions with K_D in the 1-50 nM range, but their affinity for duplex DNA is typically 1000-fold lower. However, they display a range of sequence preferences at the level of DNA cleavage. While T4 endonuclease VII and T7 endonuclease I exhibit relatively low sequence selectivity, RuvC of *E. coli*, CCE1 of *S. cerevisiae* and YDC2 of *S. pombe* all have well-defined sequence preferences. All the enzymes studied have a number of critical acidic amino acid required for cleavage activity. This suggests that these residues coordinate a metal ion (s) that provides the hydrolytic water molecule required for phosphodiester bond cleavage. In the case of T4 endonuclease VII a number of the critical amino acids are displayed on a loop that is part of a 39 amino acid zinc-binding motif.

All the junction-resolving enzymes bind to DNA junctions in dimeric form, consistent with the paired cleavages required to produce a well-ordered resolution event. In the cases of T4 endonuclease VII and T7 endonuclease I the enzymes generate bilateral cleavage within the lifetime of the enzyme-junction complex. However, bilateral cleavage is not essential, because an active/inactive heterodimer of endonuclease VII induces unilateral cleavage in a four-way junction. The junction-resolving enzymes exist in dimeric form in free solution, but with variable rates of subunit exchange from < 1 min for T4 endonuclease VII to > 16 hr for T7 endonuclease I.

While the junction-resolving enzymes recognise the structure of branched DNA species, they also manipulate that structure. All the enzymes studied alter the global conformation of the junction, but in each case a different structure is generated. The most extreme example is that of yeast CCE1, which opens the structure into the unstacked square geometry normally adopted by four-way junctions in the absence of added metal ions, with opening of the central basepairs. The simultaneous recognition and manipulation of DNA structure provides an interesting challenge to understand both the mechanism and underlying significance within the overall context of the mechanism of the recombination process.

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Undoing the damage of homologous recombination by Xer site-specific recombination. *David Sherratt, Lidia Arciszewska, Bernard Hallet, Gavin Recchia, Mira Aroyo, François Cornet, Garry Blakely and Sean Colloms Division of Molecular Genetics, Dept of Biochemistry, University of Oxford, South Parks Rd., Oxford OX1 3QU, UK*

Homologous recombination events between circular chromosomes can generate dimeric chromosomes that will be unable to segregate to each of the daughter cells at cell division. Xer site-specific recombination functions in the stable inheritance of circular bacterial chromosomes and plasmids by converting such dimers to monomers. In the *Escherichia coli* chromosome, a recombination site, *dif*, located in the replication termination region, is acted on by two related recombinases, EcoXerC and EcoXerD. Recombination *in vivo* at *dif* is limited to cells that contain chromosome dimers and which can initiate cell division and localise a functional FtsK protein to the cell division septum. The C-terminal domain of FtsK is required for recombination at *dif*, irrespective of whether *dif* is chromosomally or plasmid located. It appears that a dimer-dependent and cell division-dependent checkpoint leads to activation of the Xer recombination process either by generating an appropriate DNA substrate or by activation of the recombination proteins. We propose that XerC strand exchange generates Holliday junction-containing recombination intermediates that are acted on directly by FtsK in order that they adopt a configuration suitable for XerD strand exchange. *In vitro*, supercoiled *dif* is not a substrate for EcoXerC and EcoXerD, though modification of the recombination proteins or the recombination site can lead to recombination proficiency. Under conditions that support Xer recombination at supercoiled *dif* or plasmid *psi*, XerC strand exchange leads to the formation of a Holliday junction containing intermediate, which XerD converts to product. Structure determinations along with genetic studies with recombinase mutants that either enhance or impair the catalytic activity of their partner recombinase provide a molecular basis for understanding how each recombinase reciprocally controls the activity of its partner recombinase. These studies highlight the central role of subtle changes in conformation of the Holliday junction intermediate in controlling recombination outcome during recombination at chromosomal *dif* and *psi*.

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V(D)J RECOMBINATION: LINKS TO TRANSPOSITION AND DOUBLE-STRAND BREAK REPAIR

Martin Gellert, Meni Melek, Tanya T. Paull, Mauro Modesti, Joanne E. Hesse, Dale A. Ramsden, and Kevin Hiom. Laboratory of Molecular Biology, NIDDK, National Institutes of Health, Bethesda, Maryland 20892-0540, U.S.A.

V(D)J recombination is initiated by the RAG1 and RAG2 proteins, which make double-strand breaks between the recombination signal sequences (RSS) and the neighboring coding DNA. In normal recombination these broken ends are then joined to make coding joints and signal joints. We have recently demonstrated that the purified RAG proteins are also capable of transposing RSS-ended fragments into new DNA sites (Hiom, K., Melek, M., and Gellert, M. (1998), Cell 94, 463-470). This transpositional recombination is very similar to the reactions of better-known transposons or retroviruses. The RSS ends are inserted with a 5-bp stagger into essentially random sites in the target DNA. The existence of RAG-mediated transposition strongly supports earlier proposals that the V(D)J recombination system evolved from an ancient mobile DNA element, and suggests that repeated transposition may have promoted the expansion of the antigen receptor loci. In present-day lymphoid cells, the occasional diversion of V(D)J rearrangement into a transpositional pathway may cause some of the DNA translocations associated with lymphatic tumors.

The joining steps of V(D)J recombination require several factors that are also used in repair of radiation-damaged DNA (for example DNA-PK$_{CS}$, Ku protein, Xrcc4 and DNA ligase IV). We have found novel activities of some of these proteins.

1) In addition to its known function as a cofactor of DNA-PK$_{CS}$, Ku protein can greatly stimulate ligation of blunt or near-blunt DNA ends by mammalian DNA ligases (Ramsden, D. A., and Gellert, M. (1998), EMBO J. 17, 609-614). This may correlate with biological results showing that Ku-deficient cells have a different phenotype from those lacking DNA-PK$_{CS}$.

2) The Xrcc4 protein is known to bind to DNA ligase IV and to stimulate its activity. The radiation-sensitivity of Xrcc4-deficient cells, and their failure to join broken ends in V(D)J recombination, have been attributed to this defect. We find that Xrcc4 protein also binds to DNA, and that its biological activity correlates better with DNA binding than with its effect on ligase IV.

3) In yeast, the Mre11/Rad50/Xrs2 complex is known to be important for non-homologous DNA end-joining as well as meiotic recombination. Human homologs of Mre11 and Rad50 have been cloned, and the human Nbs1 protein is thought to be the homolog of Xrs2. We found that Mre11 is a 3′ to 5′ exonuclease, more active when associated with Rad50. Mre11 also has an endonuclease activity on single-stranded DNA (Paull, T. T., and Gellert, M. (1998), Mol. Cell 1, 969-979). In the triple complex, new activities are revealed. There is now a limited ATP-dependent DNA unwinding, an ATP-dependent incision to remove 3′-overhanging tails, and an efficient cutting of perfect hairpins. These activities suggest explanations for several biological functions of the complex.

E. Lorbach, N. Christ & P. Dröge

Conservative site-specific DNA recombination: From mechanism to application.

Gene therapy can be defined as the introduction of nucleic acids into cells for the purpose of altering the course of a medical condition or disease. A number of difficult technical hurdles needs to be overcome to achieve this goal, however. For example, foreign DNA has to be introduced into a human cell without degradation or modification. Ideally, the introduced DNA should be safely and stably integrated into a defined, well characterized locus of the genome of the recipient human cell. The efficient expression of gene products encoded by the integrated DNA is often another problem in potential gene therapy approaches. Here, we have addressed the problem of site-specific integration of foreign DNA into the human genome.

The bacteriophage λ encodes a site-specific recombinase, the so-called integrase (Int), that catalyzes the integration of the phage DNA via its attachment site (attP) into a unique locus (attB) of the bacterial chromosome. We have identified a natural target site for the Int system in the human genome, and present evidence that this site can be targeted by Int in a human Burkitt Lymphoma cell line (BL 60) *in vivo*.

E. Lorbach, N. Christ & P. Dröge. Institute of Genetics, University of Cologne, Weyertal 121, D-50931 Cologne, Germany.

Session 3

Chair: John Roth

Initiation of meiotic recombination and minisatellite instability in the yeast *Saccharomyces cerevisiae*

F. Baudat, J. Buard*, H. Debrauwere, B. de Massy, C. Mézard, Y. Okada, A. Pecina, K. Smith, C. Soustelle, P-C. Varoutas, M. Vedel, G. Vergnaud* and A. Nicolas. Institut Curie, Section de Recherche, CNRS UMR144, 26 rue d'Ulm, 75248 Paris, France. * IGM, CNRS URA2225, Bât 400, Université Paris-Sud, 91405 Orsay, France.

Genetic and physical studies in the yeast *S. cerevisiae* have shown that meiotic recombination (reviewed in ref. 1) is initiated by the formation of transient DNA double-strand breaks (DSB) throughout the genome. The DSB regions, mostly located in intergenic promoter-containing intervals, are clustered in large (39-105 kb) chromosomal domains (2). The nuclease responsible for these meiotic DSBs is the Spo11 protein (3,4). Current understanding of the control of meiotic DSB formation and the role of chromatin accessibility (5) will be summarized.

Minisatellites are tandemly repeated DNA sequences found in eukaryote genomes, particularly in mammalian genomes. In humans, the minisatellites are highly polymorphic and some, like CEB1, rearrange at a high frequency in the germline. To investigate possible mechanism(s) of rearrangement in a model system, we have integrated two CEB1 alleles (0.6 and 1.8 kb) into the *Saccharomyces cerevisiae* genome and have characterize their behavior (Debrauwere *et al.*, submitted). As observed in human cells, we found that CEB1 is destabilized in meiosis resulting in a large variety of gain or loss of repeat units. Genetical and physical analyses demonstrate that rearrangements of CEB1 result from the formation of nearby Spo11-dependent DSBs indicating that the meiotic instability of minisatellites in yeast depends on the initiation of homologous recombination. MVR-PCR analysis of homozygous and heterozygous diploids (0.6 x 1.8 kb) reveals that intra- and interallelic events leading to the gain or loss of repeat units take place, as has been observed in human rearrangements. Some events are complex with simultaneous deletions and duplications. The Msh2 Pms1 mismatch repair functions play a role in the maturation of recombination intermediates. Molecular mechanisms of minisatellite rearrangements in yeast meiosis will be discussed.

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Genetic control of DNA repeat recombination in yeast: effects of transcription and DNA repair mutations.

F. Malagón, S. Chávez, J.I. Piruat, S. González-Barrera and A. Aguilera
Departamento de Genética, Universidad de Sevilla, 41012 SEVILLA, Spain.

DNA recombination may be directly affected by other aspects of DNA metabolism such as replication and repair. A relationship particularly intriguing is that observed between recombination and transcription. Induction of recombination by transcription has been documented in prokaryotes and eukaryotes (1,2,3). It is likely that changes in local supercoiling or in chromatin structure associated with transcription may facilitate the initiation of a recombination event, although there may be other explanations. Our aim is to define and to understand the molecular nature of the effects that transcription, and hence chromatin structure, may have on mitotic recombination in yeast.

We have recently provided genetic and molecular evidence that recombination between direct repeats can be induced by transcriptional elongation impairment. Null mutations of the *HPR1* and *THO2* genes lead to an strong increase in the frequency of deletions occurring by recombination between direct repeats (>2000-fold above wild-type levels) that correlates with an impairment of transcription elongation observed in such mutants (4,5,6). The higher the impairment of transcription caused by *hpr1Δ* and *tho2Δ*, the higher the frequency of deletions. The hyper-deletion phenotype is transcription-dependent and is not observed in DNA repeats that are not transcribed. We have now evidence that reciprocal recombination is also stimulated by these mutations. The *tho2Δ* mutation increases recombination between inverted repeats above 20-fold the wild-type levels. This result suggests that transcriptional elongation impairment may induce all types of recombination, including single-strand annealing or one-ended invasion, responsible for deletions, and reciprocal recombination.

To explore whether chromatin structure might have an effect on mitotic recombination that could explain the observed effects of transcription, we have analyzed recombination of different mutants related to chromatin structure and transcription. We have observed that mutations in *SPT2*, *SPT4* and *SPT6* confer increases in the frequency of reciprocal exchange/gene conversion and deletions ranging from 1- to 15-fold above wild-type levels. Interestingly, *SPT4*, *SPT5* and *SPT6* have recently been involved in transcriptional elongation (7), presumably through their effect on chromatin structure, opening the possibility that *hpr1*, *tho2* and the *spt* mutations might increase recombination by a similar mechanism.

We have investigated the genetic and molecular basis of the increase of reciprocal exchange between inverted repeats caused by *spt6-140*. In the

inverted repeat systems that we used for our recombination assays, recombination is reduced by 4-10-fold in the *rad51*, *rad54*, *rad55* and *rad57* mutants, leading to a pattern of gene conversion and reciprocal exchange significantly different from wild type. Interestingly, *spt6-140* suppresses the Rec- phenotype of *rad51Δ* and the other *rad* mutations mentioned above, and restores the wild-type pattern of gene conversion and reciprocal exchange. These results suggest that recombination is stimulated at the level of initiation, regardless of whether it proceeds through a *RAD51*-dependent or -independent recombination pathway. We are studying whether this increase in initiation observed in the chromatin-related mutants is dependent on the transcriptional state of the inverted repeats.

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Meiotic Recombination and Chromosome Structure Nancy Kleckner
Harvard University, Cambridge, MA USA

Meiotic recombination in yeast is initiated primarily or exclusively by programmed double strand breaks (DSBs), which proceed on to strand exchange intermediates (double Holliday junctions) and thence to products (crossovers and noncrossovers). These events are subject to diverse types of regulation which ensure that they occur (a) in temporal coordination with other events of chromosomal, nuclear and cellular metabolism, (b) in spatial coordination with the structural axes of the homologs, (c) with a bias towards events between homologous nonsister chromatids ("interhomolog events") relative to intersister events, and (d) in such a way as to yield a tightly regulated distribution of crossover products between and along the chromosomes. Prior to and independent of recombination initiation, chemically intact homologs engage in recognition and pairing. In yeast, such pairing is present prior to the onset of meiosis (in vegetatively growing diploid cells), is disrupted during S-phase, and is then restored in very early prophase. These events are likely presaged by significant changes in meiotic DNA replication. Our current work in these areas will be discussed in the context of the general proposition that, throughout the meiotic process, development and modulation of meiotic interhomolog interactions is functionally and molecularly related to development and modulation of intersister interactions.

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DOUBLE-STRAND BREAK REPAIR IN HUMAN CELLS.

Stephen C. West, Peter Baumann, Fiona Benson, Claire Chappell, Adelina Davies, Eric Van Dyck, Alain van Gool, Jean-Yves Masson, Michael McIlwraith and Andrzej Stasiak*. Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts. EN6 3LD, U.K. and *Laboratory of Ultrastructural Analysis, University of Lausanne, 1015 Lausanne, Switzerland.

Double-strand breaks in DNA (DSBs) are induced by the harmful effects of ionizing radiation. These chromosomal breaks can be lethal to the cell unless repaired efficiently, and inefficient or inappropriate repair can lead to mutation, gene translocation and cancer. In lower eukaryotes, such as yeast, DSBs are primarily repaired by Rad52-dependent homologous recombination. In contrast, vertebrates repair most DSBs by Ku-dependent non-homologous end-joining (NHEJ). Using cell-free extracts prepared from human lymphoblastoid cell lines, an *in vitro* system for DNA end-joining has been developed (1). Intermolecular ligation is accurate, dependent upon DNA ligase IV/Xrcc4, and requires Ku70, Ku86 and DNA-PK$_{cs}$, the three subunits of the DNA-activated protein kinase DNA-PK.

Mammalian cells can also repair DSB's by homologous recombination (either by Rad52-dependent single-strand annealing, or by Rad51/Rad52-dependent strand invasion mechanisms). Human Rad51 protein is a structural and functional homolog of the *E. coli* RecA protein and promotes homologous pairing and strand transfer reactions in vitro. Until recently, however, little was known about Rad52. Biochemical studies now show that Rad52 forms ring structures on DNA and can stimulate Rad51-mediated pairing reactions (2, 3). Rad52 also promotes specific DNA-DNA interactions in the absence of hRad51, consistent with a direct role in single-strand annealing (SSA) pathways of recombination.

Using electron microscopy, we found that human Rad52 protein, like Ku, binds directly to DSBs, protects them from exonuclease attack and facilitates end-to-end interactions (4). These observations lead us to propose a model for DSBR in which either Ku or Rad52 binds the DSB. Ku directs DSBs into the NHEJ repair pathway, whereas Rad52 initiates repair by homologous recombination. Ku and Rad52, therefore, direct entry into alternative pathways for the repair of DNA breaks.

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hRAD51 enhances gene targeting in human cells

Rafael J. Yáñez and A. C. G. Porter
Gene Targeting Group, MRC Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital, DuCane Road, London W12 0NN, UK.

Gene targeting, the directed modification of chromosomal sequences with exogenous DNA constructs, has greatly facilitated genetic analysis in eukaryotic organisms. Gene targeting has also been applied to somatic cell genetics and could be used to repair DNA mutations causing genetic diseases. However, these latter applications require increased efficiencies in the targeting reaction to be of widespread use. The cellular machinery for homologous recombination seems to mediate gene targeting, and we are using that as a means to further our knowledge about recombination in human cells and to increase the frequency of targeting.

Genetic analysis in yeast and higher eukaryotes has shown that several members of the *RAD52* epistasis group of genes mediate homologous recombination. One of these genes, *RAD51*, is essential for cell proliferation in higher eukaryotes, and RAD51 protein is a key player in homologous recombination *in vitro*. Therefore, we sought to study the role of *hRAD51 in vivo* by overexpression in human cells. Stable transfection of a *hRAD51* transgene led to a 3-fold increase in RAD51 levels with no obvious deleterious effect. Further studies showed that gene targeting is increased 2 to 3-fold at two different loci in cells overexpressing *hRAD51*. We have also observed enhanced survival of *hRAD51*-overexpressing cells upon gamma irradiation, presumably reflecting increased repair of double-strand breaks by homologous recombination.

Our studies show that *hRAD51* is involved in homologous recombination in human cells *in vivo*, specifically in gene targeting and survival to irradiation. They also indicate that the frequency of gene targeting can be improved by overexpression of a gene involved in homologous recombination. Given that other recombination genes have recently been characterised, it is possible that further improvements in gene targeting frequencies could be obtained by using *hRAD51* in combination with additional genes.

Session 4

Chair: David J. Sherratt

Role of mouse Rad54 in DNA recombination and repair

Raoul Tan, Jeroen Essers, Sigrid M. A. Swagemakers, Jan de Wit, Jan H. J. Hoeijmakers, and Roland Kanaar

Cell Biology & Genetics, Erasmus University Rotterdam, The Netherlands

Error-free repair of ionizing radiation (IR)-induced DNA double-strand breaks by homologous recombination requires the RAD52 group proteins, including Rad51 and Rad54, in the yeast Saccharomyces cerevisiae. A key step in recombination, formation of a joint molecule between the damaged DNA and the homologous repair template, is mediated by Rad51 and stimulated by Rad54. The biological importance of the RAD52 pathway for genome stability is underscored by its conservation from fungi to humans.

We have analyzed the phenotype associated with disruption of the mouse RAD54 (mRAD54) gene. mRAD54 knockout embryonic stem cells are sensitive to ionizing radiation, mitomycin C and methyl methanesulfonate, but not to UV light. In addition, gene targeting experiments demonstrate that the frequency of homologous recombination in mRAD54 knockout cells is reduced compared to wild-type cells. These results imply that, besides DNA end-joining mediated by DNA-dependent protein kinase, homologous recombination contributes to repair of DNA double-strand breaks in mammalian cells.

Immunofluorescence experiments show that the mRad54 protein forms nuclear foci upon treatment of the cells with ionizing radiation. Interestingly, upon irradiation mRad54 partly colocalizes with mRad51. Immunopreciptation experiments indicate that the two proteins form a stable complex, but only upon induction of DNA lesions that require mRad54 for their repair.

Rad54 belongs to the SWI2/SNF2 protein family whose members modulate protein-DNA interactions in an ATP-driven manner. We have purified the human Rad54 (hRad54) protein and show that it has ATPase activity that is absolutely dependent on double-strand DNA. Results of a topological assay suggest that hRad54 can unwind double-stranded DNA at the expense of ATP hydrolysis. Unwinding of the homologous repair template could promote the formation or stabilization of hRad51-mediated joint molecules. Rad54 appears to be required downstream of other RAD52 group proteins, such as Rad52 that assist Rad51 in interacting with the broken DNA.

Genetic control of genome stability in yeast

T. D. Petes[1], C. Butler[3], R. Craven[1], M. Dominska,[1] T. Fox[3], D. Gordenin[2], P. Greenwell[1], S. Henderson[1] R. Kokoska[1], R. M. Liskay, J. Mallory[1], T. Prolla, K. Ritchie[1], M. Resnick[2], E. Sia[1], L. Stefanovic[1], M. Strand[1], H. Tran[2], [1]Department of Biology, University of North Carolina, Chapel Hill, NC 27599-3280; [2]NIEHS, Research Triangle Park, NC 27709; [3]Department of Molecular Biology and Genetics, Cornell Univ., Ithaca, NY 14853-2703

The rate of genome alterations (single base pair changes, additions and deletions of DNA sequences, and alterations in chromosome structure or number) is likely to be rate-limiting for a number of important biological processes including speciation and the development of a cancer cell from a normal cell. Simple repetitive DNA sequences (microsatellites) are intrinsically unstable genomic regions, even in wild-type yeast cells. Below, I describe the genetic regulation of the stability of three types of microsatellites: poly GT tracts in the nuclear genome, poly GT and poly AT tracts located within the mitochondrial genome, and poly TG_{1-3} sequences located at telomeres.

Poly GT tracts in the nuclear genome

The genomes of most eukaryotes, including yeast, have many tracts of poly GT exceeding 15 bp in length. Using a frameshift assay, we showed that such tracts alter at a rate that is much higher than observed for "normal" DNA sequences (1), about 10^{-5}/cell division for a tract of 33 bp. Two types of mutations result in further destabilization of microsatellites: mutations affecting DNA mismatch repair enzymes (2) and mutations affecting DNA replication (3,4). Mutations reducing the rate of recombination have no effect on microsatellite stability (1). These results suggest that most microsatellite alterations reflect DNA polymerase slippage events.

Tumor cells often have elevated levels of genome instability. One type of instability, observed for a sub-set of tumors, is elevated instability of DNA microsatellites. Most such tumors have mutations affecting DNA mismatch repair (reviewed in ref. 5).

Microsatellite stability in the mitochondrial genome

In collaboration with Tom Fox, we developed methods of monitoring microsatellite instability in mitochondrial DNA (mtDNA). We found that the types of alterations are different from those observed for nuclear microsatellites. Alterations of mitochondrial microsatellites are primarily deletions, whereas alterations of nuclear microsatellites

are primarily insertions. In addition, poly AT sequences in mtDNA are much more stable than poly GT sequences; in the nuclear genome, these two types of microsatellites have similar stabilities. In strains heterozygous for the *MutS* homologue *msh1*, poly GT microsatellites are destabilized about 35-fold, consistent with previous studies indicating an elevation in the rate of point mutations in mtDNA in strains heterozygous for *msh1* (6).

Regulation of telomere length

We have also examined the genetic regulation of the length of the poly $G_{1-3}T$ sequence located at the ends (telomeres) of the yeast chromosomes. In wild-type strains, the terminal poly TG_{1-3} tract is 350-500 bp in length. Strains with a mutation of *TEL1*, a homolog of the human gene (ATM) mutated in patients with ataxia telangiectasia, have short, but stable, telomeric repeats (7). Mutations of *TLC1* (encoding the RNA subunit of telomerase) result in strains that have continually shortening telomeres and a gradual loss of cell viability (8); survivors of senescence arise as a consequence of a Rad52p-dependent recombination events that amplify telomeric and sub-telomeric repeats (9). We show that a mutation in *MEC1* (a gene related in sequence to *TEL1* and ATM) reduces telomere length and that *tel1 mec1* double mutant strains have a senescent phenotype similar to that found in *tlc1* strains. As observed in *tlc1* strains, survivors of senescence in the *tel1 mec1* strains occur by a Rad52p-dependent amplification of telomeric and sub-telomeric repeats. These results demonstrate that Tel1p and Mec1p share an essential function required for telomere maintenance. In addition, we find that strains with both *tel1* and *tlc1* mutations have a delayed loss of cell viability compared to strains with the single *tlc1* mutation. This result argues that the role of Tel1p in telomere maintenance is not solely a direct activation of telomerase.

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DNA Damage Repair and Recombination in *Saccharomyces cerevisiae*

Rodney Rothstein, Xiaolan Zhao, Bilyana Georgieva, Frédéric Foucault, Justin Weinstein, Naz Erdeniz, and Uffe Mortensen. Department of Genetics & Development, Columbia University, College of Phys. & Surg., New York, NY 10032-2704, USA

We are studying how the yeast, *Saccharomyces cerevisiae*, detects and responds to DNA damage and the mechanisms of DNA damage repair. Damage recognition often elicits a cell cycle checkpoint. Mutants of many of the genes that control these checkpoints are radiation sensitive, including *MEC1* and *RAD53*. Both are required in all DNA damage and replication cell cycle checkpoint pathways in G1, S and G2 phases to ensure correct transmission of the genetic material. In addition to their involvement in checkpoint pathways, Mec1 and Rad53 are both essential for cell growth.

To understand the molecular basis of the essential functions of Mec1 and Rad53, we identified and characterized a mutation, *sml1*, which permits cell growth in the absence of these two proteins. *SML1* acts downstream of genes whose overexpression can suppress *mec1* deletions. In addition, *sml1* affects various cellular processes in a manner similar to the overproduction of the large subunit of ribonucleotide reductase, *RNR1*. These include an effect on mitochondrial biogenesis, on the DNA damage response and on cell growth. An increase in *RNR* transcription does not account for these effects. *In vivo* and *in vitro* experiments show that Sml1 binds to Rnr1. We propose that Sml1 inhibits dNTP synthesis post-transcriptionally by binding directly to Rnr1 and that Mec1 and Rad53 are required to relieve this inhibition at S phase.

The *SGS1* gene is a member of the RecQ/Sgs1 DNA helicase family and was first identified as a mutation that suppressed the slow growth phenotype of *top3* mutants. Subsequently the genes responsible for Bloom and Werner syndromes in man were found to be homologous to *SGS1*. A homolog in *Schizosaccharomyces pombe*, *RQH1*, has also been identified and shown to play a role in cell cycle checkpoints in *S. pombe*. We are investigating *SGS1* gene function by testing its interactions with Top3, Top1, the Srs2 helicase as well as its recombination, meiotic and checkpoint phenotype. To examine the importance of the helicase activity, we used a novel allele-replacement method to create a genomic mutation known to inactivate the ATPase activity of the helicase (*sgs1-K706R*). Our genetic analysis of this mutant indicates that the helicase activity is important for every *SGS1* function that we have examined. For example, loss of helicase activity results in sensitivity to both HU and MMS. To examine the importance of the Sgs1/Top3 interaction, we have deleted the Top3-interaction domain in *SGS1* and found that this mutation has many of the same properties of a *top3* mutation including slow growth.

Many of the lesions described above require repair with the central recombination and repair protein, Rad52. It interacts with the RecA homolog, Rad51, and may form an even larger complex consisting of Rad54, Rad55 and Rad57. Furthermore, a genetic interaction has been detected between *RAD52* and *RFA1*, which encodes a DNA-binding subunit of the single-strand DNA-binding protein complex, RP-A. Biochemically, Rad52 protein possesses DNA-binding and strand-annealing activities *in vitro*. This may explain the *RAD52*-dependence of the single-strand annealing (SSA) recombination mechanism. Interestingly, this reaction is Rad51, Rad54, Rad55 and Rad57 independent. To understand the nature of the DNA annealing properties of Rad52, we have initiated a detailed analysis of the protein and have isolated mutations that separate the x-ray resistance of *RAD52* from its effects on recombination.

Ilegitimate integration of viral DNA into *Nicotiana* species during evolution

Yoong Lim, Roman Matyasek[§] , Lena Murad, Andrew Leitch, & Conrad Lichtenstein*, School of Biological Sciences, Queen Mary and Westfield College, University of London, London E1 4NS, England UK. Tel: +44 171 775 3049 Fax: +44 181 983 0973 *Email: c.p.lichtenstein@qmw.ac.uk. [§] Present address: Institute of Biophysiscs, Czech Academy of Sciences, Brno, Moravia, Czech Republic

Geminiviruses are small circular single-stranded (ss) plant DNA viruses which replicate in the nucleus by rolling circle replication from a circular double-stranded replicative form. The viral Rep protein, a sequence- and strand-specific endonuclease /helicase/ATPase/ligase generates the ss viral DNA circular monomers from a *cis*-essential origin mapping next to the *rep* gene. The origin and *rep* gene DNA sequences are evolutionarily related to those of prokaryotic ssDNA bacteriophage such as Ø X174.

We inhibited geminiviral replication in transgenic *Nicotiana tabacum* (tobacco) plants expressing an antisense transcript targeted against a geminiviral Rep protein [1,2] and by serendipity discovered that uninfected wild-type tobacco carries multiple direct repeats of a geminiviral origin sequence adjacent to a truncated *rep* gene [3]. We believe they arose by geminiviral DNA integration; curiously, recombination between viral and genomic DNAs though not unusual in other systems was hitherto unknown for plants.

To address whether this was a unique event we examined other plants for evidence of geminivirus related DNA (GRD). We found multiple copies of these elements only in the genomes of three related *Nicotiana* species, all in the section Tomentosae: *N. tomentosiformis, N. tomentosa,* and *N. kawakamii.* DNA sequence analysis of 18 GRD copies reveal 4 distinct, but highly related, sub-families: GRD5, GRD3 and GRD53 in tobacco; GRD5 in *N. tomentosiformis* and *N. kawakamii;* and GRD2 (related to GRD5) in *N. tomentosa* [4] .

The GRD sequences are highly methylated even in comparison to other sequences in tandem array within the tobacco genome. Sequence analysis shows that CG and CNG nucleotides are under-represented relative to free geminiviruses, suggesting the possibility of methyl-cytosine to thymine transitions during sequence evolution.

We have performed fluorescent *in situ* hybridisation (FISH) to map the chromosomal locations of these elements [5,6]. Tobacco, which is an amphidiploid cross between progenitors of *N. tomentosiformis* (the T genome) and *N.sylvestris* (the S genome) carries GRD3 on chromosome T4 and like the other GRD-positive species has GRD5 on another T chromosome.

Our data suggest that all GRD elements are descended from a unique geminiviral integration event, most likely in a common ancestor of these Tomentosae species several million years ago, presumably via illegitimate recombination with DNA amplification, deletions and rearrangements. We are currently seeking to identify how these events occurred.

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Hairpin and Duplex DNA Processing by the SbcCD Recombination Nuclease

John C. Connelly, Erica S. de Leau and David R. F. Leach. Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, UK

The SbcCD protein from *Escherichia coli* is a member of a family of recombination nucleases found in bacteriophages, eubacteria, archaebacteria, yeast, drosophila, mouse and man. Members of this family have been implicated in a remarkably wide panoply of recombination reactions ranging from meiotic recombination, via the control of replication of palindromes and tri-nucleotide repeats to non-homologous end-joining and telomere maintenance.

Evidence from electron microscopy of SbcCD has revealed a distinctive structure consisting of two globular domains linked by a long region of coiled coil, similar to that predicted for the members of the SMC family. That a nuclease should have such an unusual structure suggests that its mode of action may be interesting.

Here we show that the protein degrades duplex DNA in a 3′ to 5′ direction whether or not one of the ends in closed as a hairpin structure. This degradation releases products half the length of the original duplex suggesting simultaneous degradation from the two ends. This may provide a link to the unusual structure of the protein since our data are consistent with recognition and cleavage of DNA ends (whether or not they are closed as hairpins) followed by 3′ to 5′ nicking by two nucleolytic centres within a single nuclease molecule that releases a half length limit product.

We also show that cleavage is not simply at the point of a single-strand/double-stand transition and that despite the dominant 3′ to 5′ polarity of degradation, a 5′ single-strand can be cleaved when attached to duplex DNA.

The implications of this mechanism for the action of this family of proteins in their diverse recombinational roles will be discussed.

Session 5

Chair: Martin Gellert

Novel joints formed during illegitimate and homology-directed heterospecific recombination in *Streptococcus pneumoniae*

Jean-Pierre Claverys and Marc Prudhomme
Laboratoire de Microbiologie et Génétique Moléculaire CNRS-UPR 9007, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cedex, France. Tel: (33) 561 33 59 11; Fax: (33) 561 33 58 86; Email: claverys@ibcg.biotoul.fr.

Illegitimate recombination events observed in *Streptococcus pneumoniae* during transformation with *in vitro* engineered hybrid DNA molecules (Claverys *et al.*, 1980) were called *insertion-deletions* because insertion of heterologous DNA and deletion of chromosomal sequences were concomitant. This type of event was shown to represent about 0.5% of total recombination events during transformation with hybrid DNA (Claverys *et al.*, 1980). The appearance of insertion-deletions was strictly dependent on the presence in the hybrid donor molecule of a region of homology with the recipient chromosome. The underlying recombination mechanism can therefore be referred to as homology-directed illegitimate (HDI) recombination. A tentative model for HDI recombination postulated that pairing between homologous donor and recipient sequences occured first and favored transient pairing between heterologous flanking donor and recipient sequences, within short stretches of nucleotide identity. Simultaneous incision of both heterologous and recipient strands within the transiently paired heterologous region, followed by ligation, was then postulated to produce the observed integration of heterologous DNA and concomitant deletion of recipient sequences.

Based on this model, we developped a genetic system to generate heterospecific gene fusions *in vivo* in *S. pneumoniae*. The heterologous gene pair chosen for this study consisted of the *hexA* mismatch repair gene of *S. pneumoniae* and its *Escherichia coli mutS* homologue. The two genes exhibit only 45% identical nucleotides. To favor heterologous recombination, a *S. pneumoniae* strain harboring a 5'-truncated copy of the *erm* gene downstream of the *hexA* gene was constructed. This recipient strain was transformed with a hybrid DNA molecule carrying an intact *erm* gene immediately downstream of the *mutS* gene of *E. coli*. Selection for possible *hexA-mutS* fusion recombinants was based on the reconstitution of an intact *erm* gene, which conferred resistance to Ery. This system which proved efficient for the production of *hex-mut* chimeras was called homology-directed heterospecific (HDH) recombination.

The analysis of novel joints formed during HDI and HDH revealed that these joints were located within or very close to short stretches of sequence identity between heterologous DNA and the recipient chromosome (M.P. and J.P.C., in preparation). The latter category suggested that transient pairing within short blocks of sequence identity between heterologous and recipient strands favored incision of both strands in the immediate vicinity, which resulted in resolution of the recombination intermediate.

It should be pointed out that such a mechanism is not limited to synthetic donor DNA molecules. Insertion sequences or transposons when present on both donor and recipient DNAs can themselves provide the homology required for HDI. They can therefore promote the acquisition of sequences immediately adjacent to them, thereby leading to the acquisition of completely heterologous genes. This mechanism is therefore of great potential in terms of genome evolution. It reinforces the importance of transformation for genetic plasticity.

J. P. Claverys, J. C. Lefèvre, and A. M. Sicard. Transformation of *Streptococcus pneumoniae* with *Streptococcus pneumoniae*-lambda phage hybrid DNA: induction of deletions. *Proc. Natl. Acad. Sci. USA* 77:3534-3538, 1980.

John Roth, Renee Dawson, McKell Carter, Rafael Camacho.
Department of Biology, University of Utah, Salt Lake City, USA

Use of internal (asexual) recombination assays of bacterial recombination

The major activity of the bacterial recombination system is repair of spontaneous nicks and double strand breaks that occur spontaneously during cell growth. We describe three assays of these "internal" recombination events. The assays detect exchanges initiated by spontaneous DNA damage and thus differ from sexual assays which provide double strand fragment ends to initiate recombination.

--A duplication-segregation assay detects unequal sister-strand exchanges that cause loss of a marker (lac$^+$) between the repeated sequences. Using this assay, we have isolated mutants that are defective for duplication segregation but proficient at sexual recombination; many of these affect central metabolism, notably blocking the TCA cycle. We present evidence that these mutations act by minimizing production of reactive oxygen species, which can damage DNA.

--Unequal sister strand exchanges between short direct-order repeats at separated chromosomal sites can generate either a deletion or a duplication. Each product can be generated independently by a single half-reciprocal exchange. Both products are generated together by a reciprocal exchange. When exchanges are scored during growth of a colony one can observe products as sectors and correlate formation of recombinant types (a "tetroid"). Many events appear to cause a simple duplication OR a simple deletion suggesting that the exchange was not reciprocal. Other events show paired duplication and deletion sectors, suggesting reciprocality. Duplication-generating exchanges between 1kb sequences were reciprocal in 25% of cases; this frequency rose to 75% when 10kb recombining sequences were tested.

-- Inverse repeats at separated sites in the chromosome can, in principle, recombine to generate an inversion. Ability to form these inversion recombinants depended on the chromosomal positions of the recombining sequences. Three sorts of sequence pairs were identified: 1) those that are permissive for inversion and yield healthy inversion recombinants, 2) those that are permissive but yield slow-growing inversion recombinants and 3) those that are non-permissive for inversion. All intervals became permissive and gave healthy recombinants in strains carrying a Tus mutation (which eliminates a protein needed for termination of DNA replication a Ter sites. We propose a model in which each exchange event initiates two replication forks which must proceed around the chromosome to the dif site; for sequences at certain positions, Ter sites block this needed replication. The inversion assay may allow study of the role of long-range replication in genetic recombination.

Mechanism and Genetic Control of Interspecies Recombination : Studies with Enterobacteria

M. Radman*, S. Stambuk, M. Vulic & I. Matic,
Institute Jacques Monod, F-75251 Paris Cedex 05 and
Institut Necker, Faculté de Médecine, Université Paris-5, 156 rue de Vaugirard, 75730 Paris Cedex 15, France.

An extensive genetic (1,2) and physical (3) analysis of interspecies recombination in *Escherichia coli* between the linear Hfr DNA from *Salmonella enterica* and the circular recipient chromosome reveals some fundamental aspects of recombination between closely related (homeologous) DNA sequences. Inter-genomic homeologous recombination reflects the degree of genetic isolation between the closely related species (4). Intra-genomic homeologous recombination, involving repeated sequences, generates chromosomal rearrangements (5). It is remarkable that the mismatch repair system (MRS) determines the precision of both DNA replication and recombination, i.e., controls the genetic stability, and determines the degree of genetic isolation, i.e., the rate of divergent evolution (2,4). MutS and MutL mismatch binding proteins prevent homeologous recombination by at least two independent mechanisms (6). One is MutH-independent, but requires the UvrD helicase and the RecBCD nuclease. The other mechanism is MutH-dependent and requires newly synthesized DNA strands (GATC sequences) to act upon. This *de novo* DNA synthesis requires the primosome assembly PriA functions and is associated with, and triggered by, genetic recombination.
Only the « wild type » RecBCD recombination pathway is edited by both mechanisms; the RecF and RecE recombination pathways are edited by the sole MutH-dependent mechanism (7). Therefore, the extent of genetic isolation between related enterobacterial species is much lower in the RecE and RecF pathways than in the RecBCD pathway .
Just as the inactivation of MutS and/or MutL functions disrupts genetic barriers between the well separated species, so can overproduction of these proteins create *ad hoc* genetic barriers between the strains of the same species (4,7). The MutL function is the most rate limiting one in the editing of recombination (7). A comprehensive molecular model of the mechanism and the control of homeologous recombination will be discussed.

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* speaker

Genetic variation in Neisseria.

Thomas F. Meyer and Roland Barten
Max-Planck-Institut für Infektionsbiologie, Abteilung Molekulare Biologie,
Monbijoustrasse 2, D-10117 Berlin, Germany

Neisseria gonorrhoeae constitutes a well known paradigm of the genetic variability of a pathogenic microorganism. Numerous surface proteins, and even the lipopolysaccharide, are subject to phase and antigenic variation. The resulting variability is tremendous and allows this human pathogen not only to cope with the host immune response but also to quickly adapt its population to sudden micro-environmental changes which occur during the course of an infection. For example, the phase-variable family of opacity-associated (Opa) outer membrane proteins allows the tissue-specific recognition of distinct receptor molecules on target cells[1].

On the molecular level, two principle mechanisms account for genetic variation in *N. gonorrhoeae*, i.e. replicative stand slippage and the RecA-dependent homologous recombination[2]. The latter, representing the paradigm of pilin variation, occurs via at least three pathways, one of which involves the natural transformation competence of these microbes. Several factors essential for transformation have recently been identified. The second important mechanism underlying pilin variation, i.e. "gene conversion", is still enigmatic. We recently observed the RecA-independent formation of chromosomal repetitions and suggest that these repetitions may be preceded by circle formation. These circles may include silent (*pilS*) gene copies for the recombination with expressed (*pilE*) pilin genes. Resulting recombinations may thus show a non-reciprocal phenotype, explaining non-reciprocal gene conversion events.

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Role of recombination functions in *Salmonella typhimurium* virulence: animal and tissue culture models

Josep Casadesús[1], David A. Cano[1] and Francisco García del Portillo[2]
[1] Departamento de Genética, Universidad de Sevilla
[2] Centro de Biología Molecular "Severo Ochoa", Universidad Autónoma de Madrid

The first survey of the role of recombination functions in *Salmonella* virulence, performed in F. Heffron's laboratory, indicated that *recA* and *recBC* mutants of *S. typhimurium* are avirulent in mice; their loss of virulence was shown to be related to the inability to repair DNA damage (Buchmeier et al. 1993). We have recently started trials with recombination mutants derived from *S. typhimurium* SL1344. The mutations analyzed so far include *recA*, *recBC*, *recD*, *recF* and *recJ*, alone or combined. The model systems used are (i) the animal model, based upon oral or intraperitoneal administration of *Salmonella* to mice; (ii) the tissue culture model, using epithelial and macrophage cell lines.

In the animal model, some recombination mutations cause loss of virulence (*recA*, *recBC*, *recF*), while others do not (*recD*) or cause mild symptoms (*recJ*). Comparisons between oral and intraperitoneal administration permits the detection of subtle phenotypes (e. g. *recJ* mutants are fully virulent if administrated intraperitoneally but not if administrated orally). Certain combinations of two mutations show an additive effect (e. g. *recBC recJ* mutants are more attenuated than *recA* mutants). The phenotypes of single and double mutants can be further defined by analyzing persistence in organs such as liver and spleen. Interestingly, strains with major defects in DNA repair (e. g. *recA* or *recBC recJ*) are found in large numbers in the spleen but not in the liver. Additional phenotypes are provided by the tissue culture model, using HeLa epithelial cells, macrophages (and, in certain cases, NRK fibroblasts). Certain recombination mutations cause distinct defects: for instance, *recA*, *recBC* and *recBC recJ* mutants are unable to proliferate within macrophages but show modest proliferation defects in epithelial cell lines. Somehow surprisingly, *recF* mutants are unable to grow in both epithelial cells and macrophages. Pulsed-field mapping and IS*200* fingerprint analysis have ruled out that major genome rearrangements are required to cause disease. Thus the virulence defects of recombination mutants do not reflect an impairment to rearrange DNA using homologous sequences.

A second approach to define the roles of recombination functions in *Salmonella* virulence is suppressor analysis. In some cases, suppressor-carrying derivatives can be directly selected by growth under non-permissive conditions (e. g. isolates carrying suppressors of *recBC* are recovered from macrophage tissue cultures). To monitor the genotypes of the strains used, and to detect the presence of suppressor mutations, we have developed phage plaquing assays using *abc* and *erf* mutants of P22. These assays permit, for instance, the identification of *recBC*, *recBC sbcB* and *recBC sbcB sbcCD* strains by plaquing assays with P22 *abc* phage (Cano et al., in preparation). The suppressors found include "classical" *sbc*-like mutations, some of them similar to *sbcB* mutations selected by transductional proficiency (Benson and Roth 1994). However, novel types of suppressors, unrelated to *sbcB* and *sbcCD*, are also isolated. The latter belong to several phenotypic classes and map in three or more different loci. Two classes include only recessive mutations. Another class appears to include dominant mutations,

and may involve a prophage-borne locus. This putative prophage is not Fels.

The third approach involves multicopy suppression using a *S. typhimurium* plasmid library (each clone carrying an insert of 7-11 kb). Multicopy suppression and complementation are distinguished by phage plaquing assays similar to those described above. To date, multicopy suppressors have been found for *recBC* and *recF* mutations, but not for *recA*.

We expect that the combination of the three approaches (search for novel phenotypes, suppressor analysis and identification of multicopy suppressors) will help to define the roles of recombination functions in the interaction of *Salmonella* with its host. Preliminary evidence suggests that these roles are multiple, and that the pathogen faces various kinds of challenges in the interaction with its host. If this view is correct, oxidative damage caused by the oxidative burst in macrophages may simply be one of several challenges encountered by *Salmonella* upon host invasion. Invasion, proliferation in the vacuole of host cells and other stages of infection may involve additional threats to DNA integrity. One attractive speculation is that the bacterial cell may overcome each threat by a distinct recombination-related response, and that different recombination functions may be required in different stages of host colonization.

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Recombination of T-even Phage DNA with Foreign DNA Sequences is Initiated by Pairing of Short Homologous Segments, and it Generates Multiple Mutations in the DNA Adjacent to the Acquired Foreign DNA.

Gisela Mosig and Nancy, E. Colowick. Dept. of Mol. Biol.
Vanderbilt University, Nashville, TN 37235, USA

Our recent analyses of the essential dCTPase gene *56* and adjacent nonessential genes (*69*, *dam* and *soc*) of bacteriophages T2 and T4 suggested that nonessential genes or DNA segments have been acquired during evolution by illegitimate homologous recombination mechanisms that do not require transposase but depend on pairing of short (approximately 20 bp) complementary DNA sequences, branch migration, excision of mismatched bulges in heteroduplex regions and two modes ("join-copy" and "join-cut-copy") of recombination-dependent DNA replication. (Gary,TP, Colowick, NE, and Mosig, G. 1998, GENETICS, 148:1461-1473).

This model predicts that multiple base substitutions and frameshift mutations are generated from a single pairing event and that different multiple mutations are generated in different phages, if different foreign genes had been acquired during evolution. In contrast, random errors during DNA replication initiated from origins or from intermediates of recombination are less likely to cause similar patterns of frequent multiple mutations.

These and other predictions of our model are confirmed by sequence analyses of several different T-even phages and by recombinational analyses after crossing different T2, T4, and chimeric phages.

Most importantly, in different T-even phages in which the (acquired nonessential) genes adjacent to the essential dCTPase genes are different, the dCTPase gene base sequences differ by as much as 28%, including apparent multiple compensating frameshifts. In contrast, phages that have the same nonessential gene adjacent to the dCTPase gene, or in which a nonessential adjacent gene has been deleted, differ in dCTPase base sequence by less than 3%, with no apparent frameshift mutations. The dCTPases of all these phages are functional.

Consistent with findings in many other organisms, extensive sequence divergence of functioning dCTPase genes between present-day T2 and T4 phages inhibits formation of viable recombinants, and this inhibition is relieved in crosses with chimeric phages in which sequence identity is restored, even though neither the host's methyl-directed mismatch repair system nor the RecBCD system are implicated in T4 DNA repair (Kreuzer, KN, and Drake, J.W. 1994 In: Bacteriophage T4 ASM Press pp. 89-97).

Our results support the following model as the simplest explanation for the divergence of the dCTPase genes of different present-day T-even phages, and for the apparent poor recombination between dCTPase mutations in crosses between these phages:

1) Homologous recombination mechanisms allow illegitimate pairing of foreign genes with T-even genomes, if the DNA segments share a stretch of about 20 identical base pairs.

2) In the region adjacent to the perfect homology, misaligned heteroduplex DNA segments are formed by branch migration that enlarges the base-paired region. If the intermediates are resolved by join-copy, and by join-cut-copy recombination, foreign DNA sequences are spliced into the DNA of one of the parents.

3) Partial excision of bulges in the misaligned heteroduplex region generates multiple differences from both parental sequences (as first proposed by Streisinger, G. et al., 1964, Cold Spring Harbor Symp. Quant. Biol.31:77-84). However, only those sequences that code for a functional protein (dCTPase in our example) are selected in viable phage particles. Different functional dCTPase sequences have been selected in different T-even phages.

4) Therefore, heteroduplexes formed in crosses between different present -day T-even phages are predicted to contain many bulges whose excisions are bound to generate defective dCTPase genes, and, therefore, non-viable phages.

Our model can explain mosaic arrangements of many other genes in genomes of different phages as well as of other organisms. It puts obvious limitations on interpretations of phylogenetic trees and deduced tempos of evolution.

POSTERS

Characterization of the two Rad51 homologs of *Physcomitrella patens*

Silvia Ayora[1], Enzo Russo[2], Bernd Reiss[3], and Juan C. Alonso[1]
[1]Centro Nacional de Biotecnología, Campus Universidad Autónoma de Madrid, Cantoblanco, E-28049 Madrid, Spain. [2]Max-Planck-Institut für molekulare Genetik, Ihnestr. 73, D-14195 Berlin, Germany, [3]Max-Planck-Institut für Züchtungsforschung, Carl von Linne Weg 10, D-50829 Köln, Germany.

Rad51 protein is the eukaryotic counterpart of the *Escherichia coli* RecA protein, wich is the key protein of homologous recombination in bacteria. Two distinct intron-less Rad51 genes (Rad51A and Rad51B) were isolated from the moss *Physcomitrella patens*. The Rad51A protein shares a 98% identity to the Rad51B protein and both proteins share 67% identical to human Rad51 protein. Recombinant Rad51A and Rad51B proteins were expressed and purified from *E. coli* cells. Both Rad51A and Rad51B proteins bind preferentially to ssDNA in an ATP- and Mg^{2+}-independent fashion (K_{app} = 131 nM for Rad51A and K_{app} = 380 nM for rad51B) and showed a ssDNA-dependent ATPase activity (K_m =90 μM, Vm = 0,1 min^{-1} for Rad51A, and K_m = 54 μM, Vm = 0,03 min^{-1} for Rad51B). The biochemical properties of both Rad51A and Rad51B proteins will be presented.

A TRANSPOSABLE ELEMENT MEDIATED THE GENERATION OF A *DROSOPHILA* WIDESPREAD CHROMOSOMAL INVERSION

Mario Cáceres,[1,2] José María Ranz,[1] Antonio Barbadilla,[1] Manyuan Long,[2] and Alfredo Ruiz [1]

[1] *Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, 08193 Bellaterra (Barcelona), Spain*

[2] *Department of Ecology and Evolution, University of Chicago, Illinois 60637, USA*

The origin of chromosomal rearrangements, particularly that of the polymorphic inversions of the genus *Drosophila*, is a highly debated and still non-clarified issue. Here, we report the cloning and sequencing of the breakpoints of the cosmopolitan polymorphic inversion *2j* of *Drosophila buzzatii*. We have found large insertions at both breakpoints of the inverted chromosome that are not present in the *2 standard* (*2st*) arrangement. The presence of the insertions in 21 *2j* lines and their absence in four *2st* lines has been confirmed by PCR amplification of the regions spanning the breakpoints. The insertions correspond to middle repetitive sequences that end in inverted repeats and are flanked by 7-bp target duplications, and they presumably represent copies of a new class II transposable element. The homologous sequences inserted in opposite orientation at both breakpoints and the exchange of the two pairs of target site duplications between the copies of the transposon indicate that the inversion arose by an ectopic recombination event. Thus, this is the first direct demonstration of the involvement of transposable elements in the origin of natural inversions in *Drosophila*. However, despite the transposon-mediated origin, the sequence diversity at the breakpoint regions in three *2st* and five *2j* chromosomes suggests that the inversion is monophyletic. Finally, an expression assay has revealed that the breakage and inversion process did not affect any of the genes close to the breakpoints.

Role of Recombination Proteins in Processing DNA Palindromes Through SbcCD-Dependent Double-Strand Breaks and SbcCD-Independent Single-Strand Gaps

Gareth A. Cromie and David R. Leach. Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, UK.

Long palindromic, or near palindromic, DNA sequences form hairpin structures when transiently single stranded during DNA replication. The SbcCD nuclease of *Escherichia coli* cleaves these structures leading to the formation of a double strand break. Repair of this break requires recombination utilising RecA and RecBC and without these functions palindromes cannot be propagated in the presence of SbcCD.

The role of a range of recombination proteins in the repair of palindrome-induced double strand breaks was investigated by comparing the lysogenisation frequencies of a phage λ containing a 246 near-palindrome to a palindrome-free isogenic control phage in a range of recombination mutant backgrounds. These experiments were repeated using the same mutant backgrounds carrying an additional mutation in *sbcC*.

A wide range of recombination proteins were found to be required for palindrome viability in the presence of SbcCD. These included the components of both the recB and recF 'pathways'. The lack of an effect of a *priA* mutation on palindrome viability suggested that replication fork breakdown was not occurring, despite the creation of a replication-dependent double strand break.

In the absence of SbcCD palindrome-specific initiation of recombination was still found to occur at a high high frequency. This appeared to take the form of a RecFOR-dependent process using a substrate containing a single-stranded gap.

Purification and properties of the RecU protein from *Bacillus subtilis* 168

Ernesto Doncel[1], Silvia Ayora[1], Silvia Fernández,[1] Rudi Lurz[2] and Juan C. Alonso.[1]
[1]Departamento de Biotecnología Microbiana , Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain, and [2]Max-Planck-Institut für molekulare Genetik, Ihnestrasse 73, D-14195 Berlin, Germany

Genetic evidence suggests that the *Bacillus subtilis rec*U gene product is involved in DNA repair and recombination. The RecU product, which is absent in Gram-negative bacteria, has been detected in different Gram-positive bacteria with low dG + dC content in their DNA (1). To assign a biochemical function to the *rec*U gene product, the RecU protein was purified. N-terminal protein sequence analysis of RecU was consistent with the deduced amino acid sequence of the *recU* gene. The RecU protein has a molecular mass of 23.9 kDa and an iso-electric point of 10.0. The ability of RecU protein to act as dsDNA or ssDNA nuclease (exo- or endonuclease), DNA helicase, ATPase and to bind to single-stranded (ss) or double-stranded (ds) DNA were assayed. RecU binding to ssDNA and dsDNA were the only activities observed. The RecU-ssDNA or RecU-dsDNA complex formation proceeds in the absence of nucleotide cofactors, but the type of complexes observed depend in the presence of divalent cations. The apparent equilibrium constants of the RecU-DNA complexes in the presence of Mg^{2+}, is about 8 and 50 nM for ssDNA and dsDNA, respectively. The binding reaction shows cooperativity. The biochemical properties of the RecU protein in DNA repair and homologous recombination will be discussed.

1. Genetic recombination in *Bacillus subtilis* 168, Effect of *recU* and *recS* mutations on DNA repair and homologous recombination
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J. Bacteriol. 180, 3405-3409 (1998).

Mapping the most common sites of meiotic recombination initiation by Spo11p in the *S. cerevisiae* genome

Gerton, J.L.[1], DeRisi, J.[2], Brown, P.O.[2,3], and Petes, T.D[1]. [1]Department of Biology, University of North Carolina, Chapel Hill, NC 27599-3280, [2]Department of Biochemistry, [3]Howard Hughes Medical Institute, Stanford University Medical Center, Stanford, CA 94305-5428

One of the major advantages of sexual reproduction is meiotic recombination. This process allows beneficial mutations initially carried by two individuals to be combined into the same individual. Particular genomic loci have been observed to have above-average meiotic recombination rates, such as the MHC locus in mice and the region between *BIK1* and *HIS4* in *Saccharomyces cerevisiae*. Sites at which the level of recombination is higher than average are referred to as recombination "hotspots." In yeast, recombination is initiated by a double-strand break (DSB) created by Spo11p. We are interested in mapping where DSBs occur during meiosis on a genome-wide scale. Mapping the sites of meiotic recombination in yeast in a global manner will provide us with a better understanding of the rules governing meiotic recombination at a molecular level, and possibly help explain why elevating recombination in certain regions of the genome is evolutionarily advantageous.

We are using DNA microarrays to map meiotic DSBs. DSB DNA that is covalently attached to Spo11p is purified from total meiotic genomic DNA using glass fiber filters. This DNA is then labelled *in vitro* using a fluorescently labelled trinucleotide and mixed with a second DNA sample (total yeast genomic DNA) that has been labelled with a second fluorescent tag. This mixture of DNA is used as a hybridization probe to a microarray. The microarray is a glass slide that contains about 6,200 spots of DNA, each corresponding to a unique ORF in the yeast genome. The signal from each fluor is detected for each ORF by a laser scanner and the ratio of the two fluors determines whether a particular sequence is overrepresented in the DSB sample. Genomic DNA (restricted with PstI) enriched 50-fold for Spo11p-DSB DNA from the strain SK1 was used as a hybridization probe for a microarray. About 35 regions have hybridization ratios that indicate that they have at least two times the background level of DSBs. The regions identified are distributed throughout the genome on 12 of the 16 chromosomes. Because the PstI recognition site is G/C rich, this enzyme generates large restriction fragments in the A/T-rich genome. To improve the resolution of our meiotic DSB map (and confirm our first hybridization pattern), we plan to perform a microarray hybridization with DNA restricted with a more frequently cutting restriction enzyme. Selected loci that appear to have meiotic DSBs by the microarray hybridization data have been confirmed as authentic sites for DSBs by Southern blot. In these cases, the location of the DSBs is mapped to within a few hundred base pairs.

Homologous recombination between direct repeats induced by the site-specific Flp recombinase in the absence of RAD52.

F. Prado, S. González-Barrera and A. Aguilera
Departamento de Genética, Facultad de Biología, Universidad de Sevilla, 41012 SEVILLA, Spain

The observations that double-strand breaks (DSBs) in plasmids can be repaired by recombination, that meiotic recombination is initiated by a DSB, and that mutations in genes such as *RAD52*, involved in the repair of X-ray-induced DSBs, reduce recombination in yeast suggest that homologous recombination is initiated by a DSB. Interestingly, mitotic recombination leading to deletions between direct repeats can also be initiated by a DSB in the intervening region located between the repeats in yeast, *Xenopus* and mammals. This also occurs in the absence of RAD52 in the yeast *S. cerevisiae*.

In order to understand how mitotic recombination is initiated, we have developed a system to isolate DNA sequences that function as initiators of recombination in *S. cerevisiae*. We have constructed a DNA library of random genomic DNA fragments of 200-700 bp inserted between two 600 bp-direct repeats. We have screened for clones conferring an increase in the frequency of recombination in *rad52* strains. We isolated two clones that increase the frequency of recombination between the flanking repeats 100 times above the normal *rad52* levels. Unexpectedly, both clones contained a DNA fragment from the 2μ circle. Further analysis of the clones revealed that both contained the FRT region, target of the site-specific recombinase FLP. The hyper-recombination phenotype associated to the FRT sequence was absolutely dependent on the presence of a 2μ circle in the cell, since it was only observed in cir$^+$ strains. The 2μ DNA does not play any role in the induction of recombination by participating in any type of recombination intermediate. The FLP recombinase is the only requirement for the ability of the FRT sequence to induce a recombination event between the flanking repeats, as a GAL1 promoter-driven expression of FLP was able to promote recombination in a cir^o background.

These results show that the FLP recombinase is able to initiate recombination by cutting one single FRT site. This recombination event is non-conservative and presumably occurs by single-strand annealing and/or one-ended invasion, as it is observed in both *rad52* and *RAD52* strains, respectively (1). We discuss the capacity of FLP recombinase to initiate homologous recombination and the possible mechanisms that may lead to a deletion event between repeats.

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MEIOTIC DOUBLE-STRAND BREAKS IN YEAST ARTIFICIAL CHROMOSOMES CONTAINING HUMAN DNA

Grzegorz IRA 1, 2 and Jan FILIPSKI 2

1) Nicholas Copernicus University, Torun, Poland 2) Institut J. Monod, CNRS, Universités Paris 6 et 7, Paris, France

Meiotic recombination has most intensively been studied in fungi. The long reproductive cycle of higher Eukaryota, germ cells that can not be propagated in culture and the inability to recover the products of individual meioses make study of meiotic recombination in these organisms difficult. However, evidence suggests that mammalian and fungal meiotic recombination are basically similar. Firstly, in both yeast and mammalian meiotic cells the homologous chromosomes align, form synaptonemal complexes and undergo at least one crossover per chromosome. Secondly, many genes of the RAD52 epistasis group, involved in both recombinational DSB repair and meiotic recombination, are conserved between yeast and higher vertebrates. Meiotic recombination in Saccharomyces cerevisiae is initiated at double strand breaks (DSBs). The DSBs occur more frequently at some chromosomal loci than in others. These loci coincide with the hot-spots of meiotic recombination. In yeast DSB formation is catalysed by Spo11, the meiosis-specific, topoisomerase-like protein. As genes coding for homologous proteins were identified in C. elegans and D. melanogaster it is likely that DSB initiate meiotic recombination in other eucariotic organisms also. Klein et al (1997) suggested that meiotic DSBs occur in human DNA cloned in YACs, at the same sites which are recombination hot-spots in human cells. In order to verify this hypothesis we transferred YAC A85D10 carrying a human beta globin gene cluster into the rad50S yeast mutant strain that enables meiotic DSB mapping. Two hot-spots of recombination have previously been identified in humans in this gene cluster. The first lies between delta and beta globin gene, and the second was found 30 kb downstream of the beta globin gene.

We have shown that : a/ the DSB appearing in the studied YAC in the diploid yeast strain at the onset of meiosis, are localized close to, or are co-localized with, the DNase-hypersensitive sites, and b/ these DSBs are apparently co-localized with the recombination hot-spots in humans (Ira at al., 1998). More precise mapping has revealed that these DSBs lie at, or close to, sequences that are recombination-prone in most organisms : (TG)n, tandem repeats, and perfect 160-bp palindrome. Deletion breakpoints of several unrelated cases of beta thalassemia have been described in these regions. Our results suggest that indeed, the human and yeast recombination machineries recognize the same sequences. This finding strenghtens the idea that the molecular mechanisms of meiotic recombination in yeast and vertebrates are fundamentaly similar. The finding, that the DNA sequences corresponding to the recombination hot spots in humans are also recombination-prone in yeast, may be used to precisely locate the sites of genetic rearrangements in human chromosomes. (supported by GREG 520634 and KBN 6 P04A 035 16)

The association between gene conversion and crossing-over. Inbar, O., Bitan, G. and M. Kupiec. Department of Molecular Microbiology and Biotechnology, Tel Aviv University, Ramat Aviv 69978, Israel.

Homologous recombination is a universal process that plays a role in generating diversity during meiosis, and is an important DNA repair mechanism in vegetative cells. Recombination results in the transfer of genetic information from one DNA molecule to an homologous one (gene conversion) and in the reciprocal exchange of DNA fragments between chromosomes (crossing-over). Gene conversion and crossing-over events show a nonrandom association which has lead to the assumption that they are mechanistically related. One of the characteristic features of eukaryotic genomes is the presence of large amounts of repetitive DNA. Reciprocal recombination between dispersed repeats may result in chromosomal aberrations, such as deletions, translocations, etc. that can affect the reproductive fitness of an organism or lead to cancer. Therefore, in order to maintain the genome integrity, crossing-over must be prevented during recombinational repair of DNA lesions involving dispersed repeats.

Using an inducible, double-strand-break initiated sytem for recombination, we have started to dissect the mechanism by which broken chromosomes are repaired. In our system, no genetic selection is applied in order to monitor recombination, and the whole cell population undergoes a synchronous recombination event. We have found that crossing-over can be uncoupled from gene conversion, in a way that depends on the length of homology of the interacting substrates and is affected by the mismatch repair system. These results provide a mechanism to explain how chromosomal recombinational repair can take place without altering the stability of the genome.

We have also studied the mechanism by which sequence homology is searched for during the recombinational repair, using strains in which the broken chromosome could be repaired by recombination with one of two different homologous partners. Our results provide a dynamic view of recombination, and do not support models in which repair is carried out by a one-handed invasion mechanism.

Stimulation of homologous recombination in plants by expression of the bacterial resolvase RuvC

Gil Shalev, Yaron Sitrit, Naomi Avivi-Ragolski, Conrad Lichtenstein* and Avraham A. Levy

Department of Plant Sciences, The Weizmann Institute of Science, Rehovot 76100, Israel. * School of Biological Sciences, Queen Mary and Westfield College, University of London, London E1 4NS, UK

Targeted gene disruption exploits homologous recombination (HR) as a powerful reverse genetic tool for example, in bacteria, yeast and transgenic knockout mice, but has not been applied to plants owing to the low frequency of HR. To increase the frequency of HR in plants, we constructed transgenic tobacco lines carrying the Escherichia coli RuvC gene, fused to a plant viral nuclear localization signal. We show that RuvC, encoding an endonuclease which binds to and resolves recombination intermediates -Holliday junctions, is properly transcribed in these lines, and stimulates HR. We observed a 12 fold stimulation of somatic crossover between genomic sequences, a 11 fold stimulation of intrachromosomal recombination, and a 56 fold increase for the frequency of extrachromosomal recombination between plasmids cotransformed into young leaves via particle bombardment. We discuss possible mechanisms leading to this increase as well as the possible applications.

Site-specific recombination mediated by a conjugative relaxase by sequential action of its two catalytic tyrosyl residues

Matxalen Llosa, Guadalupe Grandoso, Pilar Avila, Amalia Cayón, Miguel Angel Hernando, and Fernando de la Cruz.

Departamento de Biología Molecular, Universidad de Cantabria, C. Cardenal Herrera Oria s/n, 39011 Santander, Spain.

Protein TrwC is the relaxase responsible for the initiation and termination reactions of DNA processing during plasmid R388 conjugation (Llosa et al. 1995, 1996). In addition, it is the only known relaxase capable of catalyzing site-specific recombination at *oriT* (Llosa et al. 1994) in the absence of conjugation. This activity is presumably the result of the cut-and-paste activities of the enzyme together with as yet unidentified additional factors.

We have addressed a detailed analysis of the relaxase catalytic center. By homology comparisons a set of four conserved tyrosines in the sequence of the N-terminal relaxation domain of TrwC were the candidates to act as catalytic residues. Site-directed mutagenesis was used to change each of the tyrosines to phenylalanine. Mutation of two of them was required to abolish cleavage and strand-transfer reactions catalyzed by TrwC on oligonucleotides containing the *nic* site. Thus both residues could independently be involved in the formation of oligonucleotide-protein covalent complexes that constitute known intermediates of these reactions. The hypothesis was confirmed by the observation of the two specific peptide-oligonucleotide adducts after protease digestion of TrwC and mutant derivatives. Finally only one of the mutations abolished the nicking reaction on a supercoiled DNA containing R388 *oriT*. This mutation had the strongest effect on the ability to complement a *trwC*⁻ mutant for conjugation. Together these data suggest a model of DNA processing by sequential action of both tyrosines: one tyrosyl residue would catalyze the initial cleavage reaction, while the other would be used for the second strand-transfer reaction.

The reactions described can be easily visualized as part of a recombination process. Such reactions, however, are necessary but not sufficient to achieve recombination. We have a system to easily monitor this recombination *in vivo* and we are now analyzing the requirements for this reaction. We don't obtain recombinant molecules by *in vitro* incubation of TrwC with the plasmid substrate followed by electroporation; presumably certain host factors are needed to initiate the reaction. We are delimiting the minimal TrwC peptide as well as the minimal *oriT* sequences required to obtain efficient site-specific recombination and hope in the future to be able to use this system as a biotechnological tool for targeted chromosomal integration.

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Genetic stability and DNA rearrangements associated to a 2 x 1.1 kb-Long Perfect Palindrome in *E. coli* and *S. cerevisiae*

Francisco Malagón and Andrés Aguilera
Departamento de Genética, Facultad de Biología, Universidad de Sevilla, 41012 Sevilla, Spain.

It is well established that DNA sequences containing large regions of palindromic symmetry cannot be propagated in wild-type *E. coli* strains. The palindrome can be partially or completely deleted (instability) or can confer lethality to the DNA molecule in which it is present (inviability). Recently it has been shown that inviability of long palindromes is dependent on *sbcC* and *sbcD* (1). Nevertheless, long palindromes are unstable in *sbcC*- strains (2). Instability associated with short inverted repeats (IRs) has also been shown in eukaryotes such as *S. cerevisiae* and mice (3).

In order to determine the levels of stability of long perfect IRs in yeast and to design a system for the genetic screening of mutants putatively affected on endonuclease activities acting on palindrome and/or cruciform DNA structures we have constructed a perfect IR of 2 x 1.1 kb based on the URA3 sequence of *S. cerevisiae*. We report the analysis of stability of this IR construct on both *E. coli* and *S. cerevisiae*.

We show that circular plasmids containing the 2 x 1.1 kb IR can be propagated in *E. coli sbcC*- strains. An important fraction of these palindrome-containing plasmids can be recovered from *E. coli* strains either as linear molecules with hairpins at their ends or as head-to-head dimers, both in a RuvC and RusA independent manner. These results suggest that large palindromes may form cruciforms in *E. coli*. Palindrome-associated DNA rearrangements may occur by a process that does not require a known cruciform-resolvase activity. Our data support a replication-dependent model for the induction of DNA rearrangements by perfect palindromes (4).

In wild-type *S. cerevisiae* strains, however, the 2 x 1.1 kb perfect IR is relatively stable. We discuss possible reasons to explain the differences of stability of perfect IRs in *E. coli* and *S. cerevisiae*.

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Nucleotide sequence analysis and genetic characterization of the *recR* gene of *Streptomyces*

Ana Isabel Peláez,[1] Rosa M. Ribas-Aparicio,[2] and M. Rosario Rodicio[1]

[1] Departamento de Biología Funcional (Area de Microbiología) and Instituto Universitario de Biotecnología (IUBA), Universidad de Oviedo, 33006-Oviedo, *Spain*
[2] Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas del Instituto Politécnico Nacional, *Mexico*

Streptomyces belongs to the high G+C branch of Gram positive eubacteria. Its chromosome is a linear DNA molecule with long inverted repeats at the ends (Lin *et al.*, 1993). In this genera, genetic instability is a particularly striking feature (Volff and Altenbuchner, 1998). The chromosomal ends and adjacent regions frequently undergo large deletions which may be accompanied by amplifications or by circularization of the chromosome. Genetic instability has been connected with homologous recombination through recombinational DNA repair, since RecA protein reduces the level of chromosomal deletions and is required for amplification (Volff and Altenbuchner, 1997).

In *Escherichia coli*, the RecR, RecO and RecF proteins function, together with RecA, at an early stage of homologous recombination. Acting in pairs (RecR-RecO and RecR-RecF), they have an important role in modulating the assembly and disassembly of RecA filaments (Umezu *et al.*, 1994; Shan *et al.*, 1997; Webb *et al.*, 1997). In *Streptomyces*, apart from the *recA* gene, that was isolated from different species (including *S. lividans*; Nuβbaumer and Wohlleben, 1994), only the *recF* homolog was identified by DNA sequence of the *dnaA-gyrA* region of the *S. coelicolor* chromosome (Calcutt, 1994). In addition, we have isolated the *Streptomyces* gene equivalent to *recR* from *S. lividans* and *S. coelicolor*. The gene encodes a protein of 204 aa, with a predicted molecular weight of 22 kDa. The deduced amino acid sequence shows significant similarity to the RecR proteins of *Escherichia coli* and *Bacillus subtilis*. Like these proteins, *Streptomyces* RecR contains potential helix-harpin-helix, zinc finger and ATP-binding motifs. The *recR* genes of *E. coli* and *B. subtilis* are immediately preceded by a small ORF of unknown function. An equivalent ORF is also present in *Streptomyces*. The *recR* region of *S. lividans* is 98% identical to that of *S. coelicolor* and strongly hybridizing sequences were detected in the genome of eight other *Streptomyces* strains. By gene disruption experiments, a *recR* mutant of *S. lividans* was obtained. The mutant is more sensitive to ultraviolet light and methyl methanesulfonate than the wild type strain. Effect of the mutation on homologous recombination is now under study.

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Properties of the Bacillus subtilis 168 RecN protein

Rosario Sabariegos[1], Silvia Ayora[1], Germán Rivas[2] and Juan C. Alonso[1].

[1]Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain, and [2] Departamento de Estructura y Función de Proteínas, Centro de Investigaciones Biológicas, CSIC, Velazquez, 144, 28006 Madrid, Spain.

Genetic evidence suggests that the *Bacillus subtilis recN* gene product is involved in DNA repair and recombination. The RecN protein (predicted molecular mass 64.4 kDa) was purified toward homogeneity. The RecN protein, wich has a molecular mass of ~500 kDa, co-purified with short ssDNA segments. The RecN protein possesses a weak DNA-dependent ATPase and an endonuclease activity. RecN is an ATP-independent sugar non-specific nicking enzyme that produces 3' hidroxyl and 5' monophosphate termini. RecN nicks supercoiled DNA to yield at first relaxed circles and then linear DNA. The linear DNA is subsequently hidrolysed with a weak sequence specificity (5'- G/Y C/GCG$^{\downarrow}$C/TG/YC/RC/TA/T) to yield DNA fragments that differ from each other by - 20-nt, and 5'-phosphotetranucleotides, in dsDNA, and 5'- phosphodinucleotides, in ssDNA, as end products. Linear DNA smaller in size of ~500 pb are poor recN substrates. RecN degrades ssDNA distributively and nicks dsDNA processively. The ssDNA generated by RecN may be relevant to reactions that occur during SOS induction. We propose that the RecN activity processes a duplex circular DNA to circular or linear ssDNA that could be utilised in DNA strand exchange.

DNA END-JOINING: ARE ALL ENDS REPAIRED EQUAL?

Julianne Smith, Céline Baldeyron and Dora Papadopoulo
UMR 218 du CNRS, Institut Curie-Section de Recherche, Paris, 75231 France

In eukaryotes, two major pathways have evolved for the repair of DNA double-strand breaks (DSB), homologous recombination and DNA end-joining. *In vivo*, the process of DNA end-joining has been studied at a mechanistic level in several systems, most notably in the yeast *Saccharomyces cerevisiae*. However, little is known about the process in human cells. Thus, we have used a plasmid rejoining assay to examine the ability of human cell lines to join different DNA ends *in vivo*. To dissect finely this process, repair of complementary 5' and 3' extensions as well as several types of non-homologous termini were examined (i.e. blunt, blunt / 5' extension, 5' extension / 3' extension, non-complementary 5' extensions).

Our results indicate that in human cells the repair efficiency of all DSB examined was approximately the same, although the accuracy of the joining event varied dramatically. Interestingly, DSB with complementary and blunt termini as well as blunt / 5' extension were repaired frequently without loss of nucleotides. Thus, in contrast to *S. cerevisiae*, human cells have the capability to repair homologous and non-homologous termini with the same fidelity. However, in human cells, accurate joining of non-homologous ends appears to be limited to blunt ends, as the fidelity of repair of termini with 5' extension / 3' extension or non-complementary 5' extensions was significantly reduced. Sequence analysis of repair events associated with nucleotide loss also revealed a difference between the repair of blunt ends and that of other non-homologous termini. These results indicate that in human cells there are differences in the repair of non-homologous DSB as a function of the type of termini. The possibility that this is due to the existence of different end-joining pathways is supported by our recent findings which demonstrate that in cell lines derived from patients with Fanconi anemia, a genetic instability disorder, the accuracy of joining blunt termini is selectively altered.

Mechanistic study of ATP hydrolysis by recombination proteins: analogy with G-proteins

Oleg N. Voloshin, Lijiang Wang, Fred Dyda, Kiyoshi Mizuuchi & R. Daniel Camerini-Otero
Genetics and Biochemistry Branch and Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD 20892.

Switching between active and inactive conformations of the RecA protein is regulated by ATP hydrolysis. We show that Gln194 and Arg196 of RecA are catalytic amino acids taking part in ATP hydrolysis and functionally resembling the corresponding residues engaged in GTP hydrolysis by two distinct classes of G proteins. The role of DNA and high salt concentrations in the stimulation of the ATPase activity of RecA is similar to the role of the recently described RGSs (Regulators of G protein Signaling) in activating the GTPase of heterotrimeric G proteins and consists in stabilizing the highly mobile region involved in hydrolysis. Thus, recombinases and signaling proteins share common stereochemical regulatory mechanisms.

We also demonstrate that 30-amino acid peptides derived from the C-terminus of the MuA protein stimulate the ATPase activity of the MuB protein. It is unlikely that the peptides provide a catalytic amino acid in *trans* for ATP hydrolysis (as recently shown for small G-proteins such as Ras, by GTPase Activating Proteins (GAPs)), since point substitutions for arginines and glutamine do not abrogate the stimulation of ATP hydrolysis.

The *Saccharomyces cerevisiae* Mlh1 protein is the master organizer of MutL protein-protein interactions

Ting-Fang Wang, Neil Hunter and Nancy Kleckner
Dept. of Molecular and Cellular Biology, Harvard University
7 Divinity Avenue, Cambridge, MA 02138, USA

Genetic analyses in mammalian and yeast cells suggest that the bacterial MutL protein homolog Mlh1 is important for meitoic DNA recombination. However there is no information regarding the biochemical role(s) of Mlh1 during meiosis. Two-hybrid screening and co-immunoprecipitation techniques have been used to identify yeast genes encoding proteins that interact with Mlh1. The results of this analysis indicates that Mlh1 is a master organizer of MutL interactions. The genome of S. cerevisiae encodes four MutL proteins, Mlh1-3 and Pms1. Mlh1 forms heterodimers with each of the other three yeast MutL homologs; in contrast, none of the four proteins exhibits self-self interactions or interactions with members of the family other than Mlh1. Functional analysis of diploid strains lacking one or more Mlh1 family members further suggests distinct, non-overlapping roles for the three MutL heterodimers during meiosis. The Mlh1/Pms1 heterodimer mediates mismatch correction of hetroduplexes, while the Mlh1/Mlh3 heterodimer is required for full level of crossing-over irrespective of repair. Mlh1/Mlh2 heterodimer doesn't appear function in either mismatch correction or crossover pathways; its exact function is under investigation.

"RAD50 and recombination in Arabidopsis"

M.E. Gallego and C. White

CNRS UMR 6547, Université Blaise Pascal, Clermont Ferrand, France.

white@cicsun.univ-bpclermont.fr

Based on the sequence of a cDNA fragment (a kind gift from R. Quatrano) from Arabidopsis encoding a polypeptide with homology to the C-terminal part of the Rad50 protein of yeast, we have isolated and sequenced full length cDNA and genomic clones of the Arabidopsis RAD50 gene. The gene consists of 27 exons encoding a putative polypeptide of 1317 amino acids. A single copy of this gene is found in the Arabidopsis genome and it has been mapped to a unique locus with the Versailles INRA Arabidopsis YAC collection.

Northern analysis shows a single mRNA species of a length consistent with that derived from the cDNA sequence. This single mRNA species is detected in Arabidopsis RNA preparations from suspension cells and also from roots, flower buds and mature flowers, although not in RNA from stems and leaves.

Yeast lacking the native RAD50 gene have been transformed with the Arabidopsis Rad50 homologue under the control of both constitutive and inducible yeast promoters. No significant differences in gamma-ray, nor in MMS sensitivity were seen between the transformed and control yeast strains. Diploid yeast strains designed to test for alteration of the meiotic phenotypes have been constructed and the results of this work will be presented.

Antisense transformants of Arabidopsis suspension cells have been produced and show increased sensitivity to MMS. Homologous/illegitimate recombination mechanisms are being examined in these lines and the results of this work will be presented. This work is also being carried out in parallel in Arabidopsis plants.

LIST OF INVITED SPEAKERS

Andrés Aguilera — Dpto. de Genética, Facultad de Biología, Universidad de Sevilla, Avda. Reina Mercedes 6, 41012 Sevilla (Spain). Tel.: 34 954 55 71 03. Fax: 34 954 55 71 04. E-mail: aguilo@cica.es

Juan Carlos Alonso — Dpto. de Biotecnología Microbiana, Centro Nacional de Biotecnología, Campus Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 91 585 45 46. Fax: 34 91 585 45 06. E-mail: jcalonso@cnb.uam.es

Daniel Camerini-Otero — Genetics and Biochemistry Branch, NIDDK, National Institutes of Health, Bldg. 10, Rm. 9D15, 10 Center Dr, MSC 1810, Bethesda, MD. 20892-1810 (USA). Fax: 1 301 496 9878. E-mail: camerini@ncifcrf.gov

Josep Casadesús — Dpto. de Genética, Facultad de Biología, Universidad de Sevilla, Apartado 1095, 41080 Sevilla (Spain). Tel.: 34 95 455 7105. Fax: 34 95 455 7104. E-mail: genbac@cica.es

Jean-Pierre Claverys — Laboratoire de Microbiologie et Génétique Moléculaire, CNRS-UPR 9007, Université Paul Sabatier, 118 route Narbonne, 31062 Toulouse Cedex (France). Tel.: 33 561 33 59 11. Fax: 33 561 33 58 86. E-mail: claverys@ibcg.biotoul.fr

Michael M. Cox — Dept. of Biochemistry, University of Wisconsin, 433 Babcock Drive, Madison, WI. 53706 (USA). Tel.: 1 608 262 1181. Fax: 1 608 265 2603. E-mail: cox@biochem.wisc.edu

Martin Gellert — Laboratory of Molecular Biology, NIDDK, National Institutes of Health, Bethesda, MD. 20892-0540 (USA). Tel.: 1 301 496 5888. Fax: 1 301 496 0201. E-mail: gellert@helix.nih.gov

Roland Kanaar — Dept. Cell Biology and Genetics, Erasmus University Rotterdam, PO Box 1738, 3000 DR Rotterdam (The Netherlands). Tel.: 31 10 408 7168. Fax: 31 10 408 9468. E-mail: kanaar@gen.fgg.eur.nl

Nancy Kleckner — Dept. of Molecular and Cellular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA. 02138 (USA). Fax: 1 617 495 0758. E-mail: Kleckner@fas.harvard.edu

Stephen Kowalczykowski — Section of Microbiology, University of California, Hutchison Hall - Rm. 258, Davis, CA. 95616-8665 (USA). Tel.: 1 530 752 5938. Fax: 1 530 752 5939. E-mail: sckowalczykowski@ucdavis.edu

Conrad Lichtenstein School of Biological Sciences, Queen Mary and Westfield College, University of London, London E1 4NS (U.K.). Tel.: 44 171 775 3049. Fax: 44 181 983 0973. E-mail: c.p.lichtenstein@qmw.ac.uk

David M.J. Lilley CRC Nucleic Acid Structure Research Group, Dpt. of Biochemistry, University of Dundee, Dundee DD1 4HN (U.K.) Tel.: 44 1382 34 4679. Fax: 44 1382 201 063. E-mail: dmjlilley@bad.dundee.ac.uk

Robert G. Lloyd Institute of Genetics, University of Nottingham, Quenn's Medical Centre, Nottingham NG7 2UH (U.K.) Tel.: 44 0115 97 09 407. Fax: 44 0115 970 99 06. E-mail: bob.lloyd@nottingham.ac.uk

Thomas F. Meyer Max-Planck-Institut für Infektionsbiologie, Abteilung Molekulare Biologie, Monbijoustrasse 2, D-10117 Berlin (Germany). Tel.: 49 30 2802 63 84. Fax: 49 30 2802 66 11. E-mail: meyer@mpiib-berlin.mpg.de

Alain Nicolas Institut Curie, Section de Recherche, CNRS UMR144, 26 rue d'Ulm, 75248 Paris (France). Tel.: 33 1 42 34 64 36. Fax: 33 1 42 34 64 38. E-mail: anicolas@curie.fr

Tom D. Petes Dept. of Biology, University of North Carolina, Chapel Hill, NC. 27599-3280 (USA). Tel.: 1 919 962 1445. Fax: 1 919 962 84 72. E-mail: tompetes@email.unc.edu

Miroslav Radman Institute Jacques Monod, F-75251 Paris Cedex 05 and Institut Necker, Faculté de Médecine, Université Paris-5, 156 rue de Vaugirard, 75730 Paris Cedex 15 (France). Fax: 33 1 44 27 57 16. E-mail: radman @ccr.jussieu.fr

John Roth Dept. of Biology, University of Utah, Salt Lake City, UT. (USA). Tel.: 1 801 581 3412. Fax: 1 801 585 6207. E-mail: roth@bioscience.utah.edu

Rodney Rothstein Dept. of Genetics & Development, Columbia University, College of Phys. & Surg., New York, NY. 10032-2704 (USA). Tel.: 1 212 305 1733. Fax: 1 212 923 2090. E-mail: rothstein@cuccfa.ccc.columbia.edu

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Stephen C. West Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts. EN6 3LD (U.K.). Tel.: 44 171 269 3868. Fax: 44 171 269 3811. E-mail: west@icrf.icnet.uk

LIST OF PARTICIPANTS

Sylvia Ayora — Centro Nacional de Biotecnología, Campus Universidad Autónoma,Cantoblanco, 28049 Madrid (Spain). Tel.: 34 91 585 45 28. Fax: 34 91 585 45 06. E-mail: sayora@cnb.uam.es

Antonio Bernad — Centro Nacional de Biotecnología, Universidad Autónoma, Campus de Cantoblanco, 28049 Madrid (Spain). Tel.: 34 91 585 45 62. Fax: 34 91 372 04 93. E-mail: abernad@cnb.uam.es

Luis Blanco — Centro de Biología Molecular "Severo Ochoa", Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 91 397 84 93. Fax: 34 91 397 47 99. E-mail: lblanco@cbm.uam.es

Mario Cáceres — Dept. de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona (Spain). Tel.: 34 93 581 2729. Fax: 34 93 581 2387. E-mail: mariocs@cc.uab.es

Myriam Calonje — Centro Nacional de Biotecnología, Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 91 585 45 00. Fax: 34 91 585 45 06.

Gareth A. Cromie — Institute of Cell and Molecular Biology, University of Edinburgh, King's Buildings, Edinburgh EH9 3JR (U.K.). Tel.: 44 131 650 5372. Fax: 44 131 650 8650. E-mail: gcromie@srv0.bio.ed.ac.uk

Ernesto Doncel — Dpt. de Biotecnología Microbiana, Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 91 585 45 28. Fax: 34 91 585 45 06. E-mail: edoncel@cnb.uam.es

Peter Dröge — Institute for Genetics, University of Cologne, Im Weyertal 121, D-50931 Köln (Germany). Tel.: 49 221 470 34 07. Fax: 49 221 470 51 70. E-mail: p.droege@uni-koeln.de

M. Isabel Franco — Dpto. de Genética, Universidad de Málaga, Campus Universitario de Teatinos, 29071 Málaga (Spain). Tel.: 34 95 213 19 68. Fax: 34 95 213 19 55.

Andrés Garzón — Dpto. de Genética, Universidad de Málaga, Campus Universitario de Teatinos, 29071 Málaga (Spain). Te.: 34 95 213 1677. Fax: 34 95 213 1955. E-mail: garzon@uma.es

Jennifer L. Gerton Dpt. of Biology, University of North Carolina, Chapel Hill, NC. 27599-3280 (USA). Tel.: 1 919 962 1445. Fax: 1 919 962 1625. E-mail: gerton@cmgm.stanford.edu

Sergio González-Barrera Dpto. de Genética, Facultad de Biología, Universidad de Sevilla, Avda. Reina Mercedes 6, 41012 Sevilla (Spain). Tel.: 34 95 455 71 03. Fax: 34 95 455 71 04.

Grzegorz Ira Nicholas Copernicus University, ul. Gagarina 9, 87-100 Torun (Poland). Tel.: 48 56 6114 576. Fax: 48 56 611 44 78. E-mail: g-ira@cc.uni.torun.pl

Martin Kupiec Dpt. of Molecular Microbiology and Biotechnology, Tel Aviv University, Ramat Aviv 69978 (Israel). Tel.: 972 3 640 9031. Fax: 972 3 640 9407. E-mail: martin@ccsg.tau.ac.il

David R.F. Leach Institute of Cell and Molecular Biology, University of Edinburgh, King's Buildings, Edinburgh EH9 3JR (U.K.). Tel.: 44 131 650 5373. Fax: 44 131 650 8650. E-mail: D.Leach@ed.ac.uk

Avraham A. Levy Dpt. of Plant Sciences, The Weizmann Institute of Science, Rehovot 76100 (Israel). Tel.: 972 8 934 27 34. Fax: 972 8 934 4181. E-mail: lplevy@wiccmail.weizmann.ac.il

Matxalen Llosa Dpto. de Biología Molecular, Fac. de Medicina, Universidad de Cantabria, C. Cardenal Herrera Oria s/n., 39011 Santander (Spain). Tel.: 34 942 20 19 40. Fax: 34 942 20 19 45.

Francisco Malagón Dpto. de Genética, Facultad de Biología, Universidad de Sevilla, 41012 Sevilla (Spain). Tel.: 34 95 455 71 07. Fax: 34 95 455 71 04. E-mail: malagon@cica.es

Cristina Menéndez c/Luis Morondo 10-5°-A, 31006 Pamplona (Navarra). Fax: 34 948 16 98 93.

Gisela Mosig Dpt. of Molecular Biology, Vanderbilt University, Nashville, TN. 37235 (USA). Tel.: 1 615 322 3442. Fax: 1 615 343 6707. E-mail: MOSIGG@CTRVAX.VANDERBILT.EDU

Ana Isabel Peláez Dpto. de Biología Funcional (Area de Microbiología) e Instituto Universitario de Biotecnología (IUBA), Universidad de Oviedo, 33006 Oviedo (Spain). Tel.: 34 98 510 35 58. Fax: 34 98 510 31 48.

Rosario Sabariegos Dpto. de Biotecnología Microbiana, Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 91 561 18 00. Fax: 34 91 562 75 18.

Julianne Smith UMR 218 du CNRS, Institut Curie-Section de Recherche, 26 rue du d'Ulm, 75231 Paris (France). Tel.: 33 1 42 34 66 90. Fax: 33 1 46 33 30 16. E-mail: Julianne.Smith@curie.fr

Andrzej Stasiak Laboratoire d'Analyse Ultrastructurale, Université de Lausanne, CH-1015 Lausanne-Dorigny (Switzerland). Tel.: 41 21 692 42 82. Fax: 41 21 692 41 05. E-mail: Andrzej.Stasiak@lau.unil.ch

Oleg N. Voloshin Genetics and Biochemistry Branch and Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD. 20892 (U.K.). Tel.: 1 301 496 2038. Fax: 1 301 496 9878. E-mail: voloshin@ncisun1.ncifcrf.gov

Ting-Fang Wang Dpt. of Molecular and Cellular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA. 02138 (USA). Tel.: 1 617 495 1803. Fax: 1 617 495 8308. E-mail: tfwang@fas.harvard.edu

Charles White CNRS UMR 6547, Université Blaise Pascal, Clermont Ferrand (France). Tel.: 33 4 73 40 79 78. Fax: 33 4 73 40 77 77. E-mail: white@ cicsun.univ-bpclermont.fr

Rafael J. Yáñez Gene Targeting Group, MRC Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital, DuCane Road, London W12 0NN (U.K.). Tel.: 44 181 383 8296. Fax: 44 181 383 8303. E-mail: ryanez@rpms.ac.uk

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